

Research Article

## ***In vitro* Anti-oxidant Activity and HPLC-DAD System Based Phenolic Content Analysis of *Codiaeum variegatum* Found in Bangladesh**

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### Abstract

**Purpose:** This study evaluated the *in vitro* antioxidant potential of two varieties of *Codiaeum variegatum* leaves (spiral (CP) and royal like (BP)) extracts.

**Methods:** The different antioxidant assays, including DPPH free radical scavenging, nitric oxide scavenging, hydrogen peroxide, reducing power, total antioxidant activity, protection of lipid peroxidation and RBC membrane stabilization activity, were studied. Moreover, high-performance liquid chromatography (HPLC) coupled with diode-array detection was used to identify and quantify the phenolic compounds in the royal like (BP) leaves extract.

**Results:** *Codiaeum variegatum* extracts showed effective DPPH free radical scavenging, hydrogen peroxide radical scavenging and nitric oxide scavenging activity. However, reducing power of ferric ion was not significant compared to the standard antioxidant activity. In addition, *Codiaeum variegatum* extracts exhibited protection against lipid peroxidation. The total antioxidant activity was increased dose dependently when compared with standard drug ascorbic acid. (-)-Epicatechin, *p*-coumaric acid, rutin hydrate and ellagic acid were identified in the extract. Among the phenolic compounds, ellagic acid was abundantly present in the extract.

**Conclusion:** Our investigation suggests that *Codiaeum variegatum* leaves contain high amount of phenolic compounds which may responsible for its biological activities in folkloric medicine.

### Introduction

Medicinal plants are traditionally used in folk medicine as natural remedies from ancient era and their usages are increasing day by day. Some of them also served as a source for basic lead molecules of modern medicine. More than 70% of the developing world's population depends on traditional medicinal system.<sup>1</sup> Medicinal plants are rich source of active chemical constituents such as polyphenols and flavonoids, glycosides, alkaloids, and tannins.<sup>2</sup> Most often, a desired biological response is not due to the presence of one component but the presence of a mixture of bioactive plant components. Medicinal plants are rich source of antioxidants which has the potential to ameliorates oxidative damage in tissues and prevents degenerative diseases such as cardiovascular diseases, cancer, diabetes and aging.<sup>3,4</sup> Traditional medical practitioners use a variety of medicinal plants for treatment of different ailments in Bangladesh. Bangladesh is rich in flora and fauna, more than 500 potential medicinal plants are listed including their chemical constituents and traditional uses.<sup>5</sup> Garden crotons (*Codiaeum variegatum*) are a group of beautifully variegated leafy perennial ornamental shrubs found almost everywhere in Bangladesh. It belongs to the

family *Euphorbiaceae* which is an important plant family for diverse biological activities.<sup>6</sup> Its native habitats include India, Philippines, Sri Lanka, Thailand, Indonesia, Malaysia and some other Pacific Islands.<sup>7</sup> Apart from its ornamental values, *Codiaeum variegatum* is also used for several medicinal purposes. Root decoction is taken for the treatment of gastric ulcers. Its leaves contain antibacterial and anti-amoebic properties and cures diarrhea.<sup>8</sup> A recent report also suggests that *Codiaeum variegatum* extracts are active against influenza virus and a bioactive cyanoglucoside was isolated as the active component.<sup>9</sup> *Codiaeum variegatum* also possesses alkaloids, anthraquinones, flavanoids, terpenes, steroid, phenol, saponins, tannins, phlobatannin and cardenolide<sup>7</sup> and showed potent cytotoxicities in brian shrimp lethality bioassays.<sup>10</sup> Phytochemical components, especially polyphenols (such as flavonoids, tannins, phyenyl propanoids, phenolic acids etc) are known to be responsible for the free radical scavenging and antioxidant activities. Phenolic compounds are very well known plant constituents because of their scavenging ability.<sup>11</sup> It is demonestrated that polyphenolic compounds possess inhibitory effects on mutagenesis and carcinogenesis. *In*

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*vitro* studies also suggested that polyphenols may exert their inhibitory effects by acting as prooxidants on cancer cells or may inhibit the formation and growth of tumors by induction of cell cycle arrest and apoptosis.<sup>11</sup> However, no reports have been found on any antioxidant activities and analysis of phenolic compounds in *Codiaeum variegatum* extracts. As a part of our ongoing investigation on natural antioxidants and biological activities from local medicinal plants of Bangladesh,<sup>10,12,13</sup> in this paper, we evaluated the antioxidant activity of *Codiaeum variegatum* leaves extracts and analyzed the phenolic compounds present using HPLC-DAD system.

