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Der Pharma Chemica, 2014, 6(1):411-417
(http://derpharmachemica.com/archive.html)

ISSN 0975-413X
CODEN (USA): PCHHAX

In vitro cytotoxicity and molecular docking studies on *Acanthophora spicifera*

Meenakshi Sundaram Muthuraman[1*], Sathiyamoorthi Mani[2], Udhayachandran Thangaraj[2] and Aravind Sivasubramanian[3]

[1]*Department of Biotechnology, School of Chemical & Biotechnology, SASTRA University, Thirumalaisamudram, Thanjavur, Tamil Nadu, India*
[2]*Department of Biotechnology, Srimad Andavan Arts and Science College, Tiruchirapalli, Tamil Nadu, India*
[3]*Department of Chemistry, School of Chemical & Biotechnology, SASTRA University, Thirumalaisamudram, Thanjavur, Tamil Nadu, India*

ABSTRACT

Acanthophora spicifera, the red algae which is used as fresh vegetables in some parts of Vietnam and the Philippines was subjected to different solvent extraction (Hexane, Ethylacetate and Ethanol) and the ethanol extract of the Acanthophora spicifera was screened against the following strains Escherichia coli, Proteus vulgaris, Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeuroginosa, Bacillus subtilis and also subjected to the in-vitro cytotoxic assay. From the data obtained it was evident that the extract showed significant increase in the death rate of Ehrlich Ascites carcinoma cell lines. At the dose level of 100µg/ml, 36.46% of cell death was noticed. With GC-MS (Gas chromatography Mass spectrometry) technique, 42 compounds were identified. Five of the compounds were docked with the BCL-2 protein by using Auto Dock software. The compound Phenol, 3-methyl-5-(1-methylethyl)-, methylcarbamate showed least binding energy of -9.3. In this study, it is confirmed that the Acanthophora spicifera has significant anti-cancer activity.

Keywords: Anti-microbial activity, *Invitro* cytotoxicity assay, Ehrlich Ascites carcinoma, GC-MS, BCL-2 protein.

INTRODUCTION

Algae are relatively undifferentiated organisms which, unlike plants, have no true roots, leaves, flowers or seeds.Seaweeds are classified as Green algae (Chlorophyta), Brown algae (Phaeophyta), Red algae (Rhodophyta) and some filamentous Blue-green algae (Cyanobacteria). Most of the seaweeds are red (6000 species) and the rest known are brown (2000 species) or green (1200 species).

The Red algae are one of the oldest groups of eukaryotic algae, and also one of the largest, with about 5,000–6,000 species of mostly multicellular, marine algae, including many notable seaweeds..

Acanthohora spicifera is seaweed that grows in upright clumps of spiny branches, 1-2 mm in diameter. It can be pale yellow, brownish, dark green, or reddish (often dark green in intertidal areas and high wave motion areas). This species grows on the reef in intertidal, lagoon and reef flat habitats, or it can be free-floating. These Red Algae have some medicinal properties.

Much previous research has studied the biological effects of natural marine products, including their antioxidant, antibacterial, anti-malarial, antiviral, anti-inflammatory and cytotoxic activities [1-6]. However, only a few studies have examined the biological potential of the marine algae (seaweeds) [7-10].

Kezia *et al.*, [11] investigated the *in vitro* and *in vivo* antitumor properties of a sulfated polysaccharide isolated from the seaweed *C. feldmannii*. It showed *in vivo* antitumor effect. The extract from a red alga, *Amphiroa zonata* exhibited strong cytotoxicity to all human leukemic cell lines [12]. Four red algae showed appreciable antitumor activity against cancer cells [13].

MATERIALS AND METHODS

Collection of seaweed material

The red alga *Acanthohora spicifera* was collected in the coastal area of Rameswaram, India during the month of December 2009. The collected seaweed material was identified and authenticated by Dr V.T. Sridharan, Head, Department of Botany, National College Trichy. The whole seaweed material was shade dried and coarsely powdered

Preparation of extracts

About 650gm of powder material was soaked in 500ml of hexane chloroform and ethanol for at least 48 hrs with frequent shakings. Later the filtrate was evaporated and dried in a water bath.

