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IN-VITRO BIOACTIVITY OF MARINE SEAWEED, *CLADOPHORA RUPESTRIS*

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ABSTRACT

Marine algae are rich source of biologically active compounds. This study was done to check the antioxidant, antimicrobial and antidiabetic activity of *Cladophora rupestris*. In phytochemical analysis, lipid, protein, alkaloid, flavonoids are found to be present. In the antioxidant activity, methanol, ethyl acetate & ethanol extract showed inhibition of 78%, 72% & 63% in 5 mg/ml respectively. Well diffusion method was done to check the antimicrobial property against human and shrimp pathogenic bacteria. MIC test was done to check the inhibition of the pathogenic bacteria at minimum concentration of crude extracts. In α-amylase inhibitory assay, methanol, ethanol and ethyl acetate extract showed highest inhibition of 72%, 65% & 70% at 1000 µg/ml. In α-glucosidase inhibitory assay, methanol and ethyl acetate showed highest inhibition of 67%, 61% and 64% at 1000 µg/ml.

KEYWORDS: ***Cladophora rupestris*, Antioxidant, α- amylase, α- glucosidase**

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INTRODUCTION

Seaweeds are one of the marine living resources and excellent source of vitamin A, B, B_{12}, C, D & E as well as the minerals such as Ca, P, Na and K. The chemical composition of the algae varies with species, physiological status and environmental conditions. There are large numbers of different types of antimicrobial compounds that play an important role in natural defence of all kinds of living organisms [1] . Marine organisms are considered as good candidate as an alternative source of bioactive compounds. Che (2004) surveyed occurance and presence of organic compounds from marine organisms that possessed antiviral properties and antiviral activity [2]. Diabetes mellitus is a chronic disease. It occurs when pancreas is unable to produce enough insulin or the body cannot utilize the insulin it produces which results in the increase concentration of glucose in the blood [3]. It is considered as one of the major health problem all over the world. It affects about 171 million people worldwide in 2000 and the number is raised to be increased to atleast 366 million by 2030 [4]. Current therapies and medicines were used in the treatment of diabetes contain several side effects. The presently discovered drugs would not least progression of the disease [5]. Moreover several antioxidants for diabetics have carcinogenic effects [6]. As a result there is necessary to search a better antidiabetic agent. One therapeutic approach for treating the diabetes is to decrease the postprandial hyperglycemia. The absorption is done by suppress the absorption of glucose through the inhibition of carbohydrate hydrolyzing enzymes, α-amylase & α- glucosidase in the digestive tract [7]. Inhibitors of these enzymes delay carbohydrates digestion and prolong overall carbohydrate digestion time, causing a reduction in the rate of glucose absorption. Marine organisms are rich source of biologically active metabolites [8]. Various studies suggested that bioactive compounds isolated from marine organisms have anti-cancer, antimicrobial, antifungal, anti-inflammatory and other pharmacological activities. The reports available on the medicinal importance of algae are very few. Therefore this study intends to determine *Cladophora rupestris* extracts, its effects on blood glucose level. Inhibition of α- amylase & α- glucosidase, enzymes involved in the digestion of carbohydrate, can significantly decrease the postprandial increase of blood glucose after a mixed carbohydrate diet therefore can be important strategy in the management of blood glucose level in Type 2 diabetic patients [7]. There are no previous reports of α- amylase & α- glucosidase inhibitory activity of *Cladophora rupestris* extracts. Hence the present study concerned with the GC-MS analysis, phytochemical analysis, antioxidant activity, antimicrobial activity & α- amylase & α- glucosidase enzyme inhibition studies together to determine the anti-diabetic activity of *Cladophora rupestris.*

MATERIALS & METHODS

Porcine pancreatic α-amylase, α-glucosidase from baker's yeast, p-nitrophenyl-a-D-glucopyranoside, dintrosalicyclic acid was purchased from Sigma (India). Butylated hydroxytoluene, 2,2-diphenyl-1-picrylhydrazyl was purchased from Himedia (India).

