GAVIN PUBLISHERS

Journal of Pharmaceutical and Pharmacological Sciences

Research Article

Samuel P, et al., J Pharma Pharma Sci 02: JPPS-138.
DOI: 10.29011/2574-7711.100038

Bioprospecting of *Salicornia europaea*L. a Marine Halophyte and Evaluation of Its Biological Potential with Special Reference to Anticancer Activity

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Citation: Samuel P, Kumar VJ, Dhayalan DR, Amirtharaj K, Sudarmani DNP (2017) Bioprospecting of *Salicornia europaea* L. a Marine Halophyte and Evaluation of Its Biological Potential with Special Reference to Anticancer Activity. J Pharma Pharma Sci 02: 138. DOI: 10.29011/2574-7711.100038

Received Date: 02 August, 2017; **Accepted Date:** 25 August, 2017; **Published Date:** 02 September, 2017

Abstract

The present study was initiated with an intention to bring outthe phytochemical profile of the marine halophyte *Salicornia europaea*L., the chemical finger print of the halophyte was made by GC-MS.The phytochemicalsin the crude extract was evaluated for cytotoxic study against MCF7. The marine halophytes were collected, washedand chopped in to 5cm long and shade dried for 20-25 days in a dark room. The dried and grounded plant materials were subjected to Soxhlet extraction.Two solvents vizmethanol and ethyl acetate were used to prepare decoction of the plant.The extracts were screened using GC-MS and dried using rotary vacuum evaporator. The dried phytochemicals were evaluated for cytotoxic studies against MCF7 by MTT assay. The chemical screening by GC-MS had unveiled the presence of 32 compounds in highly polar solvent namely ethyl acetate and 29 in methanol a polar solvent.The structure of the phytoconstituents were retrieved from Pub Chem and compared in NIST library. The anti-cancer study showed promising results. The IC_{50} values of the individual extracts were evaluated and fortunately ethyl acetate extract exhibit minimum IC_{50} value and it was estimated to be 97.9µg/ml were as methanol extract exhibit 117.1µg/ml. Hence it leads to conclude that *S. europaea* L.has promising source of lead compounds that could be used to treat cancer.

Keywords: GC-MS; IC_{50} Value; MCF7; MTT Assay; Salicornia europaeaL; Soxhlet Extraction

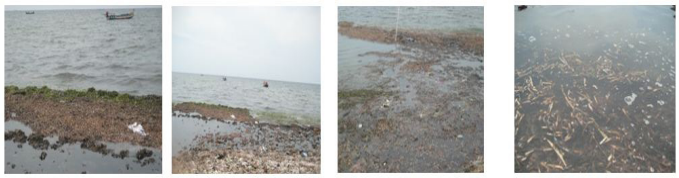
Introduction

Cancer is one of the life threatening and major health issues across the globe. According to a statistic, cancer is accounted for11.4 million new cases and 7.4 million deaths in 2004.Cancer incidence in South East Asia region was 1.7 million in 2004 (The global burden of disease 2004 update). Cancer has become one of the ten leading causes of death in India [1].Conventional methods of cancer treatment hold several undesirable side effects and hence an alternative source for obtaining anti-cancer drugs are must require. The promising sources of these alternative drugs are from plants[2]. Few reports were available on the usage of this marine halophyte as food[3,4],source of soluble polysaccharides[5]andanti-cancer drug source[6-9].Hence, we decided to exploit this plant against the hunt for anti-cancer drugs.

Materials and Methods

Study Area Description

The present study was carried out in the marine environment of South east coastal areas of Thanjavur and pudhukottai district. The sampling spots are located on 10.20°N 79.24°E on the coast of the Bay of Bengal, South East coast of Tamilnadu. The sampling locations included are Adirampattinam, Rajamadam and Mallipattinam (Thanjavur district, Tamilnadu) and Memesal (Pudhukottai District) (Figure1).



Memesal Adirampattinam Rajamadam Mallipattinam

Figure 1: Sampling spots along the south-east coast of Tamil Nadu.