## Materials and Methods

### Collection and identification of plant materials

In this present investigation, the *Codiaeum variegatum* leaves were collected from Stamford University campus, Bangladesh and was identified at the Bangladesh National Herbarium, Mirpur, Dhaka where a voucher specimen (no: DACB 31304) has been deposited.

### Drying and grinding of plants

The collected plant parts (leaves) were separated from undesirable materials such as other plants or plant parts. The plant parts were ground into a coarse powder with the help of a grinder. The powder was stored in an airtight container and kept in a cool, dark and dry place until further analysis was commenced.

### Phytochemical screening

Phytochemical screening of the extract was performed using the following reagents and chemicals: alkaloids were determined using Dragendorff's reagent, flavonoids were determined by using Mg and HCl; tannins were determined using ferric chloride and potassium dichromate solutions and saponins were determined by the ability to produce suds. Gum was tested using Molish reagents and concentrated sulphuric acid.

### Chemicals Used

Gallic acid (GA), (+)-catechin hydrate (CH), vanillic acid (VA), caffeic acid (CA), (-)-epicatechin (EC), *p*-coumaric acid (PCA), rutin hydrate (RH), ellagic acid (EA), myricetin (MC), kaempferol (KF), and quercetin (QU) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC), methanol (HPLC), acetic acid (HPLC), and ethanol was obtained from Merck (Darmstadt, Germany). 1,1-diphenyl-2-picrylhydrazyl (DPPH), Butylated hydroxytoluene (BHT), trichloroacetic acid (TCA), Ammonium molybdate, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), sodium nitroprusside, were also purchased from Merck, Germany and ethylene diamine tetra acetic acid (EDTA), sodium phosphate, sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride from BDH, England. Ferric chloride was obtained from Thomas Baker and

Potassium ferricyanide was purchased from Guandong Chemical Reagent, China. All other reagents were of analytical grade.

### In vitro antioxidant assays

#### DPPH radical scavenging activity

**Qualitative assay:** Qualitative assay was performed according to the method previously described.<sup>14</sup> Test samples were prepared with a suitable solvent system on a TLC plate and sprayed with 0.004% w/v DPPH solution in methanol using a sprayer. The positive activity was detected by the discolored (pale yellow) spots on a reddish purple background.

**Quantitative assay:** The free radical scavenging capacity of the extracts was determined using DPPH.<sup>15</sup> A methanol DPPH solution (0.004% w/v) was mixed with serial dilutions (1 to 500 µg) of *Codiaeum variegatum* extracts and after 10 min, the absorbance was read at 515 nm using a spectrophotometer (HACH 4000 DU UV – visible spectrophotometer). Ascorbic acid was used as a standard antioxidant.

#### Nitric oxide radical inhibition assay

Nitric oxide radical inhibition was estimated using Griess reagents.<sup>15,16</sup> In this investigation, Griess-Illsovoy reagent was modified by naphthyl ethylene diamine dihydrochloride (0.1% w/v) instead of 1-naphthylamine (5%). The reaction mixture (3 ml) containing sodium nitroprusside (10 mM, 2 ml), phosphate buffer saline (0.5 ml) and *C. variegatum* extract (10 µg to 160 µg) or standard solution (BHT, 0.5 ml) was incubated at 25°C for 150 min. After incubation, 0.5 ml of the reaction mixture was mixed with 1 ml of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotization. Then, 1 ml of naphthyl ethylene diamine dihydrochloride was added, mixed and allowed to stand for 30 min at 25°C. A pink color chromophore formed in diffused light. The absorbances of these solutions were measured at 540 nm against the corresponding blank solutions. Ascorbic acid was used as a standard antioxidant.

#### Scavenging of hydrogen peroxide

A modified method based on that of Ruch et al.<sup>17</sup> was used to determine the ability of the extracts to scavenge hydrogen peroxide.<sup>15</sup> Hydrogen peroxide (43 mM) was prepared in phosphate buffered saline (pH 7.4). Standards (ascorbic acid) and extract solutions were prepared at concentrations of 50 to 250 mM. Aliquots of the standard or extract solutions (3.4 mL) were added to 0.6 mL of hydrogen peroxide solution. The reaction mixture was incubated at room temperature for 10 min, and the absorbance was determined at 230 nm. The percentage of scavenging was calculated as follows:

$$\% H_2O_2 \text{ Scavenging} = 100 \times \frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}}$$

**Reducing power**

The reducing power of *Codiaeum variegatum* extracts were determined according to the method previously described.<sup>18</sup> Different concentrations of *Codiaeum variegatum* extract (100 µg – 1000 µg) in 1 ml of distilled water was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [ $K_3Fe(CN)_6$ ] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and  $FeCl_3$  (0.5 ml, 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as a standard antioxidant.