Animals

Adult swiss albino mice (20-25gm) were procured and used for the culturing of cell lines they were housed in microlon boxes in a controlled environment (temp 25ºc and 12h dark and light cycle) with standard laboratory diet and water and libitium. Necessary institutional animal ethical committee clearance was obtained before starting the experiment.

Cells

EAC cells were obtained through the courtesy of amala cancer research center, trichur, kerala. They were maintained by intraperitoneal inoculation of 10^6 cells/mouse.

Pigment Extraction and Estimation of Chlorophylls, Carotenoids and Phycobili proteins in *Acanthohora spicifera*:

The extraction of chlorophyll and carotenoids were done for *Acanthophora spicifera.*

Procedure:

Exactly 1 g of each algal sample was taken separately and with the help of a mortar and pestle, the tissue was homogenized either in 10 ml of 80% cold acetone. The algal sample was transferred to a clean screw capped test tube and placed in an ice box and kept in deep freeze compartment of a refrigerator over night to allow complete extraction of pigments. The following day the sample was centrifuged at 3000rpm to remove cell debris and sand particles and the clear supernatant was used for optical density measurement in a spectrometer at specified wavelength using 80% acetone to zero the instrument each wavelength. The specific wavelengths are,

For Chlorophyll “A” – 665nm
For Chlorophyll “B” – 645nm
For Carotenoids – 480nm
For Phycocyanin – 615nm
For Phycocyanin - 562nm
For Allophycocyanin – 652nm.

Estimation of Chlorophylls, Carotenoids and Phycobiliproteins in *Acanthohora spicifera*:

Optical density readings were taken using 1 cm width cuvette at 665(663), 645, 480 nm for chlorophylls and carotenoids and at 562, 615, 652 nm for phycobilisomes in spectrophotometer.

CHLOROPHYLL "A" = $\frac{12.7A_{663} - 2.69A_{645} \times V}{A \times 1000 \times W}$

CHLOROPHYLL "B" = $\frac{22.9\ A_{645} - 4.68\ A_{663} \times V}{A \times 1000 \times W}$

CAROTENOIDS = $\frac{D \times V \times F \times 10}{2500}$

PHYCOCYANIN = $\frac{A_{615} - 0.474\ (A_{562})}{5.34}$

ALLOPHYCOCYANIN = $\frac{A_{652} - 0.208\ (A_{615})}{5.09}$

Antimicrobial Activity

Method

Assay of antimicrobial activity was performed by disc diffusion method. The Muller-Hinton agar plates were prepared and the organism was swabbed over it using a sterile cotton swab. The antimicrobial discs were placed on the surface of the agar plates and then, the plates were incubated at 37°C for 24 hrs. After incubation, the zone of inhibition was measured.

Microorganisms

The ethanolic extract of *Acanthohora spicifera* was tested against various organisms such as *Escherichia coli***,** *Proteus vulgaris, Staphylococcus aureus***,** *Streptococcus pyogenes***,** *Pseudomonas aeuroginosa, and Bacillus subtilis*

Invitro Studies

Short term in-vitro cytotoxicity [14]

Short term in-vitro cytotoxicity was assessed using EAC cell lines by incubating the different concentration of drugs at 37ºc for 3 hours. The tumor cells were aspirated from peritoneal cavity of tumor bearing mice using a 10 ml syringe and transferred to a test tube containing isotonic saline. The cells were then washed in normal saline and cell number was determined using a haemocytometer and adjusted at 10 x 10^6 cells/ml. for the cytotoxicity assay , different concentrations of drug (50,75&100µg/ml) were added to each and the final volume was adjusted to one ml with normal saline. Control tubes were kept with the solvent and without the solvent along with tumor cells. All the tubes were incubated at 37ºc for 3 hours. After incubation 0.1ml of 1% trypan blue dye in isotonic saline was added to each tube and the number of viable (unstained) and dead (stained) cells were counted using haemocytometer. The percent cytotoxicity (%dead cells) was calculated using the formula.

