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Characterization and evaluating of antioxidant and antihypertensive properties of green alga (*Codium fragile*) from the coast of Sfax

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Abstract
The present study was conducted to evaluate the composition and the antioxidant activity of hexane, ethyl acetate and methanol extracts of the green algae (*Codium fragile*) collected from Sfax (Tunisia) during spring 2013. Alga was cleaned from epiphytes, washed; air dried and powdered to determinate the mineral, polyphenol, flavonoids and tannin content. The antioxidant activity of the algal extracts was determined using the procedures of total antioxidant activity, free radical scavenging (DPPH-decolorization method) and ABTS radical-scavenging activity. According to the results, it was noted that this green alga has an important carbohydrate content, followed by lipids and proteins with a higher content in polyphenol, flavonoid and low levels of tannins in the three extracts of the seaweed with an interesting antioxidant *in vitro* capacity showed at the methanol extract. The tested extract showed excellent interfacial concentration-dependent properties. Overall, the results suggested that *Codium fragile* is promising source of natural antioxidants and ACE inhibitory agents and could, therefore, be used as alternative additives pharmaceutical preparations.

Keywords: Algae, mineral element, antioxidant activities

Introduction
Marine organisms are rich sources of structurally new and biologically active metabolites (Ely & *et al*. 2004) [6]. Seaweeds are known to contain reactive antioxidant molecules, such as ascorbate and glutathione (GSH) when fresh, as well as secondary metabolites, including carotenoids (α and β carotene, fucoxanthin, astaxanthin), mycosporine-like amino acids (mycosporine-glycine) and catechins (e.g., catechin, epigallocatechin, epigallocatechin), gallate, phlorotannins (e.g., phloroglucinol), eckol and tocopherols (α-, χ-, δ-tocopherols) (Yuan & al. 2005) [27].
In Asia, seaweeds have been consumed as a vegetable since the beginning of time. On average, the Japanese eat 1.4 kg per person per year. This ancient tradition and everyday habit has made possible a large number of epidemiological researches showing the health benefits linked to seaweed consumption (Hiqashi & *al*. 1999) [11].
Other marine organisms, the structure and composition of marine algae have multiple beneficial effects on the human body. Algae have been and today remains a subject of constant research and it is for this reason that there are various industries that exploit such as the pharmaceutical industry, cosmetics or food industry. Seaweeds are also used to treat skin and this is why there has various cosmetic products based on seaweed. Specialists in biology confirm that there are thousands of types of seaweed and each type has different properties.
It is known that algae, regardless of their type, are rich in vitamins and minerals. They are extracted and processed to be used in various fields.
In our studies we are interested of a green algae collection area of Sfax "*Codium fragile*" view that green algae are used mainly thanks to their benefits and their antioxidant properties. Thus they have a composition rich in vitamins A, C and E and magnesium is that is beneficial for body care.

2. Materials and methods
2.1 Collection and processing of seaweed
The seaweed was collected from Sfax (Tunisia) during spring 2013. It was picked with hand and immediately washed with seawater to remove the foreign particles, sand particles and epiphytes. Then it was kept in an ice box containing slush ice and immediately transported to the laboratory and washed thoroughly using tap water to remove the salt on the surface of the

sample. Then the seaweed was spread on blotting paper to remove excess water then air dried and powdered. Hundred grams of each algal species were extracted three times by solvents of increasing polarity (Hexane, Ethyle acetate, Methanol). Extracts were lyophilized and their weights were determined.

2.2 Protein estimation
The total proteins were estimated using the Biuret method of (Raymont & *al*.1980) [23].

2.3 Lipid estimation
The extraction of lipids was done by the chloroform-methanol mixture.

2.4 Carbohydrate estimation
The total carbohydrate was estimated by following the Phenol sulphuric acid method of.

2.5 Determination of mineral element
5 g of solids were mixed with a volume of hydrochloric acid, and nitric Perchloric report (10, 5, 1). The digestion was carried out at elevated temperature of 250˚C using a mineralization during 1h. After cooling, the samples were filtered and diluted for the determination of minerals by atomic absorption.