i. Sample Collection

The algae was collected on January 2013 between 12-2 pm on the rock surface of the sea shore at Kovalam beach, Chennai (12^0 47'3" N 80^0 15'11"E, 35^0c). After collection, the sample was kept in the cold room at -20 ^{0}c until washing and drying.

ii. Extract Preparation

The seaweeds were rinsed with distil water and washed 4-5 times for the complete removal of sand and other particles attached to the seaweed. After washing the seaweeds were kept for drying at room temperature. The dried seaweeds were crushed by an electrical grinder and the obtained powder was

stored at 7^0c until the extraction step. The powder was extracted with methanol, ethanol and ethyl acetate for 4 hours at 55^0c using soxhlet apparatus. The obtained extracts were evaporated under vaccum using a rotary evaporator. Residues were then diluted in 2 ml pure methanol, ethanol & ethyl acetate. The extracts were kept open for the proper evaporation of the solvent and the slurry remains at the bottom [9]. After drying in the room temperature, the samples were kept in the lyophilizer for freeze drying.

iii. Preliminary Phytochemical Analysis

The preliminary phytochemical analysis of the algal extracts was determined. The following tests were performed on the extracts to determine the presence of various phytoconstituents.

Detection of Alkaloids

5 mg of extract was stirred with few ml of dilute hydrochloric acid and filtered. The filtrate was tested with various alkaloid reagents [10].

Mayer's Test

Few ml of filtrate, two drops of Mayer's reagent were added side by side of the test tube. A white or creamy precipitate indicates the test as positive [10].

Wagner's Test

Few ml of filtrate , few drops of Wagner's reagent were added by the side of the test tube. A reddish-brown precipitate confirms the test as positive [11].

Detection of Carbohydrate

5 mg of extract were dissolved in 100 ml of distilled water and filtered. The filtrate were used for various tests [12].

Molish's Test

1 ml of filtrate, two drops of alcoholic solution of α-naphthol were added, mixture is shaked well and add 0.5 ml of concentrated sulphuric acid is added slowly along the sides of the test tube and allowed to stand. Violet ring indicates the presence of carbohydrates.

Fehling's Test

1 ml of filtrate is boiled with 1 ml of Fehling's solution. Red precipitate indicates the presence of carbohydrates.

Detection of Amino acids and Proteins

10 of extract was dissolved in 1 ml of distilled water and filter through Whatmann filter paper and the filtrate is subjected to tests for amino acids and proteins [13].

Ninhydrin Test

Two drops of ninhydrin solution (1 mg ninhydrin in 20 ml acetone) were added to two ml of aqueous filtrate. Purple colour indicates the presence of amino acids.

Biuret Test

2 ml of filtrate is mixed with 1 drop of 2% copper sulphate solution. Add 1 ml of 95% ethanol, followed by potassium hydroxide pellets. Pink colour indicates the presence of proteins [14].

Detection of Phenolic compounds

10 mg of extract was dissolved in 1 ml of distilled water and filtered.

Ferric chloride Test

Few drops of filtrate were mixed with 5% ferric chloride solution. Dark green colour indicates the presence of phenolic compounds [15].

Lead acetate Test

1 ml of filtrate, add 3 ml of 10% lead acetate solution were added. A white precipitate indicates the presence of phenolic compound.

Detection of Oils & Fats

Few drops of 0.5 N alcoholic potassium hydroxide solution were added to 3 mg of extract with a drop of phenolphthalein. The mixture is heated on waterbath for 2 hours. Formation of soap indicates the presence of oils & fats [16].

Detection of Flavonoids

2 mg of extract is mixed 10 ml of ethyl acetate. Boiled in water bath and cooled at room temperature. The layers are separated and the red colour of ammonia was noted [17].

Detection of Saponins

10 mg of extract is mixed with distilled water and made upto 10 ml. The mixture was shaken for 15 minutes. A layer of foam indicates the presence of saponins [16].

Detection of Phytosterols

5 mg of extract is dissolved in 1 ml of acetic anhydride. Add 1-2 drops of concentrated hydrochloric acid are added slowly along the sides of the test tube. A change of colours showed the presence of phytosterols [18].

