Journal Of Harmonized Research (JOHR)

Journal Of Harmonized Research in Applied Sciences
3(3), 2015, 165-171

ISSN 2321 – 7456

Original Research Article

IN VITRO ANTIOXIDANT ACTIVITY OF KAPPAPHYCUS ALVAREZII (DOTY) DOTY EX. P .C. SILVA

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Abstract: Antioxidant activity is one of the most studies, due to the interest of compounds both as preservatives and protectors against oxidation in food, cosmetics, and health implications because of its potential and functional group present in it. To find out the impact of solvents on antioxidant activities different solvents like ethanol, methanol, petroleum ether, benzene, ethyl acetate were used for the extraction of compounds from red algae, *Kappaphycus alvarezii.* To find out the anti-oxidant activities, DPPH radical scavenging, ABTS radical scavenging, hydroxyl assay, superoxide dismutase assay and reducing power assay were used to determine antioxidant properties of this seaweed. The anti-oxidant assay was performed at the concentration ranging from 50-800µg/ml. Among the different extracts methanol expressed the best result and benzene solvent showed the poor performance. The present studies confirm that *Kappaphycus alvarezii* received special attention and used as a source of natural antioxidant.

Key words: Antioxidant activity, radical scavenging activity, solvent extracts, ascorbic acid

Introduction: Marine algae are one of the antioxidant sourced food materials which are indicated by the presence of antioxidant activity with organic solvent extract (Santoso *et al*., 2002; Yuan and Walsh 2006; Wong *et al.*, 2009; Cox *et al*, 2010; Zakaria *et al*., 2011; Jimenez-Escrig *et al*., 2001). Marine macro algae are important source of bioactive compounds used in pharmaceutical, cosmetic and food industries (Cardozo *et al*., 2007; Carignan *et al*., 2009; Gressler *et al*., 2010; Gressler *et al*., 2011; Yan *et al*., 1998).

Seaweeds have recently received significant attention for their potential as natural antioxidants. Antioxidant activity of marine algae may arise from carotenoids, tocopherols and polyphenols. These compounds directly or indirectly contribute to inhibition or suppression of free radical generation (Athukorala *et. al*., 2003). Investigation on dimethyl sulphoniopropionate (DSMP) has recently revealed that this compound from marine algae

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Received on: September 2015
Accepted after revision: September 2015
Downloaded from: www.johronline.com

could serve as an effective antioxidant (Ganesan, *et. al*., 2005).

Methanol is a good multifunctional solvent for preliminary extraction (Harborne, 1984), it has been extensively used for marine algal antioxidant extraction (Santoso *et al.*, 2004; Matanjun and Mohamed, 2008; Kumar *et al.*, 2008). Methanol concentration influences the extraction of total phenol content (Chew *et al*., 2008) and the antioxidant activity of the marine algae (Damongilala *et al.*, 2013).

Among natural antioxidants, phenolic antioxidants are in the fore front as they are widely distributed in the plant kingdom. Plants contain diverse group of phenolic compounds, including simple phenolics, phenolic acids, anthocyanins, hydroxycinnamic acid. Polyphenols (phenolic acid, flavonoids, tannis) are widely distributed in seaweeds which are known to exhibit higher antioxidant activities (Cahyana *et al*., 1992). Many natural antioxidants originating from plant sources have been identified as free radical or active oxygen scavengers (Fujimoto and Kaneda, 1984; Vimala and Adenan, 1999; Nagai *et al*., 2003; Nagarani and Kumaraguru 2008).

Kappaphycus alvarezii, formerly termed as *Eucheuma cottonii* (Ro¨nnba¨ck, Bryceson, & Kautsky, 2002; Bryceson, 2002), is a red algae which is cultivated in many tropical countries. It is a popular species for aquaculture, being farmed at places with strong wave action and moderate water current (Prud'homme van Reine & Trono, 2001). Its main product of commercial importance is carrageenan used by human in various industrial products.