Adirampattinamis located on 10.20°N 79.24°E on the coast of the Bay of Bengal. Being close to the equator, Rajamadam is a small village located at the longitude of 79.34°N and latitude of 10.30°E. Mallipattinam is a small village in Thanjavur District in Tamilnadu State. Located on Lat. 10.27°N and Long79.31°E, Memesal is one of the Villages in Pudhukottai District in Tamilnadu. Located on Lat. 9 .56° N and Long. 79 .10°E.

Collection and Processing of plant

The fresh plant was collected from south east coast of Tamilnadu. The plant was identified by Dr. V. Ramasubramanian, Head, Department of Botany (UG), Ayya Nadar Janaki Ammal College, (Autonomous), Sivakasi. Later the plant was washed in running tap water chopped and kept shade dried for 20-25days in a dark place.

Screening of Phytochemicals

The different analytical tests were performed in order to establish the chemical profile of the extracts. The following chemical tests like Test for Flavonoids[10], Tannins[10], Saponin[10], Steroids[10], Quinones[10], Coumarins [10], Terpenoids[10], Cardiac glycosides [10]and Phlobatannins[10]were performed.

Chemical fingerprint of S. europaea L. by GC-MS

The chemical fingerprint of the halophyte was obtained through GC-MS analysis.Model No: Agilent 7890B Gas Chromatograph connected with 5977A MSD mass spectrometry. The Gas chromatography unit was equipped and coupled to a HP_5MS 5% Phenyl Methyl Silox -60°C-325°C (325°C) column and its dimension of the column is 30m×250µm×0.25µm. Prior to the initiation of the phytochemical scanning procedure the instrument was set to an initial temperature of 50ºC. At the end of this preparatory period the oven temperature was rose up to 300ºC, at the rate of an increase of 5ºC/min, and maintained for 9 min. Following are the parameters need to be ensured for the successful operation of the instrument. The temperature of the sample injection port was ensured as 250ºC and the flow rate of Helium was ensured as 1ml/min. The ionization voltage was set at 70eV. The extract samples were injected in split mode. Scan range was set at 45-450 (m/z). The structure of the phytoconstituents was retrieved using computer searches on a NIST Version-Year 2011.

Evaluation of Anticancer activity of Phytocompounds by MTT assay

Preparation of Cell line

The cell line used in the present study was Human breast adenocarcinoma cell lines (MCF7) and it was obtained from National Centre for Cell Science (NCCS), Pune. The cell line was maintained in Eagles Minimum Essential Medium containing 10% Fetal Bovine Serum (FBS). The cells were maintained under the controlled incubatory conditions like the temperature was at 37ºC, 5% CO2, 95% air and 100% relative humidity.To maintain the viability of cells in the cell line it was and passaged weeklyaccording to Mosmann [11].

Cell treatment procedure

According to Monks[12], after monolayer cells were formed it was detached with enzyme based salt. The procedure was carried out to make detached single cell suspensions from monolayer and viable cells were counted using a hemocytometer. The cells were diluted with medium containing 5% fetal bovine serum to yield a final concentration of cells and it was calculated to be 1x105cells/ml. Initially about one hundred microliters of cells were pipetted and transferred per well in 96-well platesand the satisfied plating density was calculated to be 10,000 cells/well.To promote the cell attachment certain parameters were adapted like 37°C, 5% CO2, 95% air and 100% relative humidity. Exactly after 24 h of incubation the cells grown the 96-well plates were treated with different concentrations of the test samples. The plant extract was dissolved in DimethylSulfoxide (DMSO). Once it was dissolved an aliquot of the test sample solution was further diluted to obtain a desired final maximum concentration with serum free medium.Using a microliter pipette 100 µl of these different sample dilutions were transferred to the appropriate wells which were already loaded with 100 µl of medium. Following the addition of test sample,the microtitre plates were subjected to incubation for 48 h with the following specified parameters viz 37°C, 5% CO2, 95% air and 100% relative humidity. To compare the test sample equally another preparation was maintained but that was free from test sample.