Detection of Glycosides

5 ml of 50% hydrochloric acid is mixed with 1 mg of extract. The mixture was heated for 5 minutes in waterbath. Add 5 ml of Fehling's solution and boiled for 10 minutes in waterbath. A brick red precipitate indicates the presence of glycosides [17].

Detection of Tannins

2 drops of 5% ferric chloride solution was added to 1 mg of extract. Dark green indicates the presence of Tannins [17].

iv. Antioxidant Assay

The antioxidant activity of the seaweed were studied by their ability to scavenging the free radicles using 2, 2-Diphenyl-1-Picryl hydroxyl (DPPH) reducing power [19].

Preparation of Positive Control:

The synthetic antioxidant Butylated Hydroxy Toluene (BHT) was used positive control at a concentration of 3 mg/10 ml [19].

Preparation of Test Extracts:

1 mg of extract was dissolved in 1 ml of methanol. Mix the solution and make different concentration viz. 1 mg/ml, 2 mg/ml, 3 mg/ml, 4 mg/ml and 5 mg/ml.

Preparation of DPPH (2,2-Diphenyl-1-Picryl Hydrazyl):

11.8 mg of DPPH was dissolved in 100 ml of methanol (0.3 mM concentration). The content was kept in dark condition because DPPH is light sensitive [19].

DPPH Free Radical Scavenging Assay:

The free radical scavenging activity of seaweed extract was measured by DPPH. Percentage inhibition or DPPH scavenging activity was calculated by following expression.

Percentage of scavenging = $[(A_0 – A_1)/ A_0] \times 100$

Where, A_0 = Absorbance of control

A_1 = Absorbance of sample

The samples were kept in the dark for 30 minutes and the optical density was measured at 517 nm where as positive control = 250 µl BHT + 250 µl DPPH + 2.5 ml methanol, control = 250 µl DPPH + 2.5 ml methanol and test sample = 250 µl sample + 250 µl DPPH + 2.5 ml methanol.

v. Antimicrobial Activity

Three shrimp pathogenic bacteria and three human pathogenic bacteria were collected from VIT University laboratory, Tamil Nadu. Thus the antimicrobial activity of the crude extract of *Cladophora rupestris* were determined by measuring the zone of inhibition in the agar well diffusion method. The results were compared with the standard antibiotic Ampicillin.

vi. Minimum Inhibitory Concentration (MIC) Test

Minimum inhibitory concentration of all the bacterial pathogens were determined by inoculating the crude extracts of various concentration viz. 1000 µg/ml, 750 µg/ml, 500 µg/ml and 250 µg/ml along with the pathogens and kept for incubation for 48 hours. The optical density was measured at 560 nm.

vii. In vitro α- amylase activity

250 µl of algal extract and 250 µl of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M Sodium chloride) containing α-amylase solution (0.5 mg/ml) were incubated at 25^0 c for 10 min. 250 µl of 1% starch solution in 0.02 M of sodium phosphate buffer (pH 6.9 with 0.006 M Sodium chloride) was added to each of the tube at a interval of 5 s. The reaction mixtures were then incubated at 25^0 c for 10 min. The reaction was stopped with 500 µl of dinitrosalicyclic acid colours reagent. The test tubes were then incubated in a boiling water bath for 5 min and cooled at room temperature. The reaction mixture was then diluted after adding 5 ml of distilled water and the absorbance was measured at 540 nm [20].

