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Differential solvent extraction of two seaweeds and their efficacy in controlling *Aeromonas salmonicida* infection in *Oreochromis mossambicus*: A novel therapeutic approach

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ABSTRACT

The present study uses two differential extraction methods (sequential, maceration) to identify bioactive compounds present in two seaweeds *Gracilaria folifera* and *Sargassum longifolium*. Five different solvents namely Petroleum ether, Dichloromethane, Chloroform, ethanol and water were used. The bioactive compounds present in the extracts showed antioxidant and antibacterial activities against *Aeromonas salmonicida*. Ethanol and aqueous extracts of these seaweeds were found to have better antibacterial activity against bacterial diseases in fish when compared to the other extracts. Toxicological and in vivo pathogenicity studies were performed with the highest concentration of 250 mg/l of extracts. The compounds identified by GC–MS which were found to have antibacterial activity were prepared in the form of a colloidal suspension for oral treatment. The use of algal extracts in aquaculture disease management is a cost effective and eco-friendly approach. These extracts could be used as prophylactics.

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1. Introduction

Free radicals are often formed due to the oxidation of molecules. These radicals are responsible for causing many diseases and cell damage. Protection from the oxidation of molecules and formation of free radicals are controlled by the uptake of antioxidant substances or molecules. Antioxidant compounds scavenge free radicals such as hydrogen peroxide and superoxide anion from the body and prevent the damage caused by them (Thanigaivel et al., 2014). Antioxidants are broadly classified into two classes as enzymatic and non-enzymatic. The enzymatic antioxidants include superoxide dismutase, catalase, and glutathione peroxidase which are produced endogenously. The non-enzymatic antioxidants include carotenoids, ascorbic acid, flavonoids and tannins which are obtained from natural plant sources and are helpful in the recovery of various disorders (Lee et al., 2004). Antiviral, antifungal, antimicrobial, antimalarial, hypoglycemic and antifertility activities have been detected in Rhodophyta, Phaeophyta and Chlorophyta (Cox et al., 2010; Kolanjinathan et al., 2009) The use of antioxidant molecules and their derivatives for the prevention of human and animal diseases has increased. The commercialization and consumption of the products could be increased by combining the antioxidant rich substances or natural products for the efficient release of the anti-oxidant activity to give long term benefits to the consumers. A novel method of combining the natural products having active biocompounds with potent antibacterial and antioxidant activity was adopted for studying the feasibility of its application in aquaculture to control or prevent diseases. Disease outbreaks are frequently associated with fish fitness and health. Most pathogens are opportunistic and take advantage of immunocompromised or stressed fish. Therefore the aim of alternate solutions should be to maximize fish immunity and fitness to avoid pathogenic infections. Some of the proposed solutions are the use of natural products (plant extracts) or probiotics (beneficial microbial strains) in the culture of fish and shrimp. Therefore, limiting the use of chemical products and increasing the use of natural products in aquaculture could enhance the consumption of these products and decrease the use of chemical products (Ashley 2007; Mohapatra et al., 2013; Reverter et al. 2014).

2. Materials and methods

2.1. Reagents and chemicals

Ascorbic acid, Folin–Ciocalteu reagent, Gallic acid, Quercetin, EDTA, ethanol, Dichloromethane, Petroleum ether, Chloroform, sodium carbonate, potassium acetate, Aluminium chloride, sodium phosphate, Sulfuric acid, Ammonium molybdate, TCA, Ferric Chloride, potassium ferricyanide, Ferric chloride, Ferrozine, hydrogen peroxide, Phosphate Buffer, Ferrous sulfate, sodium salicylate, DPPH, ABTS and APS were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All chemicals used were of analytical grade.

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2.2. Collection and maintenance of experimental animals

Healthy fingerlings of tilapia, *Oreochromis mossambicus* were purchased from a farm. They were transported in live condition in 20 lit containers containing freshwater with continuous battery-powered aeration. They were maintained under lab condition in 1000 lit fiber glass tanks containing fresh water with continuous aeration.

2.3. Collection of seaweeds

Brown seaweeds *Gracilaria folifera* and *Sargassum longifolium* were collected from Mandapam coastal area. Seaweeds were identified in the Central Salt and Marine Chemicals Research Institute (CSMCRI), Mandapam Camp, Rameshwaram, India. The seaweeds were washed in running tap water to remove unwanted impurities, adhering sand particles and epiphytes. They were transported to the laboratory in polythene bags. Samples were then washed thoroughly using tap water to remove salt on the surface of the sample. The samples were then shade dried for 72 h. The dried samples were kept in sealed bags in a dry and cool place to prevent it from deteriorating (Zakaria et al., 2011). The dried samples were cut into small pieces and ground into fine powder using a dry grinder. The ground samples were sieved to get uniform particle size. They were transfered to an air-tight container and stored in a freezer (−20 °C).

2.4. Preparation of extract

The ground seaweed samples were extracted with five different solvents namely Petroleum ether, Dichloromethane, Chloroform, ethanol and water by two different methods, maceration and sequential. Standard protocols described by Zakaria et al. (2011) and Vijayabaskar and Shiyamala (2012) were followed while using these methods. All the extracts were then dried under reduced pressure using a rotary evaporator. The dried samples were then scraped out for further analysis which was carried out by mixing the dry powders with the appropriate solvents. The dried yields of the seaweed extracts obtained from the differential extraction method were calculated to determine the dry weight of the sample (Heo et al., 2005).