MTT Assay

The MTT assay was performed according to Monks[12].After 48 h of incubation, 15µl of MTT (5mg/ml) in Phosphate Buffered Saline (PBS) was added to each well and incubated at 37ºC for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. The % cell inhibition was determined using the following formula.

% Cell Inhibition = 100 - Abs (sample)/Abs (control) x100

To understand the relationship between % Cell inhibition

and Log ConcentrationNonlinear regression graph was plotted and IC50 was determined using Graph Pad Prism software.

Results and Discussion

Identification of the Plant

The halophyte was identified as Salicornia europaea L (Figure 2).

Figure 2: *Salicornia europaea* L. grown in large scale along the southeast coast of Tamil Nadu.

Extraction of Phytochemicals by Soxhlet

The dried biomass of the halophyte was extracted with methanol and ethyl acetate (Figure 3) under controlled environment.

Figure 3: Phytochemical extraction of *Salicornia europaea* L.

The ethyl acetate extract of the biomass was pale yellow and methanol extract was dark brown respectively. The optical property of the extract was bit critical that the ethyl acetate extract was transparent whereas the methanol extract was opaque (Figure 4).

Figure 4: Methanol and ethyl acetate extract of *S. europaea* L.

Screening of Phytochemicals

Qualitative analysis of the ethyl acetate and methanol extracts were carried out. The study revealed the presence of two important phytochemicals called Tannins and Coumarins in methanol extract and flavonoids in ethyl acetate extract (Table 1). Essaidiet al.(2013) [13]reported the presence of phenonic acids and flavonoids. The study slightly correlated with above finding further it becomes very clear that the lack of phenonic acids might be due to the condition in which the halophytes are growing. In a detailed study conducted by Cybulskaet al. 2014[14]had confirmed fatty acids, terpenoids, flavonoids, alkaloids, steroids, tannins, saponins, quinones and coumarins. It was found that many of the constituents reported to have therapeutic values in humans. In the present study, phytochemical screening had paved the way for the confirmation of same type of phytochemicals in the halophyte thus it leads to the confirmation that there is no connectivity between phytochemicals and geographical distribution.

Phytochemicals	E. ace	Methanol
Flavonoids	+	-
Tannins	+	+
Saponins	+	+
Steroids	-	-
Quinones	-	-
Coumarins	-	+
Terpenoids	-	-
Cardiac glycosides	-	-
Phlobatannins	-	-

Table 1: Screening of phytochemicals in different Extract.

Chemical fingerprint of *S.europaea*L. by GC-MS

The list of phytochemicals present in both extracts wasscanned through GC- MS and the results were tabulated (Table 2& Table3). The analysis had confirmed the prolific nature of compounds in the selected halophyte. Further the GC-MS analysis confirmed the presence of structurally diversified compounds based on retention time (Table 4& Table 5). The detailed reports on the chromatograms are given in [Figure 5 & 6] respectively.

S.No	Name of the Compounds
1.	Aziridine, 1-ethenyl-
2.	2-Propanone, 1-hydroxy-
3.	Propanoic acid, 2-oxo-, methyl ester
4.	2(3H)-Furanone, 5-methyl-
5.	4-Cyclopentene-1,3-dione
6.	1,2-Cyclopentanedione
7.	Undecane
8.	1-Butanol, 2-methyl-, acetate
9.	Pyrrolidine, 1-(1-cyclohexen-1-yl)-
10.	Quinoline, 1,2-dihydro-2,2,4-trimethyl-
11.	Heptadecane, 2,6,10,15-tetramethyl-
12.	2,6-Difluorobenzoic acid, tridec-2-ynyl ester
13.	Phenol, 2,4-bis(1,1-dimethylethyl)-
14.	Hexadecane, 2,6,11,15-tetramethyl-
15.	Imidazolo[1,2-a]pyrimidine-2,5(1H,3H)-dione, 3,7-dimethyl-
16.	3-Hexadecanol
17.	Isopropyl myristate