The α-amylase inhibitory activity was expressed in inhibition % and was calculated as follows:

% inhibition = (Control $_{540}$ – Extract $_{540}$ / Control $_{540}$) × 100

viii. In vitro α- glucosidase activity

50 µl of sample and 100 µl of 0.1 M phosphate buffer (pH 6.9) containing α-glucosidase solution (1.0 U/ml) were incubated in 96 well plates at 25^0 c for 10 min. After pre-incubation, 50 µl of 5 mM p-nitrophenyl-a-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added to each well at 5 s interval. The reaction mixtures were incubated at 25^0 c for 5 min. After incubation, absorbance was measured at 405 nm at micro plate reader [20]. The α-glucosidase inhibitory activity was expressed as inhibition % and was calculated as follows:

% inhibition = (Control $_{405}$ – Extract $_{405}$ / Control $_{405}$) × 100

RESULTS AND DISCUSSION

i. Preliminary Phytochemical Analysis

SL. NO	TESTS	METHANOL	ETHANOL	ETHYL ACETATE
1	ALKALOIDS	+	+	+
2	CARBOHYDRATE	+	+	+
3	AMINO ACID & PROTEIN	+	+	+
4	PHENOLIC COMPOUND	+	+	+
5	OILS & FATS	+	+	+
6	FLAVONOIDS	+	+	+
7	SAPONINS	-	-	-
8	PHYTOSTEROLS	-	-	-
9	GLYCOSIDES	-	-	-
10	TANNINS	+	+	+

Note: + (Present), - (Absent)

Table 1
Preliminary phytochemical analysis of Cladophora rupestris

The preliminary Phytochemical Analysis of methanol, ethanol and ethyl acetate extracts showed the presence of Alkaloid, Carbohydrate, Amino acids & proteins, Phenolics, Oils & fats, Flavonoids, Saponins, Phytosterols, Glycosides and Tannins, but in *Cladophora rupestris* Phytosterols, Saponins and Glycosides were absent.

ii. DPPH Scavenging Assay

In the present DPPH study of seaweeds extracts, the concentrations of the crude extracts are 1 mg/ml, 2 mg/ml, 3 mg/ml, 4 mg/ml and 5 mg/ml. Scavenging capacity increase with the increase concentration. DPPH assay showed significant difference when compared with the positive control. The higher the concentration of the extract the more will be the scavenging capacity.

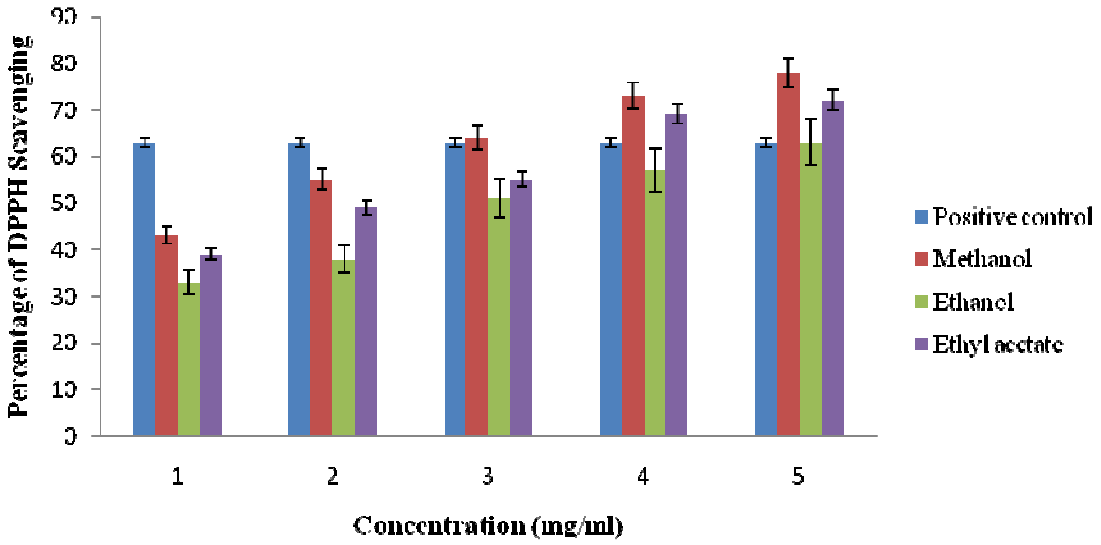

Figure 1
Inhibition percentage of crude extracts and scavenging of DPPH

As shown in fig. 1 the three extracts (methanol, ethanol and ethyl acetate) of *C.rupestris* shows good inhibitory effect in antioxidant activity at all tested concentration. At higher concentration, 5 mg/ml methanol, ethanol and ethyl acetate extract shows highest 78% (±0.22), 63% (±0.29) and 72% (±0.24) inhibitory activity in compared to the positive control 63% (±0.29). In methanol extract, the inhibitory activity shows good result and the inhibition is increased with the increased concentration. The IC_{50} values of methanol, ethanol and ethyl acetate extract was found to be 1.20, 2.75 and 2.25 in mg/ml respectively.