Materials and methods

Cultivation and Collection of *Kappaphycus alvarezii:-* The red algae, *Kappaphycus alvarezii* used in this study was cultivated by raft method (Eswaran *et al.*, 2006) in CSIR-CSMCRI, experimental cultivation farm at Thonithurai (Palk Bay, 09° 17.057′ N 079° 10.989′E) Mandapam, Southeast coast of India. After 45 days of growth period the fully grown plants were harvested. The harvested samples were dried under shade at room temperature, pulverized by a mechanical grinder and sieved through 40mm meshes. The sample was preserved at -20^{0}C deep freezer until further use.

DPPH Radical Scavenging (Blois, 1958 and Shen *et al*., 2010)

The DPPH is a stable free radical and is widely used to assess the radical scavenging activity of antioxidant component. This method is based on the reduction of DPPD in methanol solution in the presence of hydrogen donating antioxidant to the formation the non-radical form DPPH-H9 (Shen *et al*., 2010). The free radical scavenging activities of all the extracts were evaluated by 1,-diphenyl-2.-picryl-hydrazyl (DPPH). An 0.1 mm solution of DPPH in methanol was prepared, and 1 ml of this solution was added to 5ml of all extracts in methanol at different concentrations (50, 100, 200. 400 and 1000µg/ml). The mixture were shaken vigorously and allowed to stand at room temperature for 30 minutes. Then the absorbance was measured at 517 nm using a UV-VIS Spectrophotometer (Genesys 10S, USA). Ascorbic acid was used as the reference. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability to scavenging DPPH radical was calculated by using the following formula:

DPPH scavenging effect inhibition

$$= \{(A_0\text{-}A_1/ A_0) *100\}$$

Here, A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged.

Hydroxyl Radical Scavenging Activity (Halliwell *et al.*, 2000): The scavenging capacity of hydroxyl radical was measured according to the standard method. Stock solutions of EDTA (Ethylene diamine tetraacetic acid) (1mm), $FeCl_3$ (10mm), ascorbic acid (1mm), H_2O_2 (10mm) and Deoxyribose (10mm), were prepared in deionised water.

The assay was performed by adding 0.1ml EDTA, 0.01ml of $FeCl_3$, 0.1 ml H_2O_2), 0.36 ml of deoxyribose, 1.0 ml of the extract in different concentration (50, 100, 200, 400, and 800 µg/ml) dissolved in deionised water, 0.33 ml of

phosphate buffer (50 mm, pH 7.4), 0.1 ml of ascorbic acid in sequence. The mixture was then incubated at 37^0 C for 1 hour. 1.0 ml portion of the incubated mixture was mixed with 1.0ml of 10% trichloro acetic acid (TCA) and 1.0ml of 0.5% TBA (in 0.025M NaOH containing 0.025% BHA) to develop the pink chromogen measured at 532 nm. The hydroxyl radical scavenging activity of the extract is reported as % inhibition of deoxyribose degradation is calculated by using the following equation.

Hydroxyl radical scavenging activity

$$= \{(A_0 - A_1)/ (A_0)*100\}$$

Where, A_0 is the absorbance of the control reaction, and A_1 is the absorbance in the presence of all of the extract samples and reference. All the tests were performed in triplicate and the result were averaged.

Superoxide Radical Scavenging Activity (Srinivasan *et al*., 2007):- The superoxide anion radicals were generated in 3.0ml of Tris – HCL buffer (16mM, pH 8.0), contain 0.5ml of NBT (Nitro Blue Tetrazolium) (0.3mm), 0.5ml NADH (0.936mM) solution, 1.0ml extract of different concentration (5, 100, 200, 400 and 800µg/ml), and 0.5 ml Tris – buffer (16mM, pH 8.0). The reaction was started by adding 0.5ml PMS (Phenazine methosulphate) solution (0.12mM) to the mixture, incubated at 25^0C for 5 minutes and the absorbance was measured at 560 nm against a blank sample, ascorbic acid. The percentage inhibition was calculated by using the following equation.