2.5. Antioxidant and radical scavenging assays

2.5.1. Total phenolic content

The concentration of phenolic compound was measured by the Folin–Ciocalteu method. The assay was carried out following the slightly modified method of Thanigaivel et al. (2014) and Boonchum et al. (2011). 10 μl of the five different extracts (Petroleum ether, Dichloromethane, Chloroform, ethanol and aqueous extracts) of the two seaweeds were mixed with 20 μl of Folin–Ciocalteu reagent and 50 μl of sodium carbonate. Then the sample volume was made up to 1000 μl by the addition of distilled water and incubated under dark condition for 1 h. The absorbance was measured at 725 nm using a Bio spectrophotometer. Gallic acid was used as a standard, and the phenolic content was expressed as gallic acid equivalents in mg/g of dried seaweed (Boonchum et al., 2011).

2.5.2. Determination of total flavonoid content

Total flavonoid content was determined by mixing 25 μl of each extract with 700 μl of double distilled water, 350 μl of ethanol, 25 μl of Aluminium chloride, and 25 μl 25 mM of potassium acetate. It was incubated in the dark for 30 min. Then the absorbance was measured at 415 nm against a blank. Quercetin was used as standard and results were expressed as mg Quercetin equivalents (QE)/g extracts (Cox et al., 2010).

2.5.3. Determination of total antioxidant activity

The total antioxidant activity of *G. folifera* and *S. longifolium* was evaluated by Phosphomolybdenum method of Raghu et al. (2011). Antioxidant can reduce Mo (IV) to Mo (V) and the green phosphate/Mo (V) compounds which have an absorption peak at 695 nm, are generated subsequently (Bhaigyabati et al., 2011). 10 μl of sample was mixed with 1 ml of reagent solution (10 ml of sodium phosphate +10 ml of Ammonium molybdate + 10 ml of Sulfuric acid) and incubated in a boiling water bath at 98 °C for one and half hours. The absorbance was measured at 675 nm using a Bio photometer. Ascorbic acid was used as a standard. Total antioxidant activity was expressed as the number of equivalents of ascorbic acid in mg/g of extract.

2.5.4. Determination of free radicals scavenging by DPPH method

Free radicals scavenging activity of the extracts were determined using 1, 1-diphenyl-2-picryl hydrazyl (DPPH). DPPH was prepared by adding 0.78 mg in 10 ml of ethanol. 500 μl of this DPPH solution was mixed with 10 μl of extracts and 490 μl of ethanol was added (Bhaigyabati et al., 2011). Quercetin was used as standard. The absorbance was measured at 15 minutes' kinetics using a spectrophotometer at 517 nm. The blank was prepared without the addition of extract. A low absorbance of the reaction mixture indicates higher free radical scavenging activity. The scavenging ability of the DPPH radical was calculated using the following equation.

$$\text{DPPH Scavenged}(\%) = \text{control–test/control} \times 100$$

Where control is the absorbance of the control reaction and test is the absorbance of the sample in the presence of the extracts (Arulpriya et al., 2010).

2.5.5. Determination of reducing power

The reducing power of *G. folifera* and *S. longifolium* was determined according to the modified method of Chang et al. (2007). 10 μl of sample extract was mixed with 515 μl of phosphate buffer and 500 μl of potassium ferricyanide. The mixture was then incubated at 50 °C for 20 min; during this period ferricyanide was reduced to ferrocyanide. Then 500 μl of trichloroacetic acid was added to the mixture, which was then centrifuged at 6000 rpm for 10 min. The supernatant (500 μl) was mixed with 500 μl of distilled water and 100 μl of FeCl3. Absorbance was measured at 700 nm using a bio photometer to determine the amount of ferric ferrocyanide formed.

2.5.6. Determination of antioxidant activity by ABTS radical cation

ABTS (2, 2′-azino-bis3-ethylbenzthiazoline-6-sulfonic) radical cation decolorization assay: The ABTS radical scavenging assay was carried out by the modified method of Re et al. (1999). The ABTS reagent solution was prepared by adding 10 mg of ABTS tablet in 5 ml of distilled water. 0.0022 g of APS (Ammonium Persulfate) was dissolved in 5 ml of distilled water. Both the solutions were mixed and made up to 50 ml by adding distilled water. The ABTS solution was then kept in the dark for 6 h. 10 μl of sample was mixed with 1 ml of ABTS solution and 90 μl of ethanol. The absorbance was measured at 734 nm at 7 minute's kinetics. Trolox was used as standard. 100 μl of ethanol and 1 ml of ABTS mixture were used as reference. Percentage inhibition of the sample was calculated by the following equation

$$\% \text{ Inhibition} = A/A_0 \times 100.$$

'A' expresses the absorbance of sample at 7 minute's kinetics; 'A' express reference (Boonchum et al., 2011).

2.5.7. Determination of metal chelating

The ferrous chelating ability of the fractions was monitored by measuring the formation of the ferrous ion ferrozine complex. The reaction mixture containing 1.0 ml of different concentrations of the fractions

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