iii. Antimicrobial Activity (Agar diffusion method)

The antimicrobial activity of crude methanol, ethanol and ethyl acetate extract of *Cladophora rupestris* with various concentration viz. 250 µg/ml, 500 µg/ml and 1000 µg/ml against three human pathogenic bacterial strains viz. *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and three shrimp bacterial pathogenic strains *Vibrio harveyii*, *Vibrio parahaemolytical*, *Vibrio alginolyticus* were done to check the antibacterial activity and the zone of inhibition compared with the zone of inhibition of standard antibiotic, Amphicilin.

Pathogenic Bacteria		Zone of inhibition (mm)			
		250 µg/ml	500 µg/ml	1000 µg/ml	Ampicillin
1.	*Escherichia coli*	8.3	13.9	15.9	17.3
2.	*Pseudomonas aeruginosa*	9.1	12.5	15.3	16.9
3.	*Staphylococcus aureus*	10.1	13.3	16.3	18.2
4.	*Vibrio harveyii*	11.3	13.4	15.1	17.5
5.	*Vibrio parahaemolyticus*	9.8	10.4	11.3	15.3
6.	*Vibrio alginolyticus*	7.8	10.9	11.7	16.2

Table 2
Antimicrobial activity of methanol extract against human pathogenic bacteria and shrimp pathogenic bacteria

Pathogenic Bacteria	Zone of inhibition (mm)			
	250 µg/ml	500 µg/ml	1000 µg/ml	Ampicillin
1. *Escherichia coli*	4.5	11.8	12.3	17.1
2. *Pseudomonas aeruginosa*	6.6	10.3	11.9	16.6
3. *Staphylococcus aureus*	7.2	10.1	11.3	17.9
4. *Vibrio harveyii*	10.7	15.7	16.1	17.2
5. *Vibrio parahaemolyticus*	8.3	10.3	11.1	14.9
6. *Vibrio alginolyticus*	7.4	8.3	1.09	11.9

Table 3
Antimicrobial activity of ethanol extract against human pathogenic bacteria and shrimp pathogenic bacteria

Pathogenic Bacteria	Zone of inhibition (mm)			
	250 µg/ml	500 µg/ml	1000 µg/ml	Ampicillin
1. *Escherichia coli*	6.7	11.1	14.4	16.7
2. *Pseudomonas aeruginosa*	10.3	12.3	14.2	16.6
3. *Staphylococcus aureus*	10.6	12.1	13.2	15.5
4. *Vibrio harveyii*	10.1	10.9	11.3	17.8
5. *Vibrio parahaemolyticus*	8.7	12.1	13.3	18.9
6. *Vibrio alginolyticus*	10.5	12.5	14.3	20.6

Table 4
Antimicrobial activity of ethyl acetate extract against human pathogenic bacteria and shrimp pathogenic bacteria

As shown in Table 2, 3 & 4, the methanol, ethanol and ethyl acetate extracts of *Cladophora rupestris* showed good antimicrobial activity the human and the shrimp pathogenic bacteria. But methanol extract & ethyl acetate extract showed zone of inhibition in compared to ethanol extract on the tested pathogens. The diameter of the zone of inhibition was larger in higher concentration of 1000 µg/ml in compared to lower concentration of 250 µg /ml.

iv. Minimum Inhibition Concentration (MIC) Test

The Minimum Inhibitory Test (MIC) with three human pathogens and three shrimp pathogens with the crude methanol, ethanol and ethyl acetate extract to determine the growth of the different pathogens at minimum concentration. At minimum concentration (250 µg/ ml) showed better growth in compared to the highest concentration (1000 µg/ml). Increased concentration of the crude extract inhibited the growth of bacteria.