Superoxide radical scavenging activity

$$= \{(A_0 - A_1)/ (A_0)*100\}$$

Where, A_0 is the absorbance of the control reaction, and A_1 is the absorbance in the presence of all of the extract samples and reference. The entire tests were performed in triplicate and the results were averaged.

Antioxidant Activity by Radical Cation ($ABTS^+$) (Huang *et al*., 2011): ABTS assay was based on the slightly modified method of Huang *et al*., (2011). ABTS radical cation ($ABTS^+$) was produced by reacting 7mm ABTS (2, 2'-azinobis-3-ethylbenothiazoline-6-sulphonic acid) solution with 2.45 mm potassium per sulphate and allowing the mixture to stand in the dark at room temperature for 12-16 hours before use. The $ABTS^+$ solution were diluted with ethanol to an absorbance of 0.70+ 0.02 at 734 nm. After addition of 100 µl of sample or trolox standard to 3.9 ml of diluted $ABTS^+$ solution, absorbance was measured exactly after 6 minutes at 734 nm with Genesys 10S UV-VIS spectrophotometer (USA). Results were expressed as trolox equivalent antioxidant capacity (TEAC).

ABTS radical cation activity

$$= \{(A_0 - A_1)/ (A_0)*100\}$$

Where, A_0 is the absorbance of the control reaction, and A_1 is the absorbance in the presence of all of the extract samples and reference. All the tests were performed in triplicate and the result were averaged.

Reducing Power (Kumar and Hemalatha, 2011): The reducing power of the extract was determined by standard methods. 1.0 ml of solution containing 50, 100, 200, 400 and 800 µg/ml of extract was mixed with sodium buffer (5-0 ml, 0.2 M, pH 6.6) and potassium ferricyanide (5.0 ml 1.0%). The mixture was incubated at 50ºC for 20 minutes. Then 5 ml of 10% trichloroacetic acid was added and centrifuged at 980 x g (10 minutes at 50ºC) in a refrigerator centrifuge. The upper layer of the solution (5.0ml) was diluted with 5.0 ml of distilled water and ferric chloride and absorbance read at 700nm. The experiment was performed thrice and results were averaged.

Statistical Analysis: All the data were expressed as means ± standard deviation (SD). Statistical analysis was calculated by two way ANOVA using the statistika 6 computer software applications. Number of the replicate experiments carried were, n=3. Differences were considered to be statistically significant if $p<0.05$.

Results and Discussion: ABTS (2, 2'-azinobis-3-ethyl benzothiazoline -6- sulfonic acid) assay is an excellent tool for determining the antioxidant activity of phytochemical products (Leong *et al*., 2002). Zhu *et al*., (2002) reported that alcoholic extract of *T. conoides* behave as both primary and secondary antioxidants. ABTS radical scavenging activities of four red

seaweeds were reported by Sachindra *et al*., (2007). The methnolic extract of *K. alvarezii* possessed 116.41 % followed by ethanol as 110.46% (Table.1).. The IC_{50} value of ABTS found high in methanol extract and it was 32.41 µg/ml and the lower value was noted in petroleum ether 28.48.

Table 1. Effect of different solvent extract of *Kappaphycus alvarezii* on ABT assay