**Determination of total antioxidant capacity**

The antioxidant activity of the extracts of *Codiaeum variegatum* were evaluated by the phosphomolybdenum method according to the procedure previously described.<sup>15</sup> The assay is based on the reduction of Mo (VI)–Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH. 0.3 ml extract was combined with reagent solution (0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer (HACH 4000 DU UV – visible spectrophotometer) against blank after cooling to room temperature. Methanol (0.3 ml) was used as the blank. The antioxidant activity is expressed as the number of equivalents of ascorbic acid.

**Lipid peroxidation assay**

Thiobarbituric acid reactive substances were determined by using previously described method.<sup>18</sup> The reaction mixture contained in a final volume of 1.0 ml, 500 µl of liver microsomal fraction, 300 µl buffer containing the plant extract (50–150 µg), 100 µl of  $FeCl_3$  (1 mM) and 100 µl ascorbic acid (1 mM) to start peroxidation. Samples were incubated at 37°C for 1 hour, after that lipid peroxidation was measured using the reaction with thiobarbituric acid (TBA). The absorbance of the organic layer was measured at 532 nm. All reactions were carried out in duplicate. Ascorbic acid was served as a standard antioxidant.

**Determination of total phenolic content**

The total phenolic content of the extract was determined by the modified Folin-Ciocaltu method.<sup>19</sup> Briefly, 1.0 ml of each extract (1 mg/ml) was mixed with 5 ml Folin-Ciocaltu reagent (1:10 v/v distilled water) and 4 ml (75g/l) of sodium carbonate. The mixture was vortexed for 15 second and allowed to stand for 30 min at 40°C for color development. The absorbance was read at 765 nm with a spectrophotometer. Total phenolic content was determined as mg of gallic acid equivalent per gram

using the equation obtained from a standard gallic acid calibration curve  $y = 6.2548x - 0.0925$ ,  $R^2=0.9962$ .

**Membrane stabilizing activity****Preparation of erythrocyte suspension**

Whole blood was obtained with heparinized syringes from rats through cardiac puncture. The blood was washed three times with isotonic buffered solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4). The blood was centrifuged each time for 10 minutes at 3000 g.

**Hypotonic solution-induced rat erythrocyte haemolysis**

Membrane stabilizing activity of the extracts was assessed using hypotonic solution-induced rat erythrocyte haemolysis.<sup>15</sup> The test sample consisted of stock erythrocyte (RBC) suspension (0.50 ml) mixed with 5 ml of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing the extract (0.25- 2.0 mg/ml) or indomethacin (0.1 mg/ml). The control sample consisted of 0.5 ml of RBC mixed with hypotonic -buffered saline solution alone. The mixtures were incubated for 10 min at room temperature and centrifuged for 10 min at 3000 g and the absorbance of the supernatant was measured at 540 nm. The percentage inhibition of haemolysis or membrane stabilization was calculated according to the below mentioned formula.

$$\% \text{ Inhibition of haemolysis} = 100 \times \frac{OD1 - OD2}{OD1}$$

Where:

OD1 = Optical density of hypotonic-buffered saline solution alone

OD2 = Optical density of test sample in hypotonic solution

**High performance liquid chromatography (HPLC) system**

Chromatographic analyses were carried out on a Thermo Scientific Dionex UltiMate 3000 Rapid Separation LC (RSLC) systems (Thermo Fisher Scientific Inc., MA, USA), coupled to a quaternary rapid separation pump (LPG-3400RS), Ultimate 3000RS autosampler (WPS-3000) and rapid separation diode array detector (DAD-3000RS). Phenolic compounds were separated on a Acclaim® C18 (4.6 x 250 mm; 5µm) column (Dionix, USA) which was controlled at 30°C using a temperature controlled column compartment (TCC-3000). Data acquisition, peak integration, and calibrations were performed with Dionix Chromeleon software (Version 6.80 RS 10).