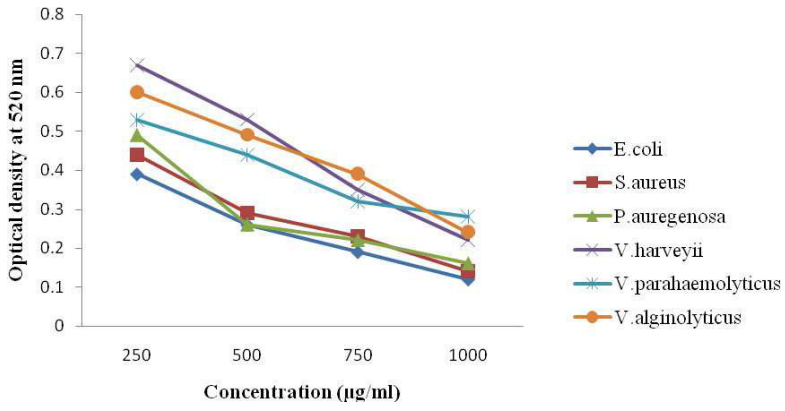

Figure 2
Minimum Inhibitory Concentration (MIC) of Human pathogenic bacteria in compared to Shrimp pathogenic bacteria using methanol extract

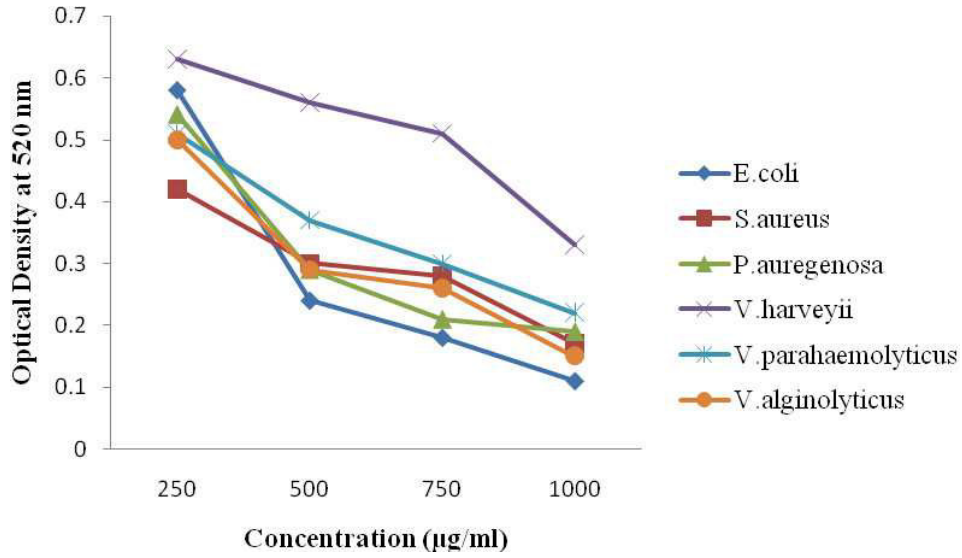

Figure 3
Minimum Inhibitory Concentration (MIC) of Human pathogenic bacteria in compared to Shrimp pathogenic bacteria using ethanol extract