18.	Eicosane, 2-methyl-
19.	Hexadecanoic acid, methyl ester
20.	Benzothiazole, 2-(2-hydroxyethylthio)-
21.	Bis(tridecyl) phthalate
22.	Hexanoic acid, 2-ethyl-, anhydride
23.	Methyl 10-trans,12-cis-octadecadienoate
24.	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-
25.	Phytol
26.	Methyl stearate
27.	Tetracosane
28.	2-methyloctacosane
29.	Heneicosane, 11-(1-ethylpropyl)-
30.	4,4'-((p-Phenylene)diisopropylidene)diphenol
31.	4,11-Dihexyl-16,16-dimethyl-1,14-dioxa-4,11-diazacycloheptadecane-3,12-dione
32.	γ-Sitosterol

Table 2: List of phytochemicals present in ethyl acetate extract.

S.No	Name of the Compounds
1.	CH3C(O)CH2CH2OH
2.	2-Propanone, 1-hydroxy-
3.	Ethanamine, 2-chloro-N,N-dimethyl-
4.	Dodecane, 4,6-dimethyl-
5.	2-Methoxy-4-vinylphenol
6.	2,4-Difluorobenzoic acid, 2-formyl-4,6-dichlorophenyl ester
7.	2-Pentadecanone, 6,10,14-trimethyl-
8.	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester
9.	Hexadecanoic acid, methyl ester
10.	3,5-Dimethoxy-4-hydroxycinnamic acid
11.	Dibutyl phthalate
12.	n-Hexadecanoic acid
13.	Hexanoic acid, 2-ethyl-, anhydride
14.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester
15.	9-Octadecenoic acid (Z)-, methyl ester
16.	cis-13-Octadecenoic acid, methyl ester
17.	Methyl stearate
18.	9,12-Octadecadienoic acid (Z,Z)-
19.	Benzenethanamine, 3,4-dimethoxy-α-methyl-
20.	3-Eicosene, (E)-
21.	Eicosanoic acid, methyl ester
22.	4,8,12,16-Tetramethylheptadecan-4-olide
23.	n-Tetracosanol-1
24.	Glycerol 1-palmitate
25.	Diisooctyl phthalate
26.	9,12-Octadecadienoic acid (Z,Z)-, 2,3-dihydroxypropyl ester
27.	Stigmasterol
28.	Cholestane, 1-vinyl-1-hydroxy-
29.	γ-Sitosterol

Table 3: List of compounds present in the GC-MS analysis ofMethanol extract.

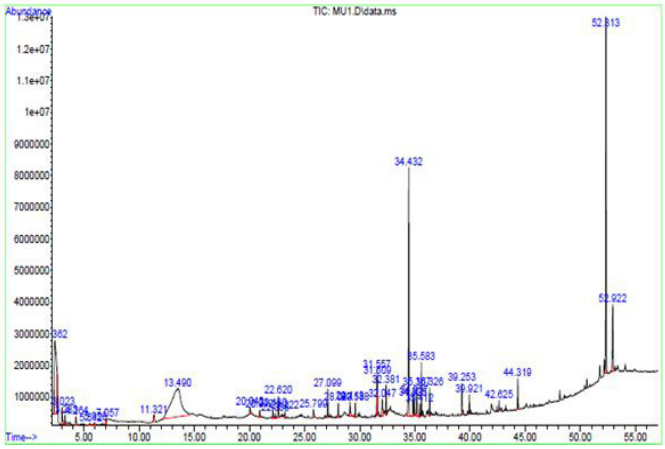


Figure 5: GC-MS chromatogram of Methanol extract of *Salicornia europaea*.

