Journal of Algal Biomass Utilization
J. Algal Biomass Utln. - Copyright © 2009 PHYCOSPECTRUM - ISSN: 2229 – 6905

In-silico studies on metabolites of Phormidium fragile against colon cancer EGFR protein

***S.Mukund, V. Sivasubramanian. N.S.Senthilkumar**

Department of Plant Biology and Plant Biotechnology, R K M Vivekananda College, Chennai, India.

*E-Mail: t.s.mukunth@gmail.com

Abstract:
This work was to find out the flavonoid derived form and Cyanobacterial metabolites from the Cyanobacterium *Phormodium fragile* to test them against colon cancer protein. The metabolites were extracted in 100% methanol from the Cyanobacterial biomass of *Phormodium fragile* isolated from effluent. The crude methanol extract was purified and analysed for the flavonoid form by HPLC and compounds from the extract was assessed by GC-MS analysis. Two predominant compounds (Quercetin) and Bis (2-ethyl hexyl pthalate) identified in the extracts were screened against the Colon cancer protein (1IVO) by in-silico docking method. Of the compounds, Quercetin was the most potent having the docking score of −8.1 Kcal/mol. This value was better than the standard drug "GEFITINIB". This work recommends the Quercetin for further in vitro and in vivo studies towards its development as anticancer drug

1. Introduction

Cyanobacteria have gained a lot of attention in recent years because of their potential applications in biotechnology. We present an overview of the literature describing the uses of cyanobacteria in industry and services sectors and provide an outlook on the challenges and future prospects of the field of cyanobacterial biotechnology. Cyanobacteria have been identified as a rich source of biologically active compounds with antiviral, antibacterial, antifungal and anticancer activities.Cyanobacteria have been known to be an enormous resource for compounds with varying bioactivities including antimicrobial, antiviral and enzyme inhibitory effects. They are potent sources of bioactive compounds have currently been used in drugs discovery. The cyanobacterial genus *Phormidium* is evenly distributed throughout the Indian coast, having many species mainly marine in nature, rich in different types of pigments specially phycobiliproteins and different types of carotenoids. The crude aqueous extract may contain large amount of water soluble pigment phycocyanins and extracellular polysaccharides.However, potent drugs to cure colon cancer are increasingly necessary. Hence, the present study was undertaken to identify the predominant metabolites present in *Phormidium fragile* isolated from effluent and also to test them against colon cancer protein using in-silicon molecular docking methods.

MATERIALS AND METHODS

Cyanobacterial Culture

Phormidium fragile cyanobacterium was obtained from the culture collection of Vivekananda Institute of Algal technology (VIAT), Chennai (isolated from effluent). Biomass was obtained by growing algal cultures in 20L of water and 0.25g /L of NPK fertilizer was added with a facility to pump the culture with aeration pump. The algae was grown for 20 days and harvested and then the biomass was harvested for the extraction of intracellular metabolites.

Gas chromatography-Mass spectroscopy

Preparation of extract: 1 µl of the methanolic extract of *Phormidium fragile* was employed for GC/MS analysis.
Instruments and chromatographic conditions**:** GC-MS analysis was carried out on a GC QP 2010 [SHIMADZU] comprising a AOC-20i auto sampler and gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: column Elite-1 fused silica capillary column (30 × 0.25 mm ID ×1EM df, composed of 100% Dimethyl poly siloxane), operating in electron impact mode at 70 eV; helium (99.999%) was used as carrier gas at a constant flow of 1ml/min and an injection volume of 0.5 EI was employed (split ratio of 10:1) injector temperature 240°C; ion-source temperature 200°C. The oven temperature was programmed from 110°C (isothermal for 2 min), with an increase of 10°C/min, to 200°C/min, then 5°C/min to 280°C/min, ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 40 to 550 Da.

Identification of components**:** Interpretation on mass spectrum of GC-MS was done using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained.

