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Phytochemical screening and in silico studies of flavonoids from *Chlorella pyrenoidosa*

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ABSTRACT

The recent explosion of interest in the bioactivity of the flavonoids of microalgae is due to the potential health benefits of the polyphenolic components that are major dietary constituents. The present study focuses on the phytochemical screening and in silico studies of flavonoids. Total flavonoids content in *Chlorella pyrenoidosa* was estimated in two modes of cultivation (Autotrophic and Heterotrophic) and its implication in anti-proliferation and anti-inflammatory activity was assessed through in silico approach. H-Ras p21(PDB-4L9S) and Lipoxygenase (PDB-3V99) involved in proliferation pathway and inflammatory pathway were selected as the target proteins for in silico studies. Seven compounds were selected for molecular docking. Pharmacokinetic properties of these compounds were calculated using online tools and docking was performed using Auto Dock Vina. By comparing and analyzing their binding energies in Maestro Schrodinger, suite, it was observed that Epigallocatechin gallate exhibited least binding energy of −9.1 kcal/mol and hence has anti-inflammatory activity. Catechin has best binding affinity with H-Ras p21 and hence has anti proliferative activity.

1. Introduction

Microalgae are a key natural resource for a vast array of compounds viz. biodiesel, nutraceuticals, including proteins, vitamins, minerals, carotenoid pigments, such as xanthophylls and carotenes, Flavonoids etc. Subspecies of *Chlorella* are known to have several bioactive secondary metabolites which can have bacteriostatic, bactericidal, antioxidant anti-proliferative, antifungal, antiviral and antitumor activity [1,2]. Recent exploration of anti-proliferative and anti-angiogenic properties of *Chlorella pyrenoidosa*,a unicellular fresh water green alga,paves way for exploring its use in treating inflammation and proliferation associated with various diseases.

Flavonoids are the largest groups of phenolic compounds are known to contain a broad spectrum of chemical and biological activities including antioxidant and free radical scavenging properties [3]. Flavonoids include flavonols, flavones, catechins, proanthocyanidins, anthocyanidins and isoflavonoids. In the recent times, flavonoids have gained increasing interest as they exhibit beneficial health effects due to their potential antioxidant [4], anti-inflammatory and anti-cancer activities. Information is scarce on the presence of secondary metabolites, responsible for anti-proliferative and anti-inflammatory properties of *C. pyrenoidosa*. Hence in this study, autotrophic and heterotrophic cultures of C. *pyrenoidosa* were grown and HPLC analysis was carried out for the algal biomass for analyzing various flavonoids. The aim of the present study is to assess total flavonoids content and to study the role of flavonoids as anti-proliferative and anti-inflammatory agents using in silico analysis.

In this study above mentioned properties of flavonoids were evaluated by in silico methods with lipoxygenases-an enzyme related to oxidation of various fatty acids and Ras proteins which is a member of a super family of small GTPase involved in cell growth. The 5-lipoxygenase protein enzyme (5LO) and its leukotriene metabolites have long been known to be important modulators of inflammation in other disease states [5]. The ras oncogene p21 antigen (p21) has been identified in several epithelial malignancies, including breast, colon, bladder, and prostate [6].

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2. Materials and methods

2.1. Inoculum preparation

2.1.1. Phototrophic culture

Chlorella pyrenoidosa (NCIM NO: 2738) was obtained from National Centre for Industrial Microorganisms (NCIM), Pune, India. *C. pyrenoidosa* is used for culturing in phototrophic mode by using BG11 as medium [7]. 800 ml of media was taken in four different conical flasks and 10% of inoculum was added to each flask. The cultures were grown for one week at room temperature under continuous light illumination of 55 μmol $m^{-2}s^{-1}$. The prevailing conditions like oxygen supply and carbon dioxide supply were monitored by providing air continuously at 2 l min^{-1} and CO_2 for 10 min daily.

2.1.2. Heterotrophic culture

100 ml of the phototropic culture is taken as the inoculum for growing the heterotrophic culture of *Chlorella pyrenoidosa.* It is added to one liter of modified BG11 medium with glucose as carbon source in absence of light. It was grown in dark for one week by providing aeration in a controlled manner 2 l min^{-1}. Sub culturing of the same was done again to obtain pure heterotrophic culture of *C. pyrenoidosa.*

2.2. Sample preparation for HPLC analysis from phototrophic and heterotrophic cultures

An accurately weighed 2 g of dried biomass obtained from phototrophic and heterotrophic cultures of *C. pyrenoidosa* were taken each and the samples were extracted with 2 ml of hexane for 30 min at 20 °C temperature. The tubes were centrifuged at 4500 g for 10 min and the supernatant was recovered. The extraction was repeated with 2 ml of hexane and the supernatants were collected [8]. The remaining residue was subsequently extracted twice with ethyl acetate of 2 ml for 30 min at 20 °C temperature and the supernatants were again collected. Subsequently, the residues were further extracted twice with water 2 ml each time for 30 min at 80 °C and the supernatants were combined. The hexane, ethyl acetate and together with aqueous extracts were all stored at −10 °C before using them for biochemical analysis and HPLC analysis.