2.6 Measurement of total phenolic content
The total phenolic content was analysed using the Folin–Ciocalteu method as described previously (Singh, Muthy, & Jayaprakasha. 2002) [24] with slight modifications. Algal extracts, or standard solutions, were pipetted into 2 ml tubes and to each 1.58 ml water and 100 µl Folin–Ciocalteu reagent was added. After 8 min incubation, 300 µl 20% (w/v) sodium carbonate solution was added to stop the reaction. The vortexed mixture was left at room temperature in the dark for 2 h and the absorbance was read at 760 nm using a Spectrophotometer. A standard calibration curve (20–500 mg/l) was prepared using the same procedure as above. Total phenolics were expressed as gallic acid equivalents (GAE, mg gallic acid/100 g extract)

2.7 Determination of flavonoid content
The total flavonoid content in spinach leaves was quantified following a colorimetric method described by (Liu & Zhu. 2007) [19].

2.8 Total condensed tannin content
Total condensed tannin content was determined according to the method of (Julkunen-Titto 1985) [14]. Briefly, a 50 µl aliquot of each extract was mixed with 1.5 ml of 4% vanillin (prepared with methanol) and then 750 µl of concentrated HCl was added. The solution was shaken vigorously and left to stand at room temperature for 20 min in darkness. The absorbance against blank was read at 500nm. Catechin was used to prepare the standard curve and results were expressed as mg catechin equivalents (CE)/g extract.

2.9 *In vitro* antioxidant activity
2.9.1 Determination of total antioxidant activity
Total antioxidant activity of the seaweed extracts was determined according to the method of. Briefly, 1, 5 ml of sample was mixed with 5.0 ml of reagent solution (0.6 M) sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Reaction mixture was incubated at 95˚C for 90 min under water bath. Absorbance of all the sample mixtures was measured at 695 nm. Total antioxidant activity is expressed as the number of equivalents of gallic.

2.9.2 DPPH free-radical scavenging activity
DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging activity was measured by the method of (Szabo & *al*. 2007) [25]. The reaction mixture contained 1.5´10-7 M methanolic solution of DPPH and various concentrations of the test substances and were kept in dark for 50 min. Optical Density (OD) of the samples was measured at 517 nm against a blank and IC_{50} values were calculated using the following equation given by (Duan & al. 2006) [5]:
Scavenging effect (%) = [1- (A sample 517/ A control 517)]× 100
Where: A control is the absorbance of the control (DPPH solution without sample), a sample is the absorbance of the test sample (DPPH solution plus test sample) and a sample blank is the absorbance of the sample only (sample without any DPPH solution). Ascorbic acid was used as a positive control.

2.9.3 ABTS radical-scavenging activity
ABTS was dissolved in water to make a concentration of 7 mM. ABTS was produced by reacting the ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12– 16 h before use. For the test of samples, the ABTS stock solution was diluted with phosphate-buffered saline 5 mM (pH 7.4) to an absorbance of 0.70 at 734 nm. After the addition of 1.0 ml of diluted ABTS to 20µl of sample, the absorbance reading was taken 5 min after the initial mixing (Jeong & al. 2010) [17]. This activity is given as percent ABTS scavenging that is calculated as:
% ABTS scavenging activity= [(control absorbance - sample absorbance)/ (control absorbance)]*100

2.10 *In vitro* angiotensin converting enzyme (ACE) inhibitory assay
ACE inhibitory activity was assayed as reported by Nakamura *et al*. A volume of sample solution (80 µl) containing different concentrations (0.2–0.8 mg ml^{-1}) of SVSP was mixed with 200 µl of 5 mM HHL, and then preincubated for 3 min at 37 ˚C. The sample of polysaccharide and HHL were prepared in 100 mM borate buffer (pH 8.3) containing 300 mM NaCl. The reaction was then initiated by adding buffer. After incubation for 30 min at 37 ˚C, the enzymatic reaction was stopped by adding 250 µl of 0.05 M HCl. The liberated hippuric acid (HA) was extracted with ethyl acetate (1.7ml) and then evaporated at 95˚C for 10 min by rotary evaporation. The residue was dissolved in 1ml of distilled water and the absorbance of the extract at 228 nm was determined using a UV-visible spectrophotometer. ACE-inhibitory activity was calculated as follows:

$$\text{ACE inhibition}(\%) = [\frac{B- A}{B- C}] \times 100$$

where A refer to the absorbance of HA generated in the presence of ACE inhibitor, B refer to the absorbance of HA generated without ACE inhibitors (100 mM borate buffer pH 8.3 was used instead of SVSP), and C to the absorbance of HA generated without ACE (corresponding to HHL autolysis in the course of enzymatic assay). The IC_{50} value, defined as the concentration of SVSP (mg/ml) required to inhibit 50% of

ACE activity, was calculated for each sample using non-linear regression from a plot of percentage ACE inhibition versus sample concentrations.