**Chromatographic conditions**

The phenolic composition of the leaves of *C. variegatum* ethanol extract was determined by HPLC-DAD, as described previously with some modifications.<sup>13,20</sup> The mobile phase consisted of acetonitrile (solvent A), acetic acid solution pH 3.0 (solvent B), and methanol (solvent C). The system was run with the following gradient

elution program: 0 min, 5%A/95%B; 10 min, 10%A/80%B/10%C; 20 min, 20%A/60%B/20%C and 30min, 100%A. There was a 5 min post run at initial conditions for equilibration of the column. The flow rate was kept constant throughout the analysis at 1 ml/min and the injection volume was 20 µl. For UV detection, the wavelength program was optimized to monitor phenolic compounds at their respective maximum absorbance wavelengths as follows: λ 280 nm held for 18.0 min, changed to λ 320 nm and held for 6 min, and finally changed to λ 380 nm and held for the rest of the analysis and the diode array detector was set at an acquisition range from 200 nm to 700 nm. The detection and quantification of gallic acid (GA), (+)-catechin hydrate (CH), vanillic acid (VA), caffeic acid (CA), and EC was done at 280 nm, of *p*-coumaric acid (PCA), Rutin hydrate (RH), and ellagic acid (EA) at 320 nm, and of myricetin (MC), quercetin (QU), and kaempferol (KF) at 380 nm, respectively.

### Statistical Analysis

All data are presented as mean ±Standard deviation (SD). IC<sub>50</sub> values for scavenging of free radicals by the extracts

were calculated from dose-response curve by using default analyzing tab of Graph Pad Prism Software (USA).

### Results and Discussion

Plant derived secondary metabolites are receiving great attention in recent years due to their diverse biological activities. It is believed that the use of plants for medicinal purposes have been associated with less side effects. In the present study, we have applied a wide range of established *in vitro* assays to evaluate the antioxidant and free radical scavenging activities of *Codiaeum variegatum* extracts. Preliminary phytochemical analysis of *Codiaeum variegatum* extracts revealed the presence of alkaloids, gums, tannins and saponins in the extracts (Table 1). Phenolic compounds and flavonoids were reported to be associated with antioxidant properties, acting as scavengers of singlet oxygen and free radicals.<sup>21,22</sup> Gum, tannin and saponin containing plants are also rich sources of antioxidants.<sup>23-25</sup>

**Table 1.** Phytochemical screening of *C. variegatum* extracts.

Extract	Alkaloids	Gum	Flavonoids	Tannins	Saponins
Extract of <i>C. variegatum</i> (CP)	++	+++	--	+++	++
Extract of <i>C. variegatum</i> (BP)	++	++	+	+++	+

### DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity

Phytochemical screening of the extracts indicated the presence of alkaloids, saponin, gum and tannins (Table 1). The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity of *Codiaeum variegatum* are given in Table 2 and Figure 1. In the TLC-based qualitative antioxidant assay using DPPH spray, the extract of

*Codiaeum variegatum* showed prominent free radical scavenging properties as indicated by the presence of a yellowish spot on a reddish purple background on the TLC plate. The IC<sub>50</sub> values of the extracts were found to be 40.93 µg/mL and 73.16 µg/mL for CP and BP respectively whereas IC<sub>50</sub> for ascorbic acid was 17.17 µg/mL, which is a well known antioxidant.

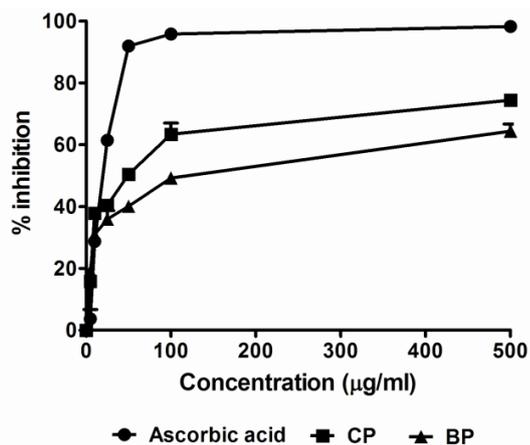
**Table 2.** IC<sub>50</sub> values of *C. variegatum* extracts in different antioxidant assays such as DPPH method, NO<sup>-</sup> scavenging method, H<sub>2</sub>O<sub>2</sub> scavenging, Scavenging of TBARS method and total phenolic content. Values are expressed as average of duplicate experiments.

Sample	IC <sub>50</sub> (µg/mL)				Total phenolic content (mg of gallic acid equivalent per g of dry extract)
	DPPH scavenging method	NO <sup>-</sup> scavenging method	H <sub>2</sub> O <sub>2</sub> scavenging method	Scavenging of TBARS	
Extract of <i>C. variegatum</i> (CP)	40.93 µg/mL	186.2µg/mL	148.5 µg/mL	148.5 µg/mL	35.73
Extract of <i>C. variegatum</i> (BP)	73.17 µg/mL	159.9 µg/mL	148.3 µg/mL	169.0 µg/mL	97.28
Ascorbic acid	17.17 µg/mL	91.15 µg/mL	124.9 µg/mL	115.8 µg/mL	-