2.3. Biochemical analysis

Both phototrophic and heterotrophic biomass of C. *pyrenoidosa* was used individually for performing biochemical analysis to test for the presence of flavonoids.

2.3.1. Test for flavonoids

5 ml of dilute ammonia solution was added to a portion of the hexane, ethyl acetate and aqueous extracts of both photo and heterotrophic samples followed by addition of concentrated H_2SO_4. A yellow color in each extract indicated the presence of flavonoids. The yellow color disappears on standing. Few drops of 1% aluminum solution were added to portion of each extract filtrate.

2.3.2. Determination of total phenolic compounds

According to Slinkard and Singleton [9], total soluble phenolic compounds were determined with Folin-Ciocalteu reagent using pyrocatechol as a standard phenolic compound. Briefly, 1 ml of the extract (1 mg/ml) in a volumetric flask diluted with 46 ml distilled water. One milliliter of Folin-Ciocalteu reagent was added and the content was thoroughly mixed. After 3 min, 3 ml of sodium carbonate (2%) was added and then was allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm in UV–vis spectrophotometer [Elico SL-210]. The total concentration of phenolic compounds in the extract determined as microgram of pyrocatechol equivalent (PE) per milligram of dry extract.

2.3.3. Total flavonoids content

Dowd method [10] was used to estimate the total flavonoids content. Briefly, two milliliters of 2% aluminum trichloride ($AlCl_3$) in methanol was mixed with the same volume of the extract solution (1 mg/ml). The mixture was incubated at room temperature for 10 min, and the absorbance was measured at 415 nm in spectrophotometer. The total flavonoids content determined as microgram of rutin equivalent (RE) per milligram of dry extract.

2.4. HPLC analysis

The hexane, ethyl acetate and aqueous extracts of both phototrophic and heterotrophic C. *pyrenoidosa* were subjected to HPLC analysis [Shimadzu LC-10AT vp] using PDA as detector with wavelength 270 nm using RP C18 as column in an isocratic manner with HPLC grade methanol: water in the ratio 90:10 as solvents at 30 °C. The flow rate was adjusted to 1 ml/min with sample injection volume 10 μl. The run time was set for 30min. The obtained results were used to carry out in silico studies to assess the anti-oxidant and anti-proliferation capacity of *C. pyrenoidosa*.

2.5. In silico analysis

2.5.1. Retrieval of protein structure

The X ray crystal structure of proteins 3V99 (5-lipoxygenase) at 2.25 Aº resolution and 4L9S (Signaling protein) at 1.61 Aº resolution used in this study were retrieved from RCSB Protein Data Bank [11]. They play a major role in inflammation and proliferation pathways respectively. The proteins were prepared for molecular docking by removing the water molecules and other hetero molecules from the original crystal structure. Active site analysis was performed using Swiss Protein Viewer, SPDBV [12].

2.5.2. Retrieval of ligands and ADME property prediction

3D structures of flavonoids that were identified from HPLC analysis of *Chlorella pyrenoidosa* were retrieved from NCBI Pub Chem Compounds in SDF format [13]. 2D structures were sketched using Chemspider and Molinspiration. The names and CID numbers of the compounds are Caffeine (CID: 2519), Protocatechic acid (CID: 528594), Catechin (CID: 73160), Epicatechin (CID: 72276),Epigallocatechin –gallate (CID: 65064), Caffeoyl-D-glucose (CID: 129661118), Dihydroquercetin-7, 4′-dimethyl ether (3D structure was generated using molinspiration). ADME [14] properties (i.e., absorption, distribution, metabolism and excretion) of the selected compounds was predicted by Molinspiration.

2.5.3. Grid preparation and molecular docking

Molecular Docking was performed using Autodock Vina [15] and MGL tools [16and17]. Docking Input files were created using AutoDock tools batch file of MGL tools. Docking was performed between selected macromolecules and ligands. Hydrogen atoms, Kollman charges were added to protein molecules and prepared as PDBQT files. The ligand was prepared in PDBQT by setting flexible torsion angles at all rotatable bonds, while the protein was kept as a rigid structure. The Lamarckian Genetic Algorithm (LGA), a local search algorithm was utilized for ligands conformations searching.

Configuration files were created for both the proteins by setting suitable Cartesian coordinates to generate Grid box. For protein 4L9S grid box parameters are X = 36.212, Y = −11.524, & Z = 5.085 and grid box dimensions was set at 60*60*60 Aº which covers all the amino acids in the active site. For protein 3V99 coordinates for X, Y, and Z axis were 11.446, −73.596, −24.378 and dimensions for grid box are 70*70*70 Aº.

The docked complex forming hydrogen bonds and other parameters like intermolecular energy (Kcal/mol) and inhibition constant (μM) were analyzed by Autodock tool. Ten best poses were generated for each ligand and scored using Autodock Vina scoring functions. Based on the docked energy all the ligands were ranked. The interacting residues with