2.10 Statistical Analysis

Exprimental results were expressed as means ± SD. Moreover, all measurements were replicated three times. The data were analyzed by analysis of variance ($P<0.05$) and the means were separated by Duncan's multiple range test.

3. Resultats and discussion

3.1 Chemical composition of *Codium fragile*

The carbohydrate, proteins and lipids concentration of *Codium fragile* can be seen in figure 1. It was noted that this green alga has an important carbohydrate content (20.47 ± 0.5%), followed by proteins (6.13 ± 0.23%) and a minimum concentration of lipids (2.53 ± 0.27%). The lipid content of the seaweed significantly varies throughout the year. These results are in correlation with (Manivannan & al. 2008) [20], which montionned that the green alga *Ulva lactuca* presented less values (1.6 ± 0.17%) than that obtained previous studies (Integaard & al. 1991) [13]. The literature has established that in seaweeds in general the lipid content is less than 4%. The differences could have been due to factors such as climate and geography of development of the seaweed.

3.2. Determination of mineral element

The mineral elements in *Codium fragile* can be seen in table 1. In this study, our results show that the green alga oyster thief has a highest level of magnesium, calcium and iron (20.23 mg / g DM, 10.02 mg / g DM and 10.5 mg / g DM) compared the other mineral elements. Previous studies showed that these ions play an important role in the body. For example, Ca, P and Mg play a crucial role in the formation of bones and teeth, while the Cu, Zn and Fe are involved in several antioxidant enzymes as a cofactor such as superoxide dismutase and catalase. Indeed, a study of (Anantharaman & al. 2010) [1], reported that some species of green algae such as *Ulva reticulata* and *Halimeda macroloba* collected from the coast of India have high levels of minerals (Zinc, iron, Mg, Mn, P).

3.3 Determination of total phenolic content

Analysis of samples of phenolic compounds (Figure2) revealed that brittle *Codium* fragile present a higher contents AG 40 meq / g extract for three types of solvent. The methanol extract from the green algae is the richest in these compounds (61.2 meq AG / g extract). Although the methanol has been frequently used and recommended for the extraction of phenolic compounds (Falleh & al. 2008) [9], because of its polarity and its good solubility for these composent. Recent studies have shown that most of the marine algae (*Halimeda tuna, Turbinaria conoides and Gracilaria foliifera*) contain phenolic compounds with antioxidant activities of various degrees (Gopalraj & al. 2011) [11]. Generally, brown algae contain higher levels ployphenols that green and red algae.

3.4 Determination of flavonoid content

Quantitative determination of the total flavonoids method reveals aluminum trichloride with a high content in flavonoids that can reach grades 48.8 meq QE at the methanol extract among *Codium fragile* followed by ethyl acetate and then the hexane (Figure3) consideration of these results can highlight a significant linear correlation ($p \leq 0.05$) between the content of the extracts of flavonoids and phenolic compounds. This is logical since the flavonoid compounds represent the majority of polyphenols. Thereby operate flavonoids as antioxidant compounds and this is due to the presence of the highly reactive phenolic hydroxyl groups which give the ability to overcome the seaweed state of oxidative stress (Burtin 2003) [2].

3.5 Total condensed tannin content

Analysis of algal extracts of condensed tannins (Figure4) revealed low levels of tannins in the three extracts of the green alga *Codium fragile* not exceeding 29.68 catechin meq / g extract even at the methanol extract. (Cox & al. 2010) [3] Showed that the content of tannins from the brown seaweed, *H. elongata* was significantly higher than the other species ($p < 0.05$). Brown species *H. elongata, L. saccharina* and *L. digitata* contained 38.34, 6.17 and 5.44 mg CE/g extract, respectively. The red and green species contained lower total condensed tannins than the brown seaweeds, in the range 3.19 to 4.35 mg CE/g of seaweed extract. Many studies have shown that phlorotannins are the only phenolic group detected in brown algae (Jormalainen & Honkanen, 2004; Koivikko & al. 2007) [16, 18].