The dead cells were calculated by the formula

$$\% \text{ dead cells} = \frac{\text{Total cells counted} - \text{total viable cells}}{\text{Total cells counted}} \text{X100}$$

GCMS (Gas chromatography Mass spectrometry)

This is technique used to separate the compounds present in the given sample (solvent extraction), and this is combination of gas chromatography as well as mass spectrometry technique .We can get result a chromatogram graph in this GC-MS.

Procedure

- The sample is dissolved in the organic solvent till it dissolves completely.
- Gas chromatography condition is maintained at100°C-280°C as 5°C/min.
- 2µl of sample is injected in to the column.
- The helium gas moves at 1ml/min through the column.
- The compound split in the ratio of 1:10.
- After the program is runes mass spectrometer scans the compounds separated.
- Then the peak area of the each peak is measured to find the compound present at the area with the structure.

Docking Studies

Autodock

Auto Dock is a suite of automated docking tools. The software is used for modelling flexible small molecule such as drug molecule binding to receptor proteins of known three dimensional structure. [15]

Bcl-2 protein, an anti apoptotic protein selected as target molecule. The 3D structure of the compounds present in the sample was drawn in chemsketch tool. The 3D structure of the compounds was converted into PDB file by using molecular converter and was taken as ligand molecule. The active site in the target protein was predicted by using the online tool Q-site finder. The PDB file of target protein, the PDB file of the ligand and text file of amino acids in the active site were taken as input files and auto docking was carried out. The 3D structure of the docked molecules and the bonds, residues involved the docking was viewed by using Pymol viewer.

RESULTS

The extraction of chlorophyll, carotenoids and phycobiliproteins was carried out. The extracted pigments was estimated by using spectrophotometer and the results were Chlorophyll“A”-0.3174(mg/g), Chlorophyll“B”-0.1160(mg/g), Carotenoids-0.0771(mg/g), Phycocyanin -0.1108(mg/g), Allophycocyanin -0.2581(mg/g).

The Ethanolic extract of *Acanthophora spicifera* was tested Antimicrobial activity on six different species of microorganisms such as *Escherichia coli, Proteus vulgaris, Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeuroginosa and Bacillus subtilis.* Among these *Escherichia coli, Staphylococcus aureus, Pseudomonas aeuroginosa* had high inhibition.

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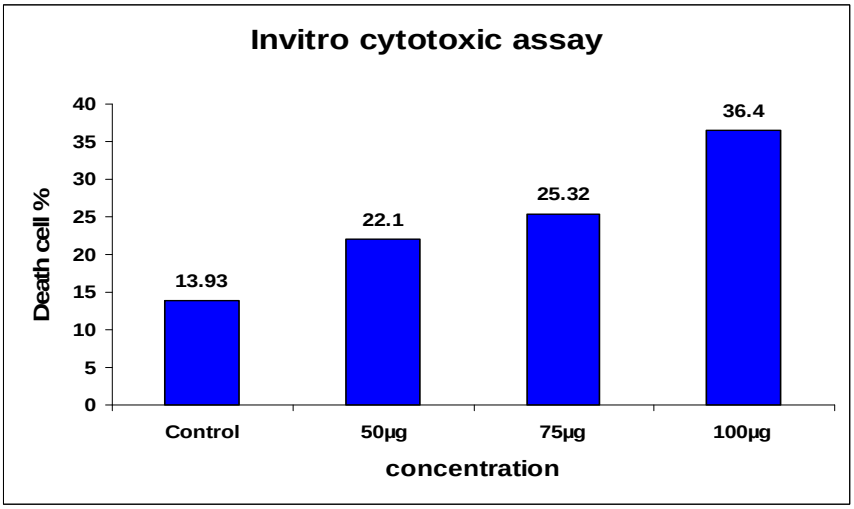
Ethanolic extract of *Acanthohora spicifera* showed significant invitro cytotoxic activity against Ehrlich Ascites carcinoma cell lines. 50µg/ml, 75µg/ml, 100µg/ml dose level are tested. Among these 100µg/ml dose of Ethanolic extract of *Acanthohora spicifera* showed 36.46% of cell death. (Fig -1).