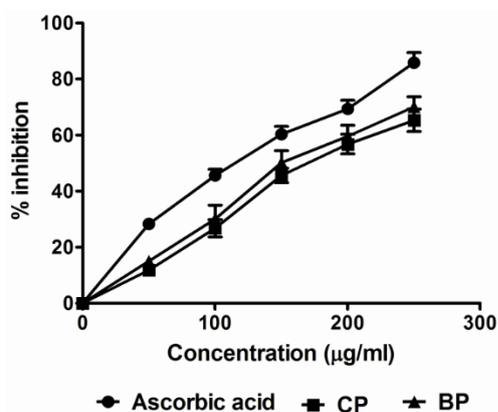
*Euphorbiaceae* plant family possess strong antioxidant activities which are greatly associated with the presence of phenolic compounds.<sup>26</sup> In this study, *Codiaeum variegatum* extracts showed significant scavenging of DPPH free radicals compared to the standard antioxidants. DPPH is a stable free radical, pink in solution, which can accept one electron from antioxidant containing plant extracts, thus neutralizes its free radical nature. The degree of decolorization of the DPPH solution can be measure in UV spectrophotometer and indicates the scavenging activity of the plant extracts.<sup>27,28</sup>

### NO<sup>-</sup> scavenging activity

Suppression of NO<sup>-</sup> release may partially be attributed to direct NO<sup>-</sup> scavenging, as all *Codiaeum variegatum* extracts decreased the amount of nitrite generated from the decomposition of sodium nitroprusside *in vitro*. The scavenging of nitric oxide by the plant extract was increased in a dose-dependent manner. The IC<sub>50</sub> value of the extract was 186.2 µg/mL and 159.9 µg/mL respectively whereas the IC<sub>50</sub> value of vitamin C was 95.15 µg/mL (Table 2 and Figure 2).



**Figure 1.** DPPH radical scavenging activity of the ethanol extract of *Codiaeum variegatum* leaves. DPPH radical scavenging activity was increased by increasing the concentration of the sample extract. Data are represented as Mean  $\pm$  SD of duplicate experiments.



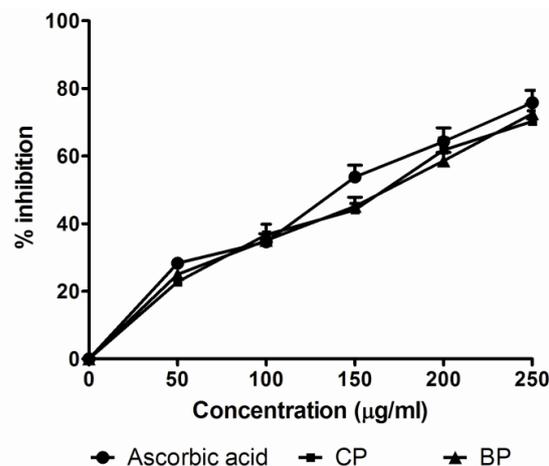
**Figure 2.** Scavenging of NO radical by *Codiaeum variegatum* leaf extracts. Data are represented as Mean  $\pm$  SD of duplicate experiments.

Nitric oxide is a gaseous water soluble molecule, implicated in inflammation, cancer and other pathological conditions.<sup>29,30</sup> Both nitric oxide and superoxide anion can cause injury to various tissues. The toxicity and damage caused by NO $\cdot$  and O $_2^{\cdot-}$  is multiplied as they react to produce reactive peroxynitrite (ONOO $^-$ ), which leads to serious toxic reactions with biomolecules.<sup>30,31</sup> The scavenging activity of reactive peroxynitrite helps to arrest the chain of reactions initiated by excess generation of NO that are detrimental to the human health.<sup>16</sup> Suppression of NO $\cdot$  release may partially be attributed to direct NO $\cdot$  scavenging, as all *Codiaeum variegatum* extracts decreased the amount of nitrite generated from the decomposition of sodium nitroprusside *in vitro*.

#### Scavenging of H $_2$ O $_2$ activity

The scavenging of H $_2$ O $_2$  by vitamin C and the extract of *Codiaeum variegatum* after incubation for 10 min was increased with increased concentration of the sample. The extract exhibited higher H $_2$ O $_2$  scavenging activity than vitamin C at similar concentrations. The IC $_{50}$  values

of the extracts CP and BP and ascorbic acid were 148.5 and 148.3  $\mu$ g/mL and 124.9  $\mu$ g/mL respectively (Table 2 and Figure 3).



**Figure 3.** Scavenging of H $_2$ O $_2$  by *Codiaeum variegatum* leaf extracts. Data are represented as Mean  $\pm$  SD of duplicate experiments.

