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Assessment of *In Vitro* antioxidant activity and phytochemical constituents of *Amphora caffeaeformis* Microalgae

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Abstract

The study aims to examine in *vitro* antioxidant activity of the extract of the brown microalga *Amphora coffeaeformis*. Antioxidant activity was evaluated by three methods: free radical scavenging, determination of total phenolic compounds and determination of total flavonoid content. The total content of phenol and flavonoid in the extract was determined as gallic acid equivalent, and rutin equivalent, respectively. The present study shows that tested algae aquatic extract demonstrated relatively strong antioxidant activity. It suggests that this microalga may be used as a good source of the natural antioxidant agent.

Keywords: algae; biological activity; *Amphora caffeaeformis.*

1. Introduction

Microalgae are eukaryotic unicellular cells that combine several advantages for the development of biotechnological applications: high biodiversity, photosynthetic yield, growth, productivity and metabolic plasticity that can be orientated using culture conditions [1,2]. Some of these metabolites are molecules of interest such as pigments, polyunsaturated fatty acids, polysaccharides, vitamins and sterols which can be introduced as dietary supplements in human nutrition and animal feed [3,4]. In addition, most of them are bioactive molecules with antiinflammatory, antibacterial, anti-UV, antifungal, anticancer, and/or antioxidant activities which may bring added value to cosmetics, nutraceuticals, or food products [5].

The demand for natural antioxidants as an alternative to synthetic antioxidants has increased [6,7]. Indeed, many synthetic antioxidants (e.g., butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT)) are considered to have a carcinogenic and/or toxic effect on animal models [8]. Among the large variety of microalgae, Cyanophyceae, Chlorophyceae, Bacillariophyceae (diatoms), and Chrysophyceae are the most studied for biodiesel production [9]. It is estimated that diatoms produce about 25% of the global primary biomass [10]. In the Sfax Solar Saltern, Bacillariophyceae dominated with Dinophyceae in the least salty ponds but they are rarely abundant in hypersaline environments [11]. Microalgae are good sources of natural antioxidants [12,13]. During the photosynthesis process, they absorb solar light which is converted into chemical energy, later used in the conversion of CO_2 into carbohydrates, and lipids, and at the same time, generating molecular oxygen, which can reach locally high concentration levels. As oxygen is easily activated by ultraviolet radiation (UV) or heat from sunlight into toxic reactive oxygen species (ROS), plants and microalgae have developed a protective mechanism that consists of the preparation of antioxidant compounds able to minimize the concentration of these ROS [13].

The phenolic compounds (phenol carboxylic acids and their derivatives, catechols, flavonoids, and carotenoids, etc.) are considered to be major contributors to antioxidant capacity in microalgae [14]. It plays a relevant role in preventing oxidative damages caused by free radicals by scavenging activity, and/or has a key role in the prevention of degenerative neuropathies or diabetes or in

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preventing cardiovascular diseases or cancers, as well as exerting anti-inflammatory, anti-viral, or anti-aging activities[15].

Diatoms are single-cell microalgae that exist in most aquatic ecosystems. Diatoms are essential members of the fresh and marine phytoplankton communities [16]. *Amphora coffeaeformis* (Agardh) Kutzing is one of the most frequently abundant species in alkaline freshwater and brackish water localities [17]. A robust antibacterial activity was identified in *Amphora* sp. which was examined against Gram-negative bacteria (*Salmonella enterica* and *Klebsiella pneumoniae*) and Gram-positive bacteria (*Micrococcus luteus* and *Staphylococcus aureus*). These results promote *Amphora* sp. utilization in aquaculture nutrition and pharmaceutical and food manufacturing [18]

Many reports confirmed that *Amphora coffeaeformis* comprises carotenoids and phenolics which had potent antioxidant activity against the free radicals and inhibition of peroxidation reactions [19]. Besides, its extract indicated a promising scavenging mode of action against hydrogen peroxide and reducing DNA lesions [20]. The physicochemical composition of *Amphora coffeaeformis* is mainly the carotenoids (astaxanthin and canthaxanthin), sulphated polysaccharides, polyunsaturated fatty acids, b-glucans, and vitamins C and E, which are known as bioactive antioxidant compounds [21, 22]. In addition, various bioactive components are to be found in *Amphora* sp. Such as hexadecanoic acid (antioxidant), phytol (anti-inflammatory), 2,6-dimethyl-4[3H]-quinazolinone (anticancer) and neophytadiene (antiviral).