3.6. Total antioxidant activity

Analysis of the antioxidant status of the algal species showed that 3 types extracts (hexane, ethyl acetate, methanol) showed high antioxidant capacity that can reach 55.99 meq AA at the methanol extract (Figure5). These results are in accordance with those of (Matanjun & al 2008) [21]. who reported that the antioxidant activity of both green algae (*Caulerpa lentillifera* and *Caulerpa racemosa*) show significant antioxidant activity.

3.7 DPPH activity

The DPPH test provided information on the reactivity of test compounds with a stable free radical because of its odd electron, 2, 2-Diphenyl-Picryl Hydrazyl radical (DPPH) gives a strong absorption band at 517 nm in visible spectroscopy (deep violet color). The efficacies of anti-oxidants are often associated with their ability to scavenge stable free radicals (Wang & al. 1999) [26]. In the present study, methanol extracts exhibited comparable DPPH radical scavenging activity with IC_{50} values 18 µg ml^{-1} compared to vitamin E (IC_{50}=12 µg ml^{-1}) (Figure 6). Recent studies conducted by Zubia & al. (2007) [28], showed that the three algal species (*Avrainvillea longicaulis*, *Chondria baileyana* and *Lobophora variegata*) have significant anti-radical activity with low IC_{50} in the range of 1.44 ± 0, 01 mg / ml, 2.84 ± 0.07 mg / ml and 0.32 ± 0.01 mg / ml respectively, the results obtained in this work indicate the existence of a remarkable and significant linear correlation between the anti-radical power methanolic extracts essentially different algae and their contents of phenol compounds (p <0.01) and flavonoid (p <0.01). Indeed, the phenolics, and flavonoids in particular are recognized as potentially having antioxidant substances with an ability to trap the radical species and reactive oxygen species (Javanovic & al. 1994) [15].

3.8 ABTS radical scavenging activity

The results of the ABTS free radical scavenging assays are listed in figure 7. Very potent potential ABTS radical scavenging activity is showed in methanol extract (IC_{50} = 4.5 mg ml^{-1}) compared to Vitamin C (18.64 mg ml^{-1}). The ABTS radical cation decolorization assay, showed quite similar results compared to those obtained in the DPPH assay (figure 6). Recently, found positive correlations between phenolic

and happy antioxidant activity tested using the Oxygen Radical Absorbance Capacity (ORAC), ABTS and the 1, 1 - Diphenyl-2-picrylhydrazyl (DPPH) assays. Malthus the Ability of a compound to scavenge ABTS • + radical can demonstrate Oxygen Radical Absorbance capacity.

3.9. *In vitro* anti-hypertensive activity

One of the therapeutic approaches to hypertension is the use of angiotensin converting enzyme (ACE) inhibitors. Thus, the renin-angiotensin system plays a pivotal role in the regulation of blood pressure, electrolytes and blood volume, and in the pathophysiology of cardiovascular diseases. In fact, the physiological function of ACE is related to the regulation of blood pressure and electrolyte homeostasis by converting angiotensin I into potent vasoconstrictor angiotensin II and by inactivating bradykinin (Ondetti & Cushman, 1982) [22].

Marine polysaccharides are recognized as important sources of ACE inhibitors, in this context, we were interested in the *in vitro* study of the effect of *Codium fragile* extract (CFE) on the angiotensin converting enzyme (ACE) inhibitory activity *in vitro* (Figure 8).

The results of the present study revealed that the ACE activity of CFE was concentration dependent. The activity increases with the raise of concentrations sample. The methanol *Codium fragile* extract has a strong inhibition of the enzyme activity of ECA *in vitro* of 66.84% ± 2.74 at a concentration of 0.8mg / ml and with a low IC_{50} of the order of (0.59mg / ml). These results are consistent with previous studies of.

The results presented in this work suggest that CFE could improve the biological properties of functional foods and it could be an attractive ingredient for future nutraceutical application against hypertension.

Table 1: Mineral composition of *Codium fragile* alga.

Mineral elements	Content (mg/100 g dry matter)
Calcium	10.02
Magnesium	20.23
Zinc	1.1
Cuivre	1.3
Fer	10.5

All values given are means of three determinations (X±SD); SD: standard deviation.