Content of Flavonoids by HPLC Method

HPLC analysis was performed using a LDC Milon Roy CM 4000 gradient pump coupled to a Hewlett Packard 1100 diode-array detector. Flavonoid separation was carried out in a 5 mm Chrompack C18 column 250 mm_4.6 mm, protected by a Chrompack C18 pre-column 3.0 mm_10 mm. Cyanobacterial extract were eluted at 1 ml/min20 µl injection volume) using as mobile phase a binary solvent system consisting in methanol, water, and phosphoric acid (100:100:1) equal volumes (about 20 µL) of each of the Standard solutions was injected and the Test solution into the chromatograph equal volumes (about 20 µL) of each of the Standard solutions was injected and the Test solution into the chromatograph . Record the chromatograms, and measure the areas for the major peaks. Calculate the percentage of each flavonoid in sample.

Retrieval of Protein Structure .The 3-D crystal structure of the targeted) was retrieved from the protein databank (PDB) (www.rcsb.org/pdb). Structural and active site studies of the protein were done by using CASTP (Computed Atlas of Surface Topography of Proteins) and pymol molecular visualization software.

Compounds Screened: Twenty seven compounds isolated from *Phormidium fragile* using GC-Ms and five compounds namely Rutin, Quercetin, Kaemferol and Luteolin and Apigenin isolated from *Phormidium fragile* using HPLC were screened against the protein .The pubchem database was used for retrieving the structure of the ligand molecules. The selected chemical structures were generated from the software (open babel). The molecular docking was performed using PyRx widely distributed public domain of molecular docking software. The inhibitor and target protein were geometrically optimized and docked using PyRx.

Docking Methods: Virtual-screening is an emerging approach and is extensively used to reduce cost, and time in drug discovery. PyRx is virtual screening software for Computational Drug Discovery (CDD), which can be used to screen libraries of compounds against potential drug targets. It uses a large body of already established open source software such as Auto Dock 4 and AutoDock Vina. These two are used as docking software.

Active Site Prediction. Active site of the target protein was predicted by using "Active site prediction tool" from SCFBio Server, which requires a. pdb file as an input and this tool explains the total number of active sites along with information on their amino acid sequence, cavity points, and the average volume of the cavity.

Ligand Binding Sites Prediction. After docking the docked structure was saved as ".pdb" file and further explored to predict the binding sites using "Ligand Explorer" software. The predicted binding sites, based on the binding energy, and amino acids make up the binding cavity. Here ligand binding site represents the site where the ligands most efficiently bind with the protein, among all the active sites.

Drug Likeliness Prediction. Ligand property was predicted by using LipinskiDrugFilters. Lipinski rule of five helps in distinguishing drug-like and non-drug-like properties and predicts high probability of success or failure due to drug likeliness for molecules. The Lipsinki filter helps in early preclinical assessment and thereby avoiding costly late-stage preclinical and clinical failures.

RESULTS

Phormidium fragile was subjected to GC-MS analysis and was analyzed for flavonoids contents by RP-HPLC, as shown in Figure 1 and Table 2.The GC–MS analysis of *Phormidium fragile* showed the presence of 27 compounds, which includes by 8-Octadecenoic acid methyl ester (31.30%) followed by 9-hexadecanoic acid methyl ester (Z) (14.83%), Hexadecanoic acid methyl ester (13.78%) and 24 compounds were distributed in different quantities. *Phormidium fragile* was analyzed for flavonoids contents by Reversed phase High Performance Liquid Chromatography (RP-HPLC).Reversed-phase HPLC for quantification of flavonoids in cyanobacterial.It has been applied especially for the identification of flavonoids derivatives.The results are confirmed using insilico docking methods. In the present investigation, flavonoids were quantified at 254nm using peak area by comparison to a calibration curve derived from the *Phormidium fragile* , the main difference was in peak eluted at 3.336 min.

All the 27 isolated compounds and 5 derivates of flavonoids were screened in docking analysis against the Colon cancer protein (EGFR (PDB 1IVO)). Of which, only three compounds namely Bis (2-ethyl Hexyl Pthalate), Phthalic acid,di(2-propylpentyl)ester and eicosane and displayed good docking scores (Table 5).

Derivates of flavonoids showed higher docking when compared with GC-MS compounds Commercial drug, GEFITINIB exhibited the docking score of – 5.5 kcal/mol as against kcal/mol for -7.9 Bis (2-ethyl Hexyl Pthalate)

(Table 4), while derivates of flavonoids showed higher docking score as compared to commercial drug with Quercetin showed maximum score with a binding energy level as -8.1 Kcal/mol.

