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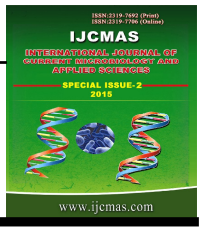

Original Research Article

Extraction, Detection and Identification of Flavonoids from Microalgae: An Emerging Secondary Metabolite

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A B S T R A C T

Wide ranges of secondary metabolites are produced by photoautotrophic micro algae and have different biological actions. With the increasing demand of natural products as anti aging factors in pharmaceuticals, neutraceuticals and cosmetic industries Flavonoids are gaining vital importance. Flavonoids with their multiple activities viz. Anti-microbial, Anti-cancer, and Anti-diabetic can serve to be of significance. In this work, microalgae were isolated from pond water and rice fields of Nasik. The medium was supplemented with L-phenylalanine for high yield of flavonoids. Extracted flavonoids were detected using $AlCl_3$ test and Shinoda test, while identification of flavonoids was done using TLC and concentration was determined spectrophotometrically. The flavonoid from the given sample was also subjected to antioxidant activity, antimicrobial activity, silver nanoparticle synthesis and immobilization. Extracted flavonoids were found to be 70µg/ml and 120µg/ml respectively from pond water and rice fields. The flavonoid was found to be Quercetin. Antioxidant activity of flavonoid was 88.9%. Flavonoids successfully synthesized silver nanoparticles with absorption maxima at 430 nm. Flavonoids were encapsulated using β- cyclodextrin for storage. This work with current science will evaluate the need of concern of the health risk which are increasing day by day and will be a solution to it.

Keywords

Flavonoids, Microalgae, Antioxidant activity, Quercetin, Encapsulation

Introduction

Micro algae are naturally present in ponds, lakes and streams, and have high potential for production of novel bioactive compounds, out of which flavonoids are of great interest in drug discovery. Bacterial diseases and cancer are leading to high mortality rates in India and worldwide. Flavonoids are secondary metabolites and have capacity to act as anti-oxidant, anti-bacterial, anti-inflammatory and anti-cancer agent. Shashank and Abhay (2013). They have been reported as potent candidates to scavenge free radicals which are harmful to cell of human body and food products (Hanaa *et al.*, 2009). They protect cell from premature aging by shielding proteins, lipids

and DNA from oxidative damage and are safe being natural in origin (Ann and Zigang, 2013). There are chemical as well as physical means of synthesis of metal nanoparticles leading to environmental pollution (Anuradha *et al.,* 2014). Flavonoids can be proved as alternative for synthesis of silver nanoparticles by biological means.

Upcoming research suggests that flavonoids are associated with health benefits and widespread belief as harmless for consumption. Flavonoids are absorbed only upto 5% in the human body and are metabolized very fast (Kevin *et al.,* 2012). So they are immobilized with cyclodextrin which increases its stability in circulation. L-phenylalanine can enhance production of flavonoids, from *Lyngbya* spp and *Oscillatoria* spp and high level of antioxidant activity with ability to synthesize nanoparticles.

Materials and Methods

Chemicals and instruments

All chemicals and materials were of analytical grade and were purchased from Himedia Company (Mumbai) and L-phenylalanine was purchased from Qualigens**.** Light binocular microscopic (Labomade), UV-VIS Spectrophotometer (Chemito), and Kjeldal apparatus were used.

Sample collection and biomass generation

Sample for Microalgae isolation was collected from two sources pond water from Botanical garden of H.P.T Arts and R.Y.K Science College, and Rice field of Village Aadgaon in Nashik. The samples were cultivated in Medium-7 (Composition: $NaHCO_3$- 15.0 gm, KH_2PO_4- 0.5 gm, $NaNO_3$- 2.0 gm, K_2SO_4- 1.0 gm, NaCl- 1.0 gm, $MgSO_4.7H_2O$- 0.1 gm, $CaCl_2.2H_2O$- 0.04 gm, $FeSO_4.7H_2O$- 0.04 gm, Distilled water- 1000 ml). It was kept in sunlight for 60 days for biomass production under continuous illumination without agitation (Kumar *et al.,* 2013; Azza *et al.,* 2013). For enhanced production of flavonoid from microalgae 100mg/L of L-phenylalanine was added to the medium and was kept for 14 days for increase in flavonoid content (Hanaa *et al.,* 2009).