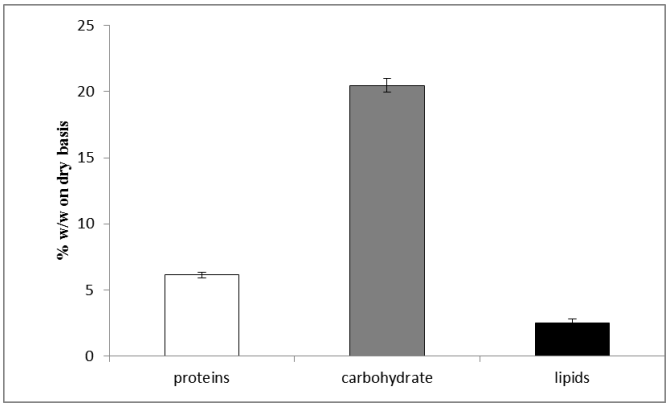

Fig 1: The proteins, carbohydrate and lipids concentration (%) of *Codium fragile.*

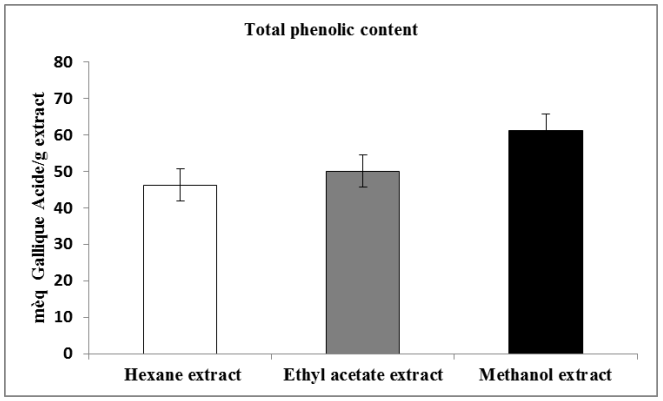

Fig 2: Total phenolic content of *Codium fragile*. Data are expressed in mean ± SEM (n = 3).

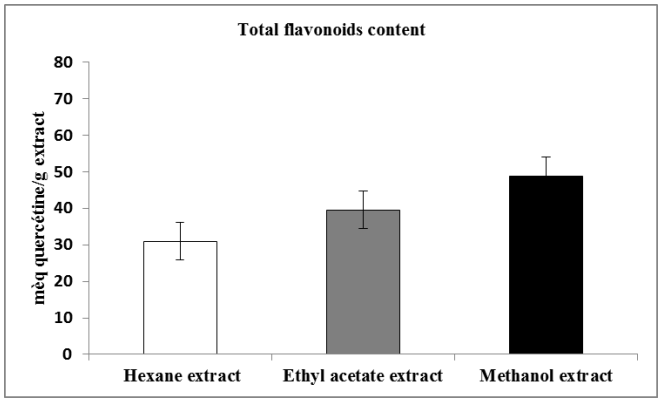

Fig 3: Total flavonoids content of *Codium fragile*. Data are expressed in mean ± SEM (n = 3).

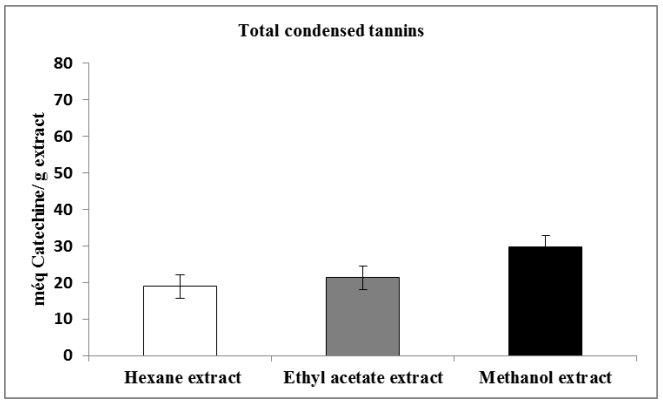

Fig 4: Total condensed tannins of *Codium fragile*. Data are expressed in mean ± SEM (n =3).