Con	Ethanol	Methanol	Petroleum Ether	Benzene	Ethyl Acetate	Ascorbic acid
50	35.67 ± 0.081	37.39 ± 0.081	21.72 ± 0.048	18.14 ± 0.048	31.74 ± 0.026	22.85 ± 0.27
100	48.21 ± 0.086	51.21 ± 0.025	40.17 ± 0.029	30.14 ± 0.021	45.91 ± 0.021	45.88 ± 0.53
200	70.49 ± 0.024	74.71 ± 0.043	49.14 ± 0.072	45.16 ± 0.056	60.21 ± 0.016	64.82 ± 0.67
400	84.14 ± 0.076	91.21 ± 0.031	64.12 ± 0.082	71.44 ± 0.026	90.21 ± 0.076	87.93 ± 0.48
800	110.46 ± 0.021	116.41 ± 0.038	94.34 ± 0.021	90.14 ± 0.04	98.41 ± 0.018	119.46 ± 0.66
F - Value	**310527.76**	**468578.14**	**535866.90**	**1773019.21**	**911498.29**	**407326.53**
P - Value	**8.6×10^{-21}**	**1.6×10^{-21}**	**9.7×10^{-22}**	**8.09×10^{-24}**	**1.15×10^{-22}**	**2.9×10^{-21}**
IC_{50}	**31.46**	**32.41**	**28.48**	**26.61**	**29.81**	**32.03**

Relatively stable organic radical DPPH (Diphenyl Picryl Hydrazyl) has been widely used in the determination of the antioxidant activity of different plant extracts (Yen and Duh, 1994; Brand-Williams *et al*., 1995). DPPH is extensively employed to evaluate the "reducing substance" and to study the functions of damaging activity of as free radical compounds (Kumar *et al*., 2008). Among the different solvent extracts of *K. alvarezii*, the minimum activity was found in benzene. At 800 µg/ml concentration methanolic extract of *K. alvarezii* possessed 128.14 % scavenging activity on DPPH and the standard ascorbic acid acidity for *K. alvarezii* was 119.26%. The IC_{50} value found to high (35.24), in methanol and in benzene it was found to be less (29.16) as shown in Table (2). Reports suggest that methanol extract of *C. rasemosa* and *K. alaverzii* possess high total phenol content and high DPPH radical scavenging activity (Chew *et al*., 2008; Ragan and Glombitza, 1986).

Table 2. Effect of different solvent extract of *Kappaphycus alvarezii* on DPPH assay

Con	Ethanol	Methanol	Petroleum Ether	Benzene	Ethyl Acetate	Ascorbic acid
50	32.64 ± 0.091	37.81 ± 0.021	26.13 ± 0.074	24.15 ± 0.047	27.54 ± 0.072	26.37 ± 0.36
100	49.41 ± 0.094	54.71 ± 0.047	34.15 ± 0.07	34.81 ± 0.028	39.42 ± 0.057	40.69 ± 0.56
200	66.81 ± 0.078	79.46 ± 0.091	66.42 ± 0.014	52.61 ± 0.036	63.61± 0.063	69.72 ± 0.29
400	91.41 ± 0.095	96.24 ± 0.078	79.25 ± 0.076	73.26 ± 0.058	79.14 ± 0.026	99.16 ± 0.57
800	105.47 ± 0.081	128.14 ± 0.071	98.41 ± 0.076	91.21 ± 0.083	93.18 ± 0.041	119.26 ± 0.83
F - Value	**5632.44**	**4838.24**	**2953.20**	**2579.61**	**9709.40**	**17968.12**
P - Value	**7.9×10^{-14}**	**1.45×10^{-13}**	**1.04×10^{-12}**	**1.7×10^{-12}**	**8.9×10^{-15}**	**7.6×10^{-16}**
IC_{50}	**30.89**	**35.24**	**30.81**	**28.43**	**29.16**	**30.34**

The hydroxyl radical is an extremely reactive free radical formed in the biological systems and it is a highly damaging species in free radical pathology, capable of damaging almost every molecule found in the living cells
(Hochestein and Atallah, 1988). Hydroxyl radical scavenging capacity of an extract is directly related to its anti-oxidant activity (19). The methanol extract of *K. alvarezii* found to be 128.18µg/ml followed by ethyl acetate 119.14 µg/ml. The lowest degree is found to be in benzene, 95.14 µg/ml. The IC_{50} value for methanol is 41.24 and for benzene are 28.84, µg/ml and it is incorporated in Table (3).