Identification and Extraction of flavonoids

Identification was done by light binocular microscopic examination (Labomade). Extraction of flavonoids was done using two methods:

A) 1gm of algal biomass was added to 5ml of distilled water which was centrifuged (Remi, India) at1000 rpm for 15 mins. The pellet was resuspended in distilled water and was incubated at 4ºC for 20 mins. Then it was transferred to water bath adjusted at 100ºC for 20 mins. The biomass was crushed using glass wool in motor and pestle and the extracts were added to 5ml of methanol and were incubated at room temperature for 48 hours. The supernatant was considered for determination of flavonoid content (Hanaa *et al.,* 2009).

B) Soxhlet apparatus- 1gm of algal biomass was mixed with 5ml of methanol and reflux condenser was run for 3 hours, the filtrate was considered for determination of flavonoid content (Massoumeh *et al.,* 2014)

Quantitative determination using $AlCl_3$ Test

To 20µl of algal extract 20µl 10% $AlCl_3$ and 20µl 1M potassium acetate with 180µl of distilled water was added and was kept at room temperature for 30mins. Optical density was read at 415nm against blank

(Massoumeh *et al.,* 2014). The calibration curve was made from standard Quercetin prepared in methanolic extracts.

Identification of flavonoid using thin layer chromatography

Thin layer chromatography was performed on 0.25mm silica gel, solvent system used was n-Hexane: Ethyl acetate: Acetic acid (31:14:5). The sheets were air dried and Developed by spraying $AlCl_3$ reagent and were visualized under UV light for fluorescence (Marica *et al.,* 2004).

Antioxidant assay: hydrogen peroxide scavenging activity

2mM H_2O_2 solution was prepared in 50mM phosphate buffer (pH- 7.4). Aliquots (0.1ml) of different fractions were transferred into the test tubes and their volumes were made up to 0.4 ml with Phosphate buffer. After addition of 0.6 ml H_2O_2 solution, tubes were vortexed and absorbance of H_2O_2 at 560nm was determined after 10 mins against a blank (Kannan *et al.,* 2014)

The abilities to scavenge the H_2O_2 were calculated using the equation - H_2O_2 Scavenging activity= [(1- absorbance of control)/absorbance of sample)] x 100

Abs control was H_2O_2 without extract and abs sample was in the presence of extract. (Azza *et al.,* 2014)

Antimicrobial activity

The antimicrobial activity of crude extract was studied on two organisms viz., *Salmonella typhi* and *Bacillus subtilis.* Standardized inoculum of $1x10^7$ cfu/ml was added uniformly to the Luria Burtinii agar plates (Jaya *et al.,* 2007) and 0.1ml crude extracts were added to the wells with Methanol as control and Quercitin as standard. The plates were incubated at 37ºC for 24 hours and the zone of inhibition was measured after 24 hours of incubation. Simic *et al.* (2012)

Synthesis of silver nanoparticles

10^{-3} mM solution of $AgNO_3$ as a metal salt precursor was added to 1mM solution of standard Quercetin and was kept for incubation at room temperature for 24 hrs, to observe color change and after 24 hrs it was subjected UV-VIS Spectrophotometer for absorption spectra with 20 nm resolution.

Encapsulation of flavonoids

Solution of β-cyclodextrin was heated at 50°C and was kept under shaking condition until complete solubilization was seen. Flavonoid containing crude extract was dispersed in β-cyclodextrin aqueous solution in suitable proportion.

The system was stirred for 3 hours at 50°C at R.T and was stored overnight at 3°C to promote precipitation of the complexes. O.D was taken at 420nm by performing $AlCl_3$ test before and after addition of β-cyclodextrin solution. Then the solvent was evaporated at 40^0C and the precipitate was collected.

Results and Discussion

Identification and Production of biomass:

Biomass production was seen within 60 days of incubation (Fig. 1). The algal cells were observed under 40X and were compared with standard photographs for identification.