Rare studies have been published concerning the utilizing *A. coffeaeformis* as a feed additive in the fish diet. It is studied the supplementation of *A. coffeaeformis* at 1%, 2% and 3% in Nile tilapia (*Oreochromis niloticus*) diets and concluded that utilizing *A. coffeaeformis* as a supplement is a promising replacement for antibiotics in Nile tilapia culture, and its optimum supplementation level ranged from 1% to 2% in fish diet [23]. Gordon et al. [24] indicated that both diatoms, Navicula cf. *lenzii Hustedt* and *Amphora luciae Cholnoky*, increased postlarval abalone (*Haliotis discus hannai*) growth and survival, particularly when provided in combination, and attributed the improvement to their complementary balanced nutritional properties. It is declared that the addition of 9% diatom powder to the sea cucumber *Apostichopus japonicus* diet enhanced both the growth rate and food conversion efficacy [25]. It is conducted *Amphora coffeaeformis* is a promising feed additive and has the potential to replace antibiotics for eliminating pathogenic bacteria and preventing diseases outbreak in Nile tilapia culture by stimulating the innate immune system response [26]

In other vertebrates, El-Sayed et al. [22] concluded that *A. coffeaeformis* extract is an antagonistic agent preventing hepatic damage and the injurious effects in rats as it exhibited the highest scavenging activity against the attack of free radicals generated as a result of oxidative stress and induced by paracetamol in liver tissues.

Antioxidant molecules from microalgae are more and more considered as a potential source of natural antioxidant compounds by the food, cosmetic and nutraceutical industries as they may bring benefits to their products. However, it is very crucial to assess properly the antioxidant activity of an algal extract owing to the wide diversity of antioxidant compounds and the mode of action combined with the diversity of ROS involved. *Amphora coffeaeformis* have been fully investigated for their taxonomy, but little data were known on its biological activity. Because of this, the present study was examined in *vitro* antioxidant activity of the water extract of the microalga *Amphora coffeaeformis*.

2. Experimental

2.1. Preparation of algal extract:

The *A. coffeaeformis* alga belongs to Bacillariophyta. It was locally isolated from the drainage water of Ismailia Governorate and was collected by El-Sayed al. [22]. The extraction was done by El-Sayed al. [22]. The extraction was done briefly by incubating the fresh vegetative algal material at 50ºC for 72 hours and then crushed into powder. The dried algal powder was then extracted several times with cold water. Consequently, the extraction process was centrifuged at 10,000 rpm for 5 minutes and then filtration was done. All filtrates were collected and allowed to evaporate under vacuum at 40ºC till complete dryness and then stored in a dark place to avoid photodegradation when exposed to light. The algal extract is rich in p-coumaric acid, catechin, gallic acid and β-carotene [22]

2.2. GC-MS analysis

A.coffeaeformis extract was sent to the Analytical Chemistry Unit at institute; Agricultural Research Center, Giza, Egypt for Gas Chromatography Mass-Spectrometry (GC/MS) analysis. The Gas Chromatography – Mass Spectrometry (GC-MS) investigation was conducted by using Agilent 7890B GC system coupled to an Agilent 5977A MSD with a capillary column (0.6 m x 100 μm x 0 μm) (Agilent Technologies, Sant a Clara, CA, USA). Helium gas was used as carrier gas

with constant flow rate of 1.5 mL/min. The injector temperature and ion source temperature were established at 250 °C and 230 °C respectively. The oven temperature was established at 40 °C for 2 min, then 10 °C/min to 180 °C for 5 min followed by 10 °C/min to 250 °C for 10 min. The total GC process time was 38 min. The GC/MS was run in Scan/SIM mode and identification of sample's constituents was achieved by Agilent Mass Hunter software (NIST14.L).