FIGURE 1. **GC-MS ANALYSIS of *PHORMIDIUM FRAGILE***

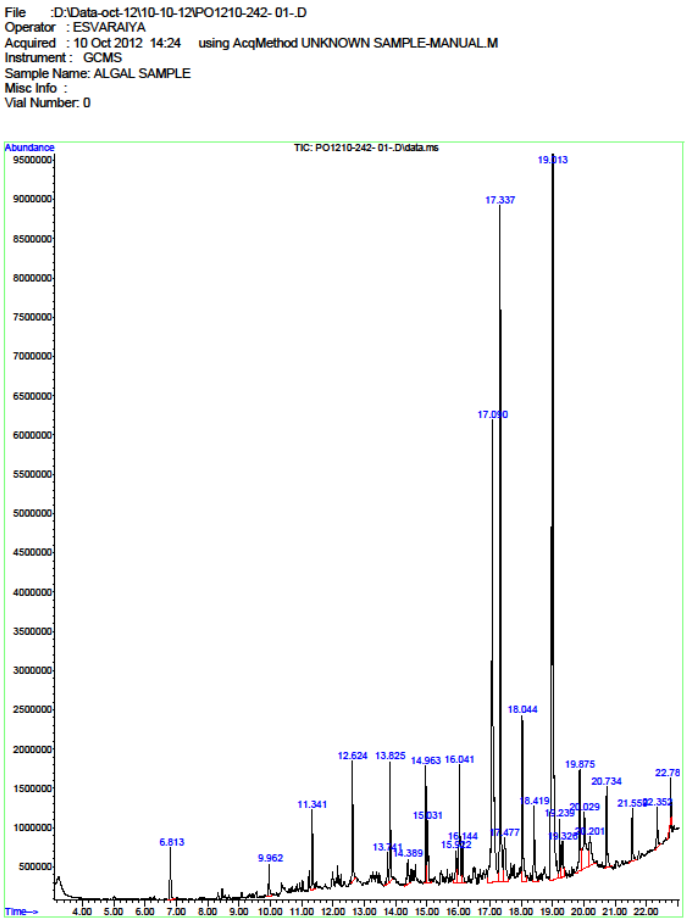


TABLE 1 DISTRIBUTION OF FLAVONOIDS IN PHORMIDIUM FRAGILE

S.NO	FLAVONOIDS(COMPOUNDS)	QUANTITY (MG/ML)
1.	**RUTIN**	**0.022**
2.	**QUERCETIN**	**0.724**
3	**KAEMFEROL**	**0.05**
4	**LUTEOLIN**	**0.021**
5	**APIGENIN**	**0.075**

TABLE 2. LIPINSKI DRUG FILTERS OF FLAVONOIDS FROM PHORMODIUM FRAGILE EXTRACT

COMPOUND NAME	LOGP	MOLECULAR WEIGHT(G/MOL)	HYDROGEN DONOR/ACCEPTOR	MOLAR REFRACTIVITY
RUTIN	1.3	610.5175	1,1.6	136.886
QUERCETIN	1.5	302.2357	5,7	73.910
KAEMFEROL	1.9	286.2363	4,6	72.246
LUTEOLIN	1.4	286.2363	4,6	72.476
APIGENIN	1.7	270.2369	3,5	70.812

TABLE 3 LIPINSKI DRUG FILTERS OF GC-MS COMPOUNDS OF PHORMODIUM FRAGILE

COMPOUND NAME	LOGP	MOLECULAR WEIGHT(G/MOL)	HYDROGEN DONOR/ACCEPTOR	MOLAR REFRACTIVITY
BIS(2-ETHYL HEXYL PTHALATE)	7.4	390.55612	0,4	107.4
9-HEXADECENOIC ACID METHYL ESTER	6.5	268.43478	0,2	83.64
PHTHALIC ACID, DI(2-PROPYLPENTYL)ESTER	1.9	390.5561	4,6	114.57
EICOSANE	10.4	286.2363	0,0	52.440
PHYTOL	4.3	296.00	1,1	95.561