The isolated microalgae belong to *Oscillatoria* species (Fig. 2) from botanical garden sample and *Lyngbya species* (Fig. 3) from rice field sample.

Enhancement in flavonoid production

There was increase in flavonoid content in both the samples after addition of 100mg/L of L-phenylalanine (Table1).

Extraction and quantitative determination of flavonoids

Extraction using Soxhlet apparatus yielded higher concentration of flavonoids i.e. 110 µg/ml and 120 µg/ml from Botanical garden and Rice field isolates as compared to chemical extraction method (Table 2) (Graph 1).

Identification of flavonoids using TLC

After spraying the sheet using $AlCl_3$ reagent, yellow fluorescence was observed under U.V. light. The RF value obtained for Botanical garden sample was 0.33 and that for Rice field sample was 0.30.The RF value of standard was 0.30 (Fig. 4).

Antioxidant activity of flavonoid

The antioxidant activity of standard Quercitin was 82.48%. The antioxidant activity of flavonoids extracted from botanical garden sample using Soxhlet apparatus was 85.18% and that using chemical extraction was 81.81%.

The antioxidant activity of flavonoid extracted from rice field sample using Soxhlet apparatus was 88.67% and that of chemical extraction was 87.96% (Graph 2).

Antimicrobial activity of flavonoids

The antimicrobial activity of extracted flavonoids was tested on two pathogenic micro-organisms viz. *Salmonella typhi* and *Bacillus subtilis* (Table 3).

Synthesis of silver nano particles

Formation of brown color indicates formation of silver nanoparticles (Fig. 5). Spectral scan shows higher optical density at 430 nm indicates presence of silver nanoparticles (Fig. 6).

Encapsulation of flavonoids using β-cyclodextrin

The extracted flavonoids were encapsulated using β- cyclodextrin. The confirmation of encapsulation was done by estimating the concentration of flavonoids in reaction mixture before the addition of cyclodextrin and after the addition of cyclodextrin. Decrease in optical density shows successful encapsulation of flavonoids (Table 4).

According to research Microalgae are large reserves of bioactive compounds including flavonoids. Due to increase in resistance to antibiotics Flavonoids have been of vital significance to mankind in recent years. They have been reported to be strong active contributors to the health benefits of humans for use in beverages such as tea and wine, foods such as fruit and vegetables, and even, recently, chocolate. Their proven effectiveness as antioxidants and free radical scavengers (Bose *et al.,* 2010) to their metal complexing capabilities, and to their ability to bind with a high degree of specificity to proteins. Study is carried out on production of flavonoids (Hanaa *et al.,* 2009) along with microbial and enzymatic transformations of flavonoids different designs have been made for production of algal biomass which are made of transparent tubes, sleeves or containers and where light source may be natural or artificial (Daniel, 1993) where in this work biomass production was done in plastic trays with source of illumination, extraction was done using methanol and Kjeldhal apparatus.

Table.1 Enhancement of flavonoid content

Sample	Initial flavonoid content (µg/ml of crude)	Final flavonoid content (µg/ml of crude)
Oscillatoriaspecies	20	66
Lyngbya species	42	110

Table.2 Comparison between the extractions techniques used for flavonoids

Extraction technique	Water sample	
	Botanical garden water (µg/ml of crude)	Rice field water (µg/ml of crude)
Chemical extraction	66	110
Using Soxhlet apparatus	70	120

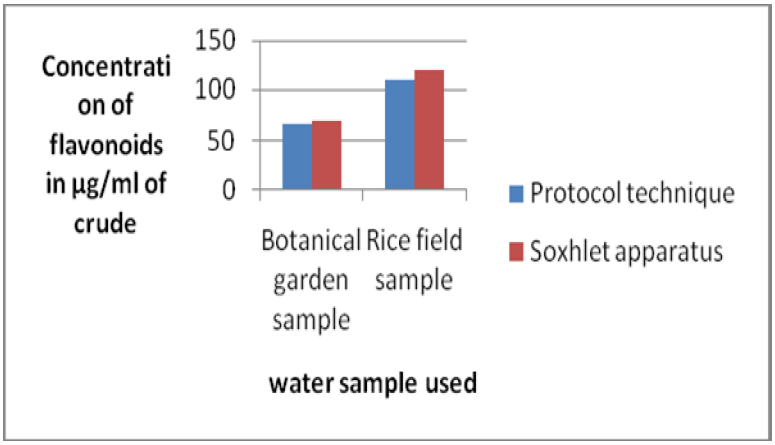
Table.3 Antimicrobial activity of extracted flavonoids