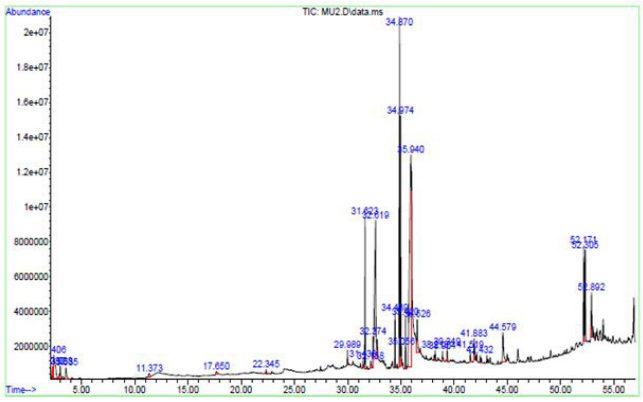


Figure 6: GC-MS analysis of ethyl acetate extract of Salicornia europaea.

The present study was correlated to the report given by Iscaet al. 2014[15],Zhaoet al. 2014[16]had also confirmed the presence of triterpenes and saponins in the solvent extract of the halophyte. Similarly, in a study carried out by Samuelet al. 2016[9] reported that this halophyte is the source of prolific chemicals which is further confirmed in the present study. The study correlates with Das Neves Costaet al. 2015[17]who had reported the selection of solvent system plays a critical step in the extraction procedure.Radwan [18]had studied the phytochemical profile of S. fruticosa L. In the report he had unveiled that there are 23 compounds by GLC

but in the present study fractionated 32 and 29 compounds respectively. Thus it gave further evidence that Salicornia europaea L. is a source of prolific and structurally diversified chemicals.

Cytotoxic activity of Phyto chemicals

The concentrated crude sample obtained from ethyl acetate and methanol was subjected to evaluate the anti-cancer activity. Before the analysis was begun the crude samples was diluted and taken in the following concentration 12.5µg, 25µg, 50 µg, 100µg and 200µg (Table2 and 3).

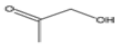
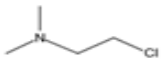
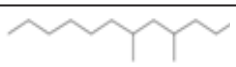
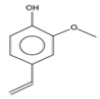
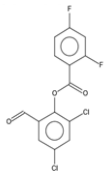
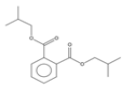
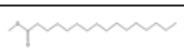
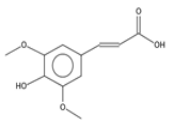
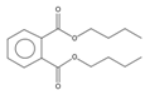
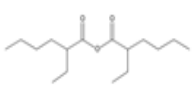
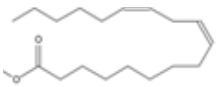
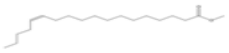
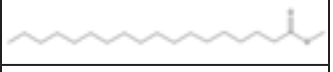
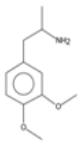
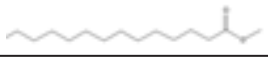
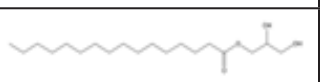
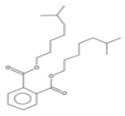
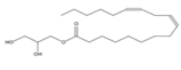
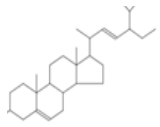
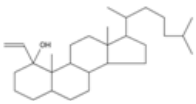
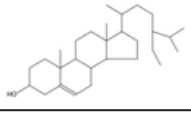
S.No	Molecular formula	Molecular weight	Structure
1.	$C_4H_8O_2$	88	
2.	$C_3H_6O_2$	74	
3.	$C_4H_{10}ClN$	107	
4.	$C_{14}H_{30}$	198	
5.	$C_9H_{10}O_2$	150	
6.	$C_{14}H_6Cl_2F_2O_3$	330	
7.	$C_{18}H_{36}O$	268	
8.	$C_{16}H_{22}O_4$	278	
9.	$C_{17}H_{34}O_2$	270	
10.	$C_{11}H_{12}O_5$	224	
11.	$C_{16}H_{22}O_4$	278	
12.	$C_{16}H_{32}O_2$	256	
13.	$C_{16}H_{30}O_3$	270	
14.	$C_{19}H_{34}O_2$	294	
15.	$C_{19}H_{36}O_2$	296	
16.	$C_{19}H_{36}O_2$	296	
17.	$C_{19}H_{38}O_2$	298	
18.	$C_{18}H_{32}O_2$	280	
19.	$C_{11}H_{17}NO_2$	195	
20.	$C_{20}H_{40}$	280	
21.	$C_{21}H_{42}O_2$	326	
22.	$C_{21}H_{40}O_2$	324	
23.	$C_{24}H_{50}O$	354	
24.	$C19H_{38}O_4$	330	
25.	$C_{24}H_{38}O_4$	390	
26.	$C_{21}H_{38}O_4$	354	
27.	$C_{29}H_{48}O$	412	
28.	$C_{29}H_{50}O$	414	
29.	$C_{29}H_{50}O$	414	