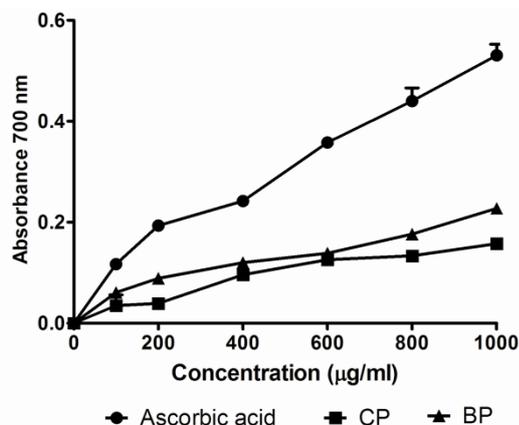
Hydrogen peroxide is toxic to cell because it may give rise to hydroxyl radicals due to the presence of iron ions.<sup>32</sup> Therefore, removing H $_2$ O $_2$  is very important for antioxidant defense in cells. Dietary polyphenols (especially compounds with the orthodihydroxy phenolic structure quercetin, catechin, gallic acid ester, caffeic acid ester) showed protection against hydrogen peroxide induced cytotoxicity in mammalian and bacterial cells.<sup>33</sup> The phenolic compounds of the *C. variegatum* extracts may be involved in removing the H $_2$ O $_2$ .

#### Reducing activity, total antioxidant capacity and total phenolic compounds

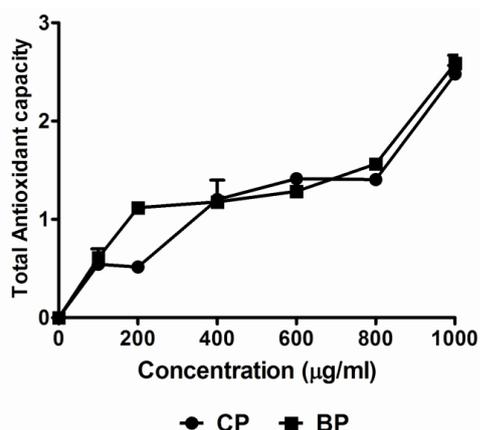
Our data on the reducing power of the tested extracts suggests low to moderate reducing properties. Like other antioxidant assays, the reducing power of *Codiaeum variegatum* extracts increased with increasing the amount of samples. Figure 4 shows the reducing ability of *Codiaeum variegatum* extracts in comparison with ascorbic acid. Total antioxidant capacity of the *Codiaeum variegatum* extract, expressed as the number of equivalents of ascorbic acid, is shown in Figure 5. Total antioxidant capacity was also increased in a dose-dependent manner.

Direct correlation between antioxidant activity and reducing power of certain plant extracts were observed previously.<sup>34</sup> The reducing properties are generally associated with the presence of reductones which showed antioxidant activity by breaking the free radical chain donating a hydrogen atom.<sup>18</sup> Our data on the reducing power of the tested extracts suggests moderate reducing properties. However, the antioxidant activity of plant extracts are attributed to various antioxidants present followed by various mechanisms such as prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical

scavenging.<sup>18,35</sup> Like the total antioxidant activity, the reducing power of *Codiaeum variegatum* extracts increased with increasing the amount of samples.



**Figure 4.** Reducing power of *Codiaeum variegatum* leaves ethanol extracts. An increase in absorbance in the reducing power method implies that extracts are capable of donating hydrogen atoms in a dose dependent manner. Data was represented as Mean  $\pm$  SD of duplicate experiments.

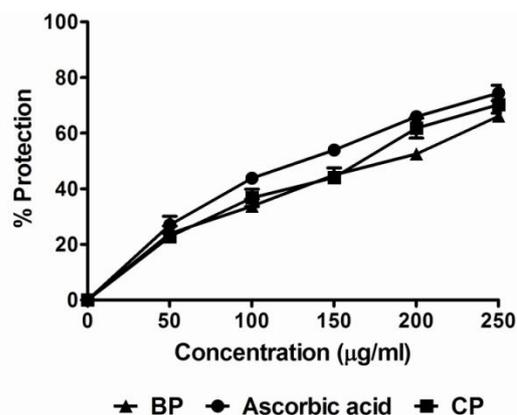


**Figure 5.** Total antioxidant capacity of *Codiaeum variegatum* leaf extracts. Data was represented as Mean  $\pm$  SD of duplicate experiments.

The amount total phenolic compound was calculated as quite high in the methanol extract of *Codiaeum variegatum* (BP) (97.28 mg of gallic acid equivalent) (Table 2). According to the results of this study, it can be revealed that the high inhibition value in the methanol extract might be due to the high concentration of phenolic compounds present in the extract.