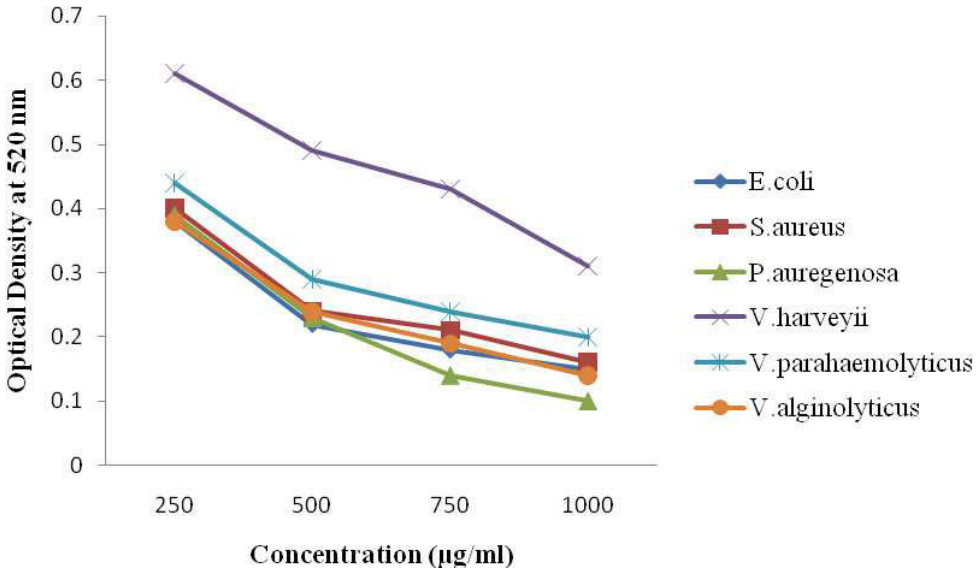

Figure 4
Minimum Inhibitory Concentration (MIC) of Human pathogenic bacteria in compared to Shrimp pathogenic bacteria using ethyl acetate extract

At 1000 µg/ml the growth of all the pathogens were inhibited in compare to other concentrations. But at 500 µg/ ml and 250 µg/ ml showed less inhibition than 1000 µg/ml. Methanol & ethyl acetate extract inhibited the growth of bacteria in higher concentration. The higher the concentration of the algal extract the less will be the growth of all the pathogenic bacteria. At higher concentration the algal extract inhibit the growth of the pathogenic bacteria. Shrimp pathogenic bacteria showed better growth than human pathogenic bacteria. The algal extract better inhibit the human pathogenic bacteria in compared to shrimp pathogenic bacteria. The MIC_{50} value of methanol extract was found for *E.coli* (375),

S.aureus (425), *P.auregenosa* (437), *V.harveyii* (745), *V.parahaemolyticus* (718) & *V.alginolyticus* (763) in µg/ml respectively. The MIC_{50} value of ethanol extract was found for *E.coli* (625), *S.aureus* (537), *P.auregenosa* (537), *V.harveyii* (732), *V.parahaemolyticus* (600) & *V.alginolyticus* (638) in µg/ml respectively. The MIC_{50} value of ethyl acetate extract was found for *E.coli* (325), *S.aureus* (287), *P.auregenosa* (294), *V.harveyii* (475), *V.parahaemolyticus* (437) & *V.alginolyticus* (316) in µg/ml respectively.

v. In vitro α-Amylase Inhibitory Activity

The three extracts of marine seaweeds of *C.rupestris* were assessed for the inhibition of α-Amylase activity in vitro. The concentration of the algal samples were 250 µg/ml, 500 µg/ml, 750 µg/ml and 1000 µg/ml and the inhibition percentage is increased with the increase concentration.