Table 4: GC-MS analysis of compounds with structure in Methanol extract.

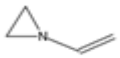
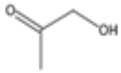
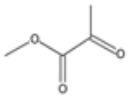
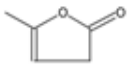
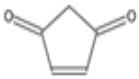
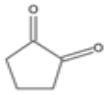
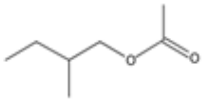
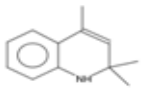
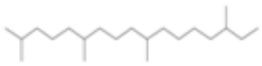
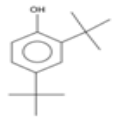
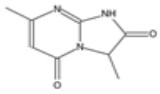
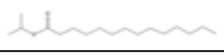
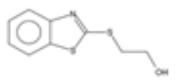
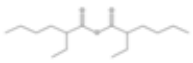
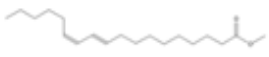
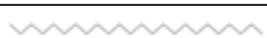
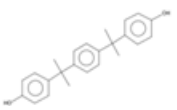
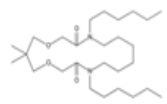
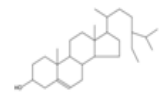
S.No	Molecular formula	Molecular weight	Structure
1.	C_4H_7N	69	
2.	$C_3H_6O_2$	74	
3.	$C_4H_6O_3$	102	
4.	$C_5H_6O_2$	98	
5.	$C_5H_4O_2$	96	
6.	$C_5H_6O_2$	98	
7.	$C_{11}H_{24}$	156	
8.	$C_7H_{14}O_2$	130	
9.	$C_{10}H_{17}N$	151	
10.	$C_{12}H_{15}N$	173	
11.	$C_{21}H_{44}$	296	
12.	$C_{20}H_{26}F_2O_2$	336	
13.	$C_{14}H_{22}O$	206	
14.	$C_{20}H_{42}$	282	
15.	$C_8H_9N_3O_2$	179	
16.	$C_{16}H_{34}O$	242	
17.	$C_{17}H_{34}O_2$	270	
18.	$C_{21}H_{44}$	296	
19.	$C_{17}H_{34}O_2$	270	
20.	$C_9H_9NOS_2$	211	
21.	$C_{34}H_{58}O_4$	530	
22.	$C_{16}H_{30}O_3$	270	
23.	$C_{19}H_{34}O_2$	294	
24.	$C_{19}H_{32}O_2$	292	
25.	$C_{20}H_{40}O$	296	
26.	$C_{19}H_{38}O_2$	298	
27.	$C_{24}H_{50}$	338	
28.	$C_{29}H_{60}$	408	
29.	$C_{26}H_{54}$	366	
30.	$C_{24}H_{26}O_2$	346	
31.	$C_{27}H_{52}N_2O_4$	468	
32.	$C_{29}H_{50}O$	414	

Table 5: GC-MS analysis of compounds with structure in Ethyl acetate extract.