#### Lipid peroxidation assay and RBC membrane stabilization assay

Activity of plant extract against non-enzymatic lipid peroxidation in rat liver microsomes is shown in Table 2 and Figure 6. Addition of Fe<sup>2+</sup>/ascorbate to the liver microsomes causes a rise in lipid peroxidation. The extract showed inhibition of peroxidation effect in all concentrations, which showed 50% inhibition at 148.5 and 169.0 µg/mL whereas the vitamin C showed at 115.8 µg/mL. The extract of *Codiaeum variegatum* at a concentration range of 0.50-2.0 mg/mL also protected the rat erythrocyte membrane against lysis induced by hypotonic solution (Table 3). In contrast, indomethacin (0.10 mg/mL) offered a significant protection of the rat red blood cells (RBC) against the damaging effect of a hypotonic solution.



**Figure 6.** Prevention of lipid peroxidation product TBARS formation by *Codiaeum variegatum* leaf extracts. Data are represented as Mean  $\pm$  SD of duplicate experiments.

**Table 3.** Membrane stabilizing activity by two varieties of *C. variegatum* extracts. Values are expressed as average of duplicate experiments.

Concentration	Absorbance BP	% Protection	Absorbance CP	% Protection
Hypotonic medium 50 mM	0.35 $\pm$ 0.01	-	0.35 $\pm$ 0.01	-
<i>C. variegatum</i> 0.25 mg/mL	0.287	17.857	0.260	25.57
0.5 mg/mL	0.280	19.857	0.223	36.285
1.0 mg/mL	0.171	51	0.203	41.857
1.5 mg/mL	0.106	69.571	0.116	66.714
2.0 mg/mL	0.059	83.142	0.050	85.601
Indomethacin (0.10 mg/mL)	0.051 $\pm$ 0.003	85.26 $\pm$ 0.41	-	-

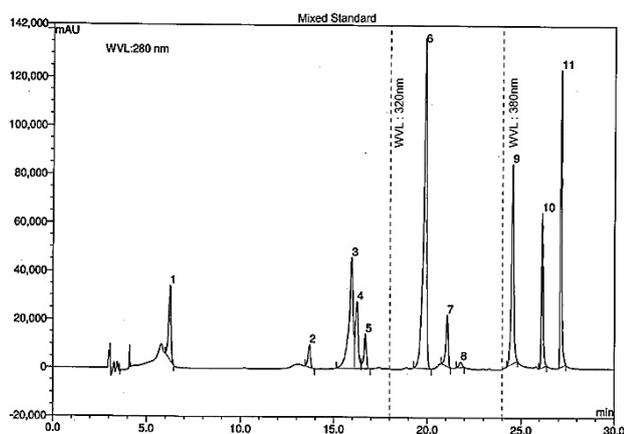
Superoxides, ferrous state of iron and H<sub>2</sub>O<sub>2</sub> are able to cause lipid peroxidation via fenton reaction in biological membrane.<sup>36</sup> During lipid peroxidation, low molecular-weight end products, probably malondialdehyde, are

formed by oxidation of polyunsaturated fatty acids which can react with two molecules of thiobarbituric acid to give a pinkish red chromogen. Exposure to hypotonic medium may cause lysis of red blood cell membrane

accompanied by haemolysis and oxidation of haemoglobin.<sup>37</sup> The haemolytic effect of hypotonic solution is related to excessive accumulation of fluid within the cell resulting in the rupturing of its membrane. Such injury to RBC membrane will further render the cell more susceptible to secondary damage through free radical induced lipid peroxidation.<sup>38</sup> The breakdown of cell membranes leads to enhance further cellular damage.<sup>39</sup> Compounds with membrane-stabilizing properties are well known for their ability to interfere with the early phase of inflammatory reactions, namely the prevention of the release of phospholipases that trigger the formation of inflammatory mediators.<sup>40</sup> As a rich source of antioxidant phenolic compounds, the extracts of this plant exhibit significant membrane stabilizing property.