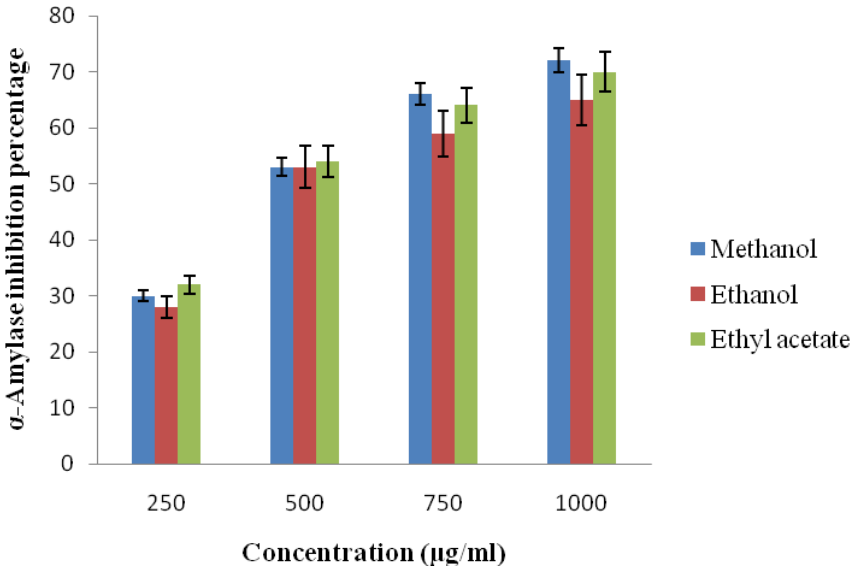

Figure 3
The enzyme inhibitory activity of different extracts of C.rupestris in α-Amylase

As shown in Fig.5, the three crude extract (methanol, ethanol and ethyl acetate) of *C.rupestris* showed good inhibitory α-amylase activity at 1000 µg/ml when compared with other concentration. At higher concentration of 1000 µg/ml the inhibitory effect of methanol extract was 72%. Inhibitory effect of crude extracts decreased in the order of: methanolic extract (72% ± 0.23) > ethyl acetate extract (70% ±0.25) > ethanol extract (65% ±0.29). The IC_{50} values of methanol, ethanol and ethyl acetate extract were found to be 475, 520 and 491 in µg/ml respectively.

vi. In vitro α-Glucosidase inhibitory activity

The three extracts of marine seaweeds of *C.rupestris* were assessed for the inhibition of α-Glucosidase activity in vitro. The concentration of the algal samples were 250 µg/ml, 500 µg/ml, 750 µg/ml and 1000 µg/ml and the inhibition percentage is increased with the increase concentration.

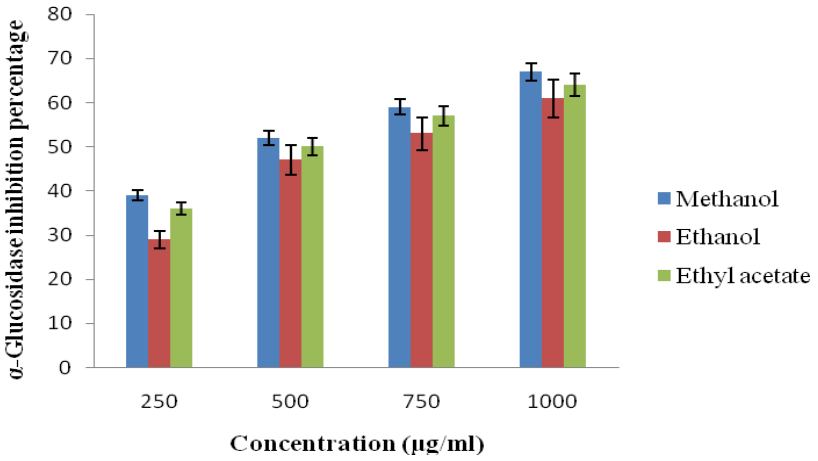

Figure 4
The enzyme inhibitory activity of different extracts of C.rupestris in α-Glucosidase

As shown in Fig.6, the three crude extract (methanol, ethanol and ethyl acetate) of *C.rupestris* showed good inhibitory α-glucosidase activity at 1000 µg/ml when compared with other concentration. At higher concentration of 1000 µg/ml the inhibitory effect of methanol extract was 64%. Inhibitory effect of crude extracts decreased in the order of: methanol extract (64% ±0.24) > ethyl acetate extract (62% ±0.22) > ethanol extract (59% ±0.36). The IC_{50} values of methanol, ethanol and ethyl acetate extract were found to be 478, 512 and 445 in µg/ml respectively.

CONCLUSION

Current therapies were used in the treatment of diabetes contain several side effects. The main aim of this study is to search a better antidiabetic agent for the management of diabetes. The results obtained in the present study determine that the marine seaweeds, *Cladophora rupestri*s had showed good inhibitory results in antioxidant and antidiabetic inhibition assays. Therefore it can be used in therapeutic use for the treatment of diabetes. Further studies are required to find out the mode of action of these crude extracts on enzymes.

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