The scavenging activity towards the superoxide radical (O_2) is measured in terms of inhibition of the generation of O_2. Superoxide and hydroxyl radicals are the two most effective representative free radicals. Superoxide radicals are formed first in cellular oxidation reaction and it

produces other kinds of cell damaging free radicals and oxidizing agents (Liu and Ng, 1999). In *K. alvarezii* the extract of the methanol solvent (141.41%) expressed a very high superoxide radical activity which is followed by ethanol solvent (127. 16%). The standard ascorbic acid value for *K. alvarezii* was (119.83%). The IC_{50} value methanol is 41.89 and for benzene are 28.92 are given in Table (4). Super oxide is produced from molecular oxygen due to oxidative enzymes of the body as well as via non enzymatic reaction such as auto-oxidation by catecholamines (Hemmani and Parihar, 1998).

Table 3. Effect of different solvent extract of *Kappaphycus alvarezii* on Hydroxyl assay

Con	Ethanol	Methanol	Petroleum Ether	Benzene	Ethyl Acetate	Ascorbic acid
50	29.41 ± 0.041	35.71 ± 0.091	30.46 ± 0.091	23.41 ± 0.041	33.71 ± 0.085	24.84 ± 0.84
100	48.46 ± 0.026	55.14 ± 0.081	45.92 ± 0.076	39.26 ± 0.042	50.4 ± 0.04	39.19 ± 0.43
200	71.42 ± 0.046	78.28 ± 0.061	67.62 ± 0.013	56.36 ± 0.069	75.19 ± 0.066	58.83 ± 0.43
400	89.61 ± 0.029	98.34 ± 0.026	86.46 ± 0.062	75.14 ± 0.028	94.31 ± 0.041	84.92 ± 0.93
800	101.14 ± 0.081	128.18 ± 0.081	99.25 ± 0.086	95.14 ± 0.026	119.14 ± 0.037	107.28 ± 0.51
F - Value	**11320. 58**	**234188.98**	**192045.69**	**182182.13**	**140424.48**	**134342.57**
P - Value	**4.8×10^{-15}**	**2.6×10^{-20}**	**5.8×10^{-20}**	**7.2×10^{-20}**	**2.05×10^{-19}**	**2.4×10^{-19}**
IC_{50}	**30.16**	**41.24**	**30.31**	**28.84**	**39.21**	**31.83**

Reducing power has been used as an antioxidant indicator for the medicinal herbs (Hsu *et al*., 2003). Reducing ability of compound generally depends on the presence of reluctant which exhibit antioxidant activity by breaking the free radical chain by donation of hydrogen atom (Rathee *et al*., 2009). In the reducing power assay, the presence of antioxidants in the sample resulted in the reduction of Fe^{3+} to Fe^{2+} by donating the electron. The extract with reducing power reveal that they are electron donators, reduce the oxidized intermediates and act as primary antioxidant substances. The reducing power value for *K. alvarezii* at 700 nm was 0.562 ± 0.028 and the standard ascorbic acid value for *K. alvarezii* was 0.441 ± 0.082. The absorbance for ethanol in *K. alvarezii* was 0.531 ± 0.0.028 shown in Table (5).

This study showed that *Kappaphycus alvarezii* possessed varying degrees of antioxidative activities in methanol extraction. Other potential benefits of this economically important carrageenophyte which can contribute to human health should be explored in future studies.

Acknowledgement: The authors express their sincere thanks to the Principal Dr. Sr. A. Jospin Nirmala Mary, for her keen interest in this study and providing facilities to carry out the experiments. The authors thank Dr. C. R. K. Reddy, Group leader, seaweed cultivation group and Dr. Vaibhav A. Mantri Scientist-in-charge, CSMCRI –MARS for their valuable support. The authors would like to thank Dr. S. Kumaravel, Scientist, Department of Food Quality and Testing, Indian Institute of Crop Processing Technology for providing
all the facilities and support to carry out the GC-MS work. This contribution has CSIR-CSMCRI PRIS registration number 152/2015.

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