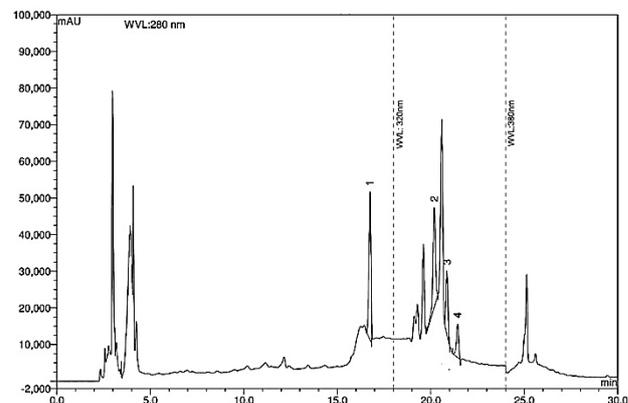
#### HPLC-DAD analysis of phenolic contents in leaves extracts

Identification and quantification of individual phenolic compounds in the large leaves of *Codiaeum variegatum* were analysed by HPLC-DAD system. In this study, we used eleven different phenolic standards, C18 column with 250 mm length, and rapid separation LC (RSLC) systems while other investigators used six standards,<sup>20</sup> C18 column with 150mm length, and HP 1090, series II, liquid chromatography systems to determine the polyphenolic contents. The wavelength between 210 and 380 nm was used for the detection of polyphenolic compounds.<sup>41,42</sup> Therefore, 280, 320 and 380 nm wavelength were selected for the detection of all standards in this study. From Figure 7, it can be observed that a good separation can be achieved within 30 min using the above condition described. Symmetrical, sharp and well-resolved peaks were observed for the eleven polyphenolic standards. The elution order and the retention times for GA, CH, VA, CA, EC, PCA, RH, EA, MC, QU, and KF were 6.25, 13.69, 15.95, 16.24, 16.69, 19.89, 21.07, 21.79, 24.54, 26.12, and 27.13 minutes respectively.



**Figure 7.** HPLC chromatogram of a standard mixture of polyphenolic compounds. Peaks: 1, gallic acid; 2, (+)-catechin; 3, vanillic acid; 4, caffeic acid; 5, (-)-epicatechin; 6, *p*-coumaric acid; 7, rutin hydrate; 8, ellagic acid; 9, myricetin; 10, quercetin; 11, kaempferol.

The chromatographic separations of polyphenols in ethanol extract are shown in Figure 8. The experimental results indicated that the extract contains a high concentration of ellagic acid (187.87 mg per 100 g of dry weight, Table 4). It was also found that moderate concentration of (-)-epicatechin, rutin hydrate and *p*-coumaric acid (26.20 mg, 56.91 mg and 15.82 mg per 100 g of dry weight, Table 4) was present in the large leaf extract. Ellagic acid is a potent antioxidant and anticancer compound. Ellagic acid showed cytotoxicity in HSC-2 oral carcinoma cells by inducing apoptosis but not toxic to normal cells.<sup>43</sup> Previously we reported that *Codiaeum variegatum* extracts showed potent cytotoxicity in brian shrimp lethality bioassays.<sup>10</sup> HPLC analysis of the *C. variegatum* extracts suggested that high ellagic acid content may be responsible for the cytotoxicity of the plant extract. However, other plant phenolics such as (-)-epicatechin, rutin hydrate and *p*-coumaric acid are also potent antioxidants and inhibitors of tumor cells. Therefore, a synergistic action of those compounds may possible while using this plant extracts.



**Figure 8.** HPLC chromatogram of *Codiaeum variegatum* extracts. Peaks: 1, (-)-epicatechin; 2, *p*-coumaric acid; 3, rutin hydrate; 4, ellagic acid.

**Table 4.** Contents of polyphenolic compounds in the *Codiaeum variegatum* large leaf extract.

Polyphenolic compound	<i>Codiaeum variegatum</i> large leaves extract	
	Content (mg/100 g of dry extract)	% RSD
EC	26.20	1.03
PCA	15.82	0.94
RH	56.91	1.96
EA	187.87	8.52

EA, Ellagic acid, PCA, *p*-Coumaric acid, RH, Rutin hydrate, EC, (-) Epicatechin

#### Conclusion

On the basis of this study, both *Codiaeum variegatum* extracts (CP and BP) showed significant antioxidant activities compared to standard compounds *in vitro*. The various antioxidant mechanisms of *Codiaeum variegatum* extract may be attributed to its strong abilities as a hydrogen donor or as scavenger of nitric

oxide and hydrogen peroxide free radicals. In addition, the antioxidant activities showed by the extracts are mainly due to phenolic compounds present in *Codiaeum variegatum* extracts. Further HPLC-DAD analysis of the extracts proposed that ellagic acid may be responsible for the antioxidant activity of *Codiaeum variegatum* extracts. Further work should be carried out using *Codiaeum variegatum* extracts to determine *in-vivo* antioxidant and anti-tumor activities. Moreover, the phenolic compounds have been of interest of health benefits, the present HPLC study could be indicative to a potential application to identify and quantify the polyphenolic compounds in any medicinal plant extract.

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### Conflict of Interest

The authors declare no conflict of interest.

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