TABLE 4. DOCKING RESULTS OF FLAVONOIDS FROM PHORMODIUM FRAGILE EXTRACT

S.No	COMPOUND WITH PUBCHEM ID	CHEMICAL FORMULA	BINDING ENERGY (KCAL/MOL).	BINDING SITE
1	RUTIN (CID 5280805)	$C_{27}H_{30}O_{16}$	-7.1	Thr 358 Thr 360
2	QUERCETIN) (CID 5280343)	$C_{15}H_{10}O_7$	-8.1	Tyr251 , Phe263
3	KAEMFEROL (CID 5280863)	$C_{15}H_{10}O_6$	-7.4	SER 324,PHE 321
4	LUTEOLIN (CID 5280445)	$C_{15}H_{10}O_6$	-7.4	LYS 185 HIS 186
5	APIGENIN (CID 5280443)	$C_{15}H_{10}O_5$	-7.2	ASN 328,THR 330
6	GEFITINIB (CID 123631) (Marketed Drug)	$C_{22}H_{24}ClFN_4O_3$	-5.5	GLN 408 ,GLY 410

TABLE 5. POTENTIAL DERIVED GC-MSCOMPOUNDS FROM PHORMIDIUM FRAGILE

S.No	Compounds	Chemical formula	Binding Energy Kcal/mol).	Binding Site
1	Bis(2-ethyl hexyl pthalate)	$C_{24}H_{38}O_4$	-7.9	Arg285, Ser285
2	Hexadecenoic acid methyl ester	$C_{17}H_{32}O_2$	-5.3	ASN 331 SER 330
3	Phthalic acid, di(2-propylpentyl)ester	$C_{24}H_{38}O_4$	-6.5	Asn 32 ,THR 330
4	Eicosane	$C_{20}H_{42}$	-5.6	ASN 331 ,LEU 332
5	Phytol	$C_{20}H_{40}O$	-5.2	Pro 257,GLU 258
6	Gefitinib(Marketed Drug)	$C_{22}H_{24}ClFN_4O_3$	-5.5	GLN 408 ,GLY 410

DISCUSSION

Expression of EGFR and its role in cancer prognosis has been investigated in many human cancers (Cavasotto *etal*., 2006). The epidermal growth factor (EGF) is the prototype of a family of peptide ligands that bind to cell membrane receptors and activate intracellular signaling pathways to control tumor cell growth, proliferation, survival, metastasis, and angiogenesis. The EGF receptor (EGFR, ErbB1, or HER1) is one of a four-membered family of transmembrane receptors that, similar to HER2, is often over expressed in cancer cells, correlating with poor prognosis. EGFR therefore represents a reasonable target for the development of novel anticancer therapies (Cho *etal., 2003).* Quercetin, a flavonoid and more specifically a flavonol, is the aglycone form of other flavonoid glycosides, such as rutin and quercitrin, found in citrus fruit, buckwheat and onions. Quercetin forms the glycosides, quercitrin and rutin, together with rhamnose and rutinose, respectively. Although there is preliminary evidence that asthma, lung cancer and breast cancer are lower among people consuming higher dietary levels of quercetin, (Van der Woude *etal*., 2005).
Quercetin is also reported to inhibit CYP450 family of enzymes (Lautraite *etal*., 2002), which play a major role in the activation of a number of suspected human carcinogens. It possess anti– inflammatory activity due to a variety of mechanisms including inhibition of COX and LOX, as well as reduction in the transcription of COX. The antitumor activity of quercetin has been related to its anti–inflammatory activity in the tumors which over express COX (Mutoh *etal*., 2000).Quercetin also causes cell cycle arrest and apoptosis by a p53 dependent mechanism (Plaumann *etal*.,1996). Inhibition of protein tyrosine kinase, (Guthrie *etal*., 1996) and inhibition of interaction of carcinogens with DNA (Kang *etal*., 1999) have been proposed as mechanisms of action of quercetin.