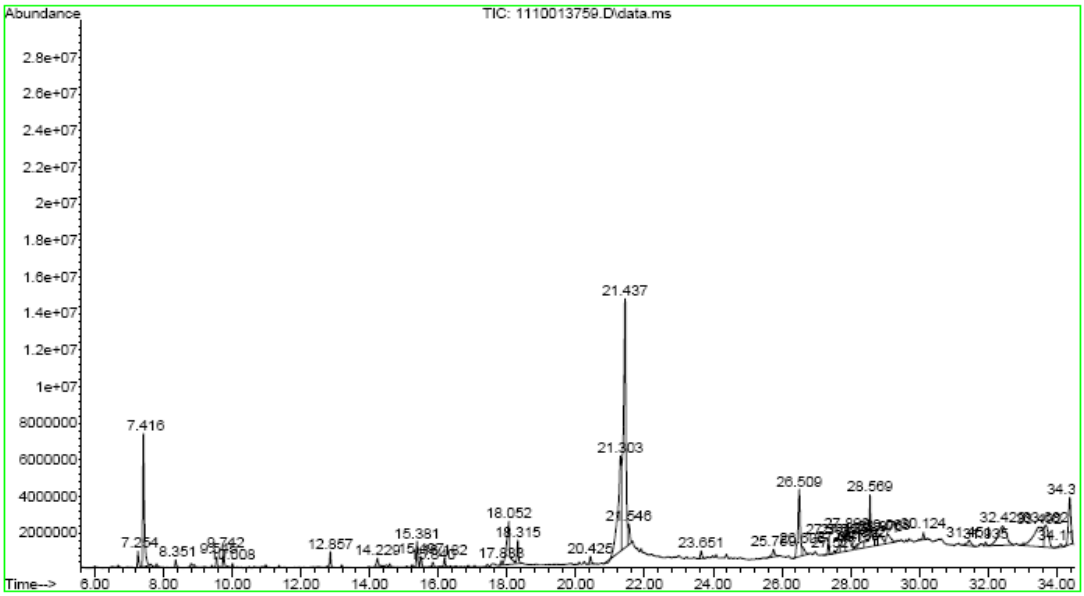
Fig -1 Short Term In-vitro Cytotoxicity Studies



GC-MS Profile of ethanol extract of *Acanthohora spicifera*

Different extract of hexane, chloroform and ethanol were prepared. The ethanol extract of *Acanthohora spicifera* was subjected to Gas chromatography/ mass spectrometry studies. There were 42 compounds identified in the extract. (Fig -1).

Fig -1 GC-MS profile of ethanol extract of *Acanthohora spicifera*



Bioinformatics Studies

Auto docking study was carried out on BCL-2 protein for the compounds present in the ethanolic extract of *Acanthophora spicifera*.

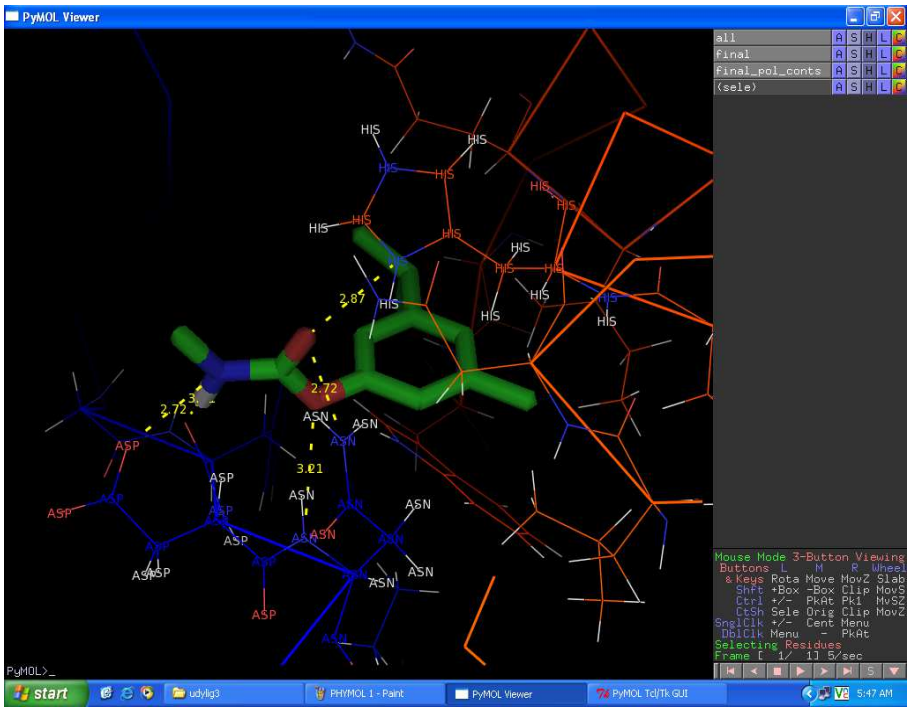
Five of the compounds were docked with the BCL-2 protein.