Antimicrobial agent	Zone of inhibition for *S.typhi (*mm)	Zone of inhibition for *B.subtilis*(mm)
Standard Quercitin	0.5	0.6
Methanol	4	4
Botanical garden (c)	0.7	0.8
Rice field(c)	0.9	0.5
Botanical garden(s)	0.6	0.7
Rice field(s)	0.9	0.1

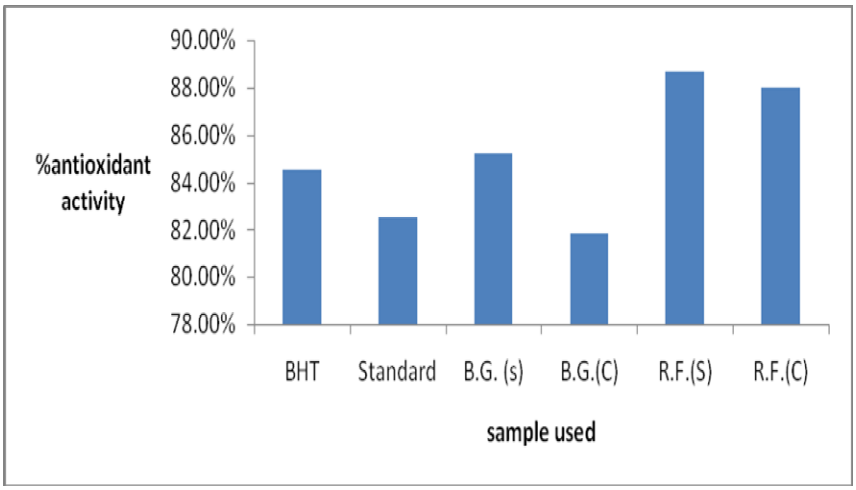
Table.4 Comparison between samples before and after addition of β-cyclodextrin

Sample	O.D. at 420 nm	
	Initial	Final
Standard	0.56	0.051
Sample	0.25	0.014

Graph.1 Quantitative determination of flavonoids



Graph.2 Antioxidant activity of extracted flavonoids, BHT: Butylated Hydroxytoluene, (B.G.(S):Botanical garden extract using Soxhlet apparatus, B.G.(C):Botanical garden extract by chemical technique, R.F.(S):Rice field extract using Soxhlet apparatus, R.F.(C):Rice field extract using chemical technique)



Attempts have been also made to genetically engineer microbial strains for large scale production of flavonoids Joseph *et al.* (2006) where in this work media optimization was done by using L-phenylalanine for high yield of flavonoids. Detection was done using thin layer chromatography and estimation was carried out using UV-VIS spectrophotometer.

Work is done on different cell lines to check the antioxidant and anticancer activity (Frank, 2013). Analytical study of the determination of flavonoids in Black Sea algae is carried out marine algae can be used for various purposes, such as: in agriculture, in pharmaceutical and food industry, as they are rich in organic substances, sugars and active principles Constanta (2010). The micro algae from rice field isolates and pond water are potent producers of flavonoids and have ability to synthesize silver nanoparticles along with anti-bacterial, anti-oxidant activity.

This work is need based looking at the demand of the market as well as human health related products

We have abundant source of flavonoids from microalgae and can be exploited as anti bacterial, anticancer and antiviral agent at industrial level. Microalgae were successfully isolated and identified. The Overall findings in this study indicate that flavonoids of *Oscillatoria and Lyngbya* spp have antioxidant and antimicrobial activity that can be further investigated as a potent drug for Pharmaceuticals and Neutraceuticals. Different extraction methods could affect the yield of total flavonoids. Flavonoids can synthesize Silver nanoparticles and can be exploited for various applications. Flavonoids immobilized with cyclodextrin can prove as more stable complex than flavonoids.

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