Quercetin can also modulate the estrogen receptor activity to inhibit the growth of breast cancer cell lines, MCF7 ((Scambia *etal*., 1993).Cancer cell lines including HT-29, the effects of flavonoids of different subclasses on cell cycle are strongly dependent on the specific structure of the compounds. Extending these studies, we here provide evidence that the nonhydroxylatedcore structure of the flavones, flavone, is a potent and selective inhibitor of proliferation of HT-29 cells. Moreover, it promotes differentiation and apoptosis in this human colon cancer cell line. Flavone, occurring in many cyanobacteria (Middleton etal., 1993) inhibits proliferation of HT-29 in a concentration-dependent manner. This topoisomerase I inhibitor is usually applied as a second-line pharmacotherapeutic in advanced colorectal cancers to promote apoptosis (Cunningham, 1999 and Whitacre 1999).It was observed using Pymol that the phenylalanine/serine and tyrosine protein kinases present in the drug was the site of binding to the receptor (PDB 1IVO) and methyl group present in the probable functional groups, which resulted in a decrease in the energy values.
The structures of colon cancer protein have been documented in recent years. Based on the literature it has been shown clearly that the secondary metabolites have been used to target the Human epidermal growth factor receptor (PDB 1IVO). Even though the antioxidant potential of these cyanobacteria was proved, *the* actual mechanism of action is not

clear. Further research is essential to determine the actual constituents of this species, which make them a good source of pharmaceutical potential through advanced techniques.

FIGURE 2. AMINO ACIDS IN THE BINDING POCKET (PHE 321 ASN 33O, SER 324, LEU327, LEU332, THR 330) RCSB LIGAND EXPLORER

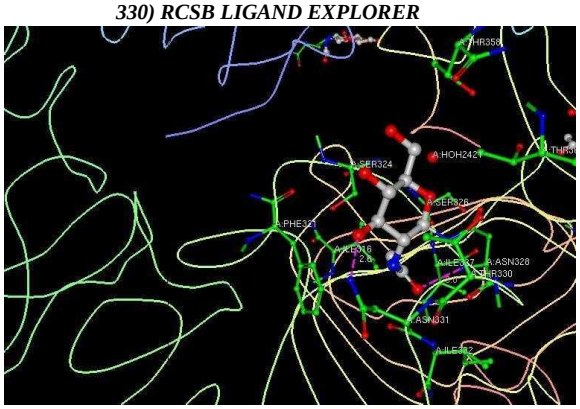


FIGURE 3. *PROTEIN –LIGAND INTERACTION OF BIS (2-ETHYLHEXYL PHTHALATE AND EGFR (PYMOL)*

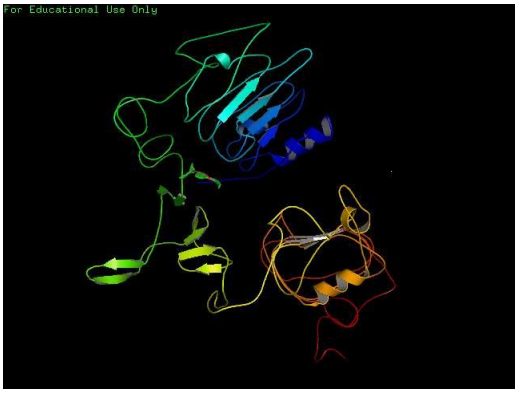
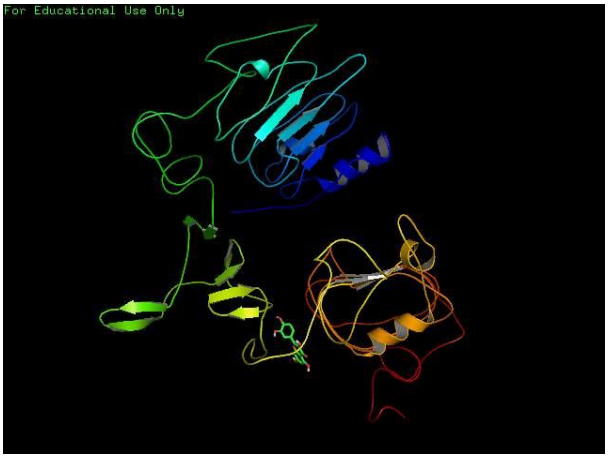


FIGURE 4. PROTEIN -LIGAND INTERACTION OF QUERCETIN AND EGFR OF COLON CANCER.



Acknowledgements

We thank the Secretary and Principal, R.K.M. Vivekananda college, Chennai 600 004, India, for providing us with the necessary infrastructure and facilities required for the study.

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