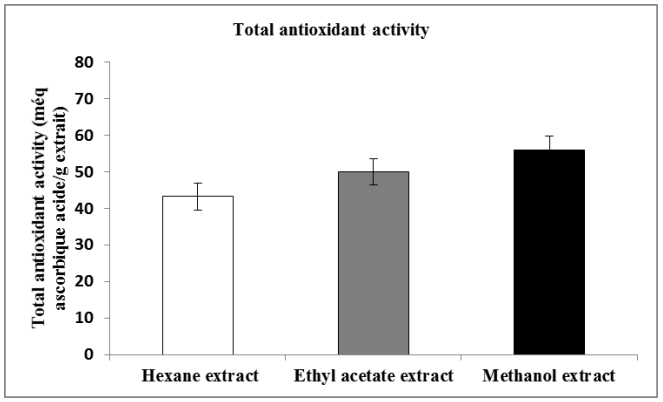

Fig 5: Total anti-oxidant activity of *Codium fragile*. Data are expressed in mean ± SEM (n = 3)

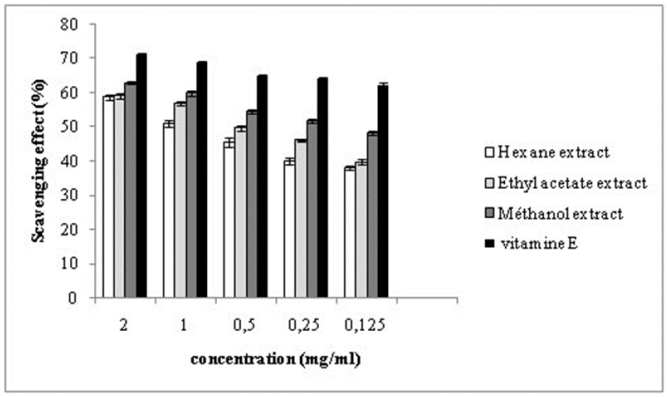

Fig 6: DPPH free radical scavenging activity of *Codium fragile* extracts. Data are expressed in mean ± SEM (n = 3).

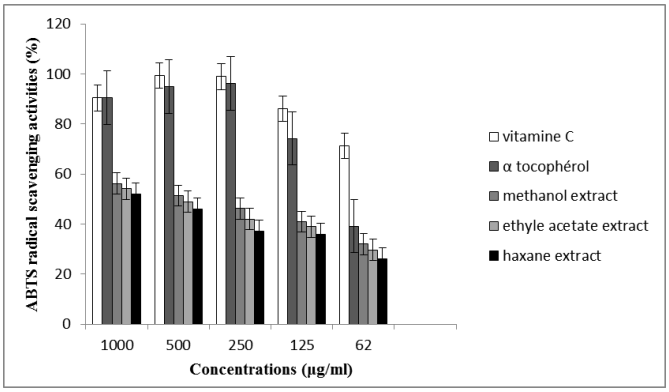

Fig 7: ABTS radical-scavenging activities of *Codium fragile* extracts. Data are expressed in mean ± SEM (n = 3).

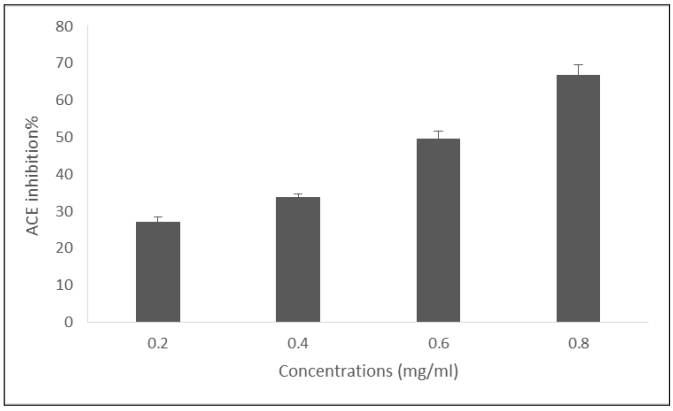

Fig 8: Anti-hypertensive activity of *Codium fragile* metanol extract. Data are expressed in mean ± SEM (n = 3).

4. Conclusion

The results showed that the three algal extracts have a strong wealth in phenolic compounds, flavonoids with low levels of condensed tannins haves a very interesting antioxidant status, allowing that should be considered as an important source of phenolic compounds that could be used as food preservatives and in other industrial and pharmaceutical field.

In addition, the best antihypertensive effect was observed for the methanolic extract, it was noticed that the extents of this biological activities of *Codium fragile* extracts are in accordance with their phenolic compounds.

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