Both the extracts were evaluated for anti-cancer potential. The cytotoxicity pattern of methanol extract and its corresponding nonlinear graph is illustrated in (Figure 7 and 8) respectively. The graph revealed the significance of relationship between dosage concentration and cytotoxicity. It was observed that the ethyl acetate extract had significant cell inhibitory activity against Human breast adenocarcinoma cell line (MCF7). Maximum cell inhibition was observed in 200µg of the compound tested prepared from ethyl acetate and methanol. The percentage of cell population inhibited at 200 µg was 81.71% (ethyl acetate) and 79.49%

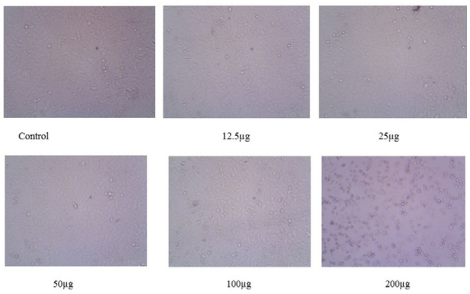


Figure 7: Cytotoxicity pattern of Methanol extract of *Salicornia europaea*.

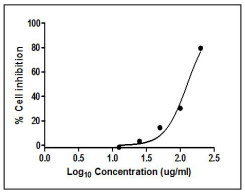


Figure 8: Nonlinear graph showing the relationship between doses of phytocompounds (Methanol) % of cell inhibition.

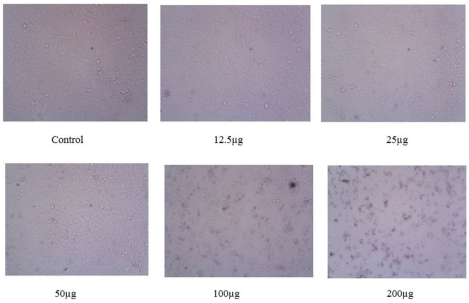


Figure 9: Cytotoxicity pattern of Ethyl acetate extract of *Salicornia europaea*.

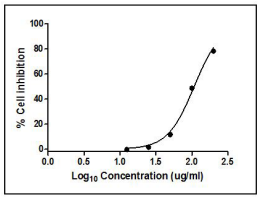


Figure 8: Nonlinear graph showing the relationship between doses of phytocompounds (Ethyl acetate) % of cell inhibition.

(methanol). Further the IC_{50} value of the compound tested against the cell line 97.9µg/ml (ethyl acetate) and 117.1µg/ml (methanol). The cytotoxicity pattern of ethyl acetate extract and its corresponding nonlinear graph is illustrated in [Figure9&10] respectively. The graph revealed the significance of relationship between dosage concentration and cytotoxicity.Lellau and Liebezeit[19] studied the cytotoxic activity of salt marsh Daphnia sp. Its activity was comparatively lesser than Salicornia europaea L. The present investigation on the halophyte reinforced that it is advantageous. The study also related to Essaidi[13]et al. 2013 in the aspect that the methanolic extract has cytotoxic activity but at the same time cytotoxic activity of the meth=anolic fraction was comparatively lesser than ethyl acetate extract.

Conclusion

The studies conclude that Salicornia europaea L. is abundant along the Adirampattinam coastal environs of Tamilnadu. Qualitative screening had led us to confirm the presence of Tannins and Coumarins in the methanolic extract and flavonids in ethyl acetate extract. Extensive phytochemical screening was carried out using GC-MS. The study yields the presence of 32 and 29 compounds in corresponding ethyl acetate and methanol extracts respectively. The ethyl acetate fraction of this halophyte had exhibited a maximum cell inhibition when it was added to the cell line in 200µg. Further the IC_{50} value also determined for the same. The value was comparatively less with that of methanolic extract.

Acknowledgement

The author expresses his thanksand gratitude to The Management and The Principal of Ayya Nadar Janaki Ammal College (Autonomous), Sivakasi for providing laboratory support with all needed chemicals and instruments for the successful completion of the work analysis and anti-cancer studies.

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