Those compounds were

- Phenol, 3-Methyl-5-(1-Methylethyl)-, Methylcarbamate
- 2-Benzyloxyethylamine
- 1,2,3-Butanetriol
- 3-Trifluoroacetoxydodecane
- Acetic acid, 3-hydroxy-6-isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydronaphthalen-2-yl ester

The compound Phenol, 3-Methyl-5-(1-Methylethyl)-, Methylcarbamate showed least binding energy of -9.3 and so that it is considered as the best candidate.

Figure-2 Pictorial Representation of Docking of Phenol, 3-Methyl-5-(1-Methylethyl)-, Methylcarbamate with Bcl-2



DISCUSSION

The red algae *Acanthophora spicifera* eaten as fresh vegetables in some parts of Vietnam and the Philippines. It is also used as animal feed. It is reported to have antibacterial, antibiotic properties. From the literature review is evident that Red algae *Acanthophora spicifera* are potent in anti microbial and anti cancer activity.

Different solvent extracts (Hexane, Ethylacetate, and Ethanol) were prepared and the ethanol extract of the *Acanthophora spicifera* was screened against the following strains *Escherichia coli, Proteus vulgaris, Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeuroginosa, Bacillus subtilis.* The activities were screened using various concentration of ethanol extract like 200µg/disc, 400µg/disc, 800µg/disc, 100µgdisc employing disc diffusion method. This results high activity was against *Staphylococcus aureus, Pseudomonas aeuroginosa, Escherichia coli.*

Various algae such as *Caulerpa racemosa, Halimeda opuntia, Gelidiela acerosa, Laurencia papillosa* and *Acanthophora spicifera,* showed antimicrobial activity against *Staphylococcus aureus* and *Bacillus subtilis* in a concentration of 50 mg/ml by the cylinder plate method. None of the algae studies showed activity against *Escherichia coli, Pseudomonas aeruginosa, Aspergillus niger* and *Candida albicans*. The content of agar in *Acanthophora spicifera* was found to be the highest (33.5%) of all the species studied [16].

The anticancer activity is carried against EAC cell lines. Ethanolic extract of *Acanthohora spicifera* was subjected to the invitro cytotoxic assay. From the data obtained it is evident that the extract showed significant increase in the death rate of Ehrlich Ascites carcinoma cell lines. At the dose level of 100µg/ml 36.46% of cell death noticed (Fig - 1). Kezia et al [11] investigated the *in vitro* and *in vivo* antitumor properties of a sulfated polysaccharide isolated from the seaweed *C. feldmannii*. Hence it can be inferred that this red algae possess significant anticancer property.

Ethanol extract of *Acanthophora spicifera* was analysed by GC-MS (Gas chromatography Mass spectrometry). There were 42 compounds identified. Five of the compounds were docked with the BCL-2 protein. The compound Phenol, 3-methyl-5-(1-methylethyl)-, methylcarbamate showed least binding energy of -9.3 and so that it is considered as the best candidate. In this study, it is confirmed that the *Acanthophora spicifera* has significant anticancer activity.

CONCLUSION

Secondary metabolites from natural sources are important for pharmacological research and drug development [17]. Despite the major advances in the modern medicine, the development of new drugs from natural products is still considered important [18]. In this direction, to conclude the present work, *Acanthophora spicifera* have high chlorophyll content and antimicrobial activity againt *Staphylococcus aureus*, *Pseudomonas aeuroginosa*, *Escherichia coli*. From *invitro* studies *Acanthophora spicifera* has significant anticancer activity.

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