2.3. Determination of the Total Phenolic Content.

The total polyphenol content of A coffaeiformis extract was determined by colorimetric spectrophotometry following the Folin-Ciocalteu method Attard, 2011 [27] using gallic acid as a standard (Fig. 1). A mixture of 100 µl of A coffaeiformis (100 µg/ml extract), 500 µl of Folin-Ciocalteu Reagent (mixture of phosphotungstic and phosphomolybdic acids is reduced to blue oxides of tungstene and molybdene) and 1.5 ml of Na2CO3 sodium carbonate (20 %) was shaken and diluted up to 10 ml with water. After 2 hours, the absorbance was measured at 630 nm (using a spectrophotometer). All determinations were carried out in triplicate. A Gallic acid stock solution of 1 mg/ml in methanol was prepared. The results were expressed in gallic acid equivalents, GAE, using a gallic acid standard curve (10-1000 µg/ml). Each of the 7 standards and 1 sample were pipetted in the plate wells in 6 replicates.

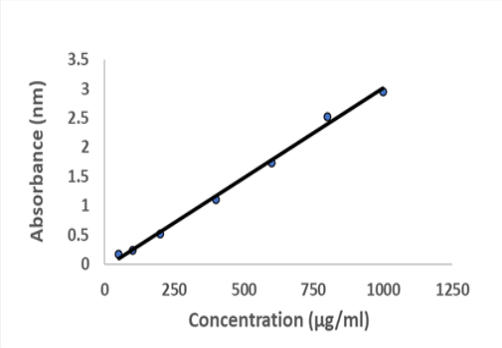

Figure (1): Gallic acid calibration curve

2.4. Determination of the Total Flavonoid Content.

Flavonoid content was determined as described by Kirnanmai et al. [28] with some modifications. An appropriate dilution of the extract was mixed with the same volume of 100 µl of 20 % AlCl3 in methanol solution (5% acetic acid in methanol). The mixture was allowed to react for 40 min and the absorbance was read at 420 nm against a blank sample without reactants. Values were determined from a calibration curve prepared with Rutin (ranging from (10-1000 µg/ml) (Fig. 2) and expressed as mg of Rutin equivalent per gram of dry weight. All determinations were carried out in triplicate. Each of the 7 standards and 1 sample were pipetted in the plate wells in 6 replicates.

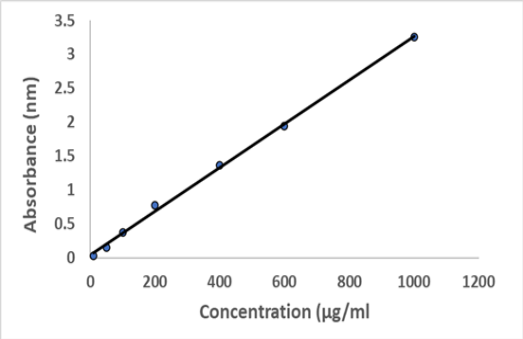

Figure (2): Rutin calibration curve

ORAC The assay was carried out according to the method of Liang et al. [29], with minor modifications.

2.4.1. Standards and samples preparation

2.4.1.1. Trolox Standard for ORAC assay:

The trolox stock solution of 1mM in methanol was prepared, and 9 serial dilutions were prepared in the concentrations of 400, 300, 200, 150, 100, 75, 50, 25 and 12.5 µM.

2.4.1.2. A. coffeaeformis preparation

the sample was provided as 4 mg/mL. From which 0.25mL was diluted with 0.75mL ethanol so that the concentration reaches 1mg/mL. 12.5 µL of the prepared sample were incubated with 75 µL fluoresceine (10 nM) for 30 min at 37C◦. Fluorescence measurement (485 EX, 520 EM, nm) was carried out for three cycles (cycle time, 90 sec.) for background measurement. Afterward, 12.5 µL of freshly prepared 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH) (240mM) were added immediately to each well. Fluorescence measurement (485 EX, 520 EM, nm) was continued for 2.5 hrs (100 cycles, each 90 sec). Data are represented as means (n=3) ± SD and the antioxidant effect of the compound/extract was calculated as µM Trolox equivalents by substitution in the linear regression equation 1. Y= 32356.3X+989769.9 (R2= 0.9957). Results of the samples are presented as µM TE/ mg sample using the linear regression equation extracted from the following calibration curve (linear dose-inhibition curve of Trolox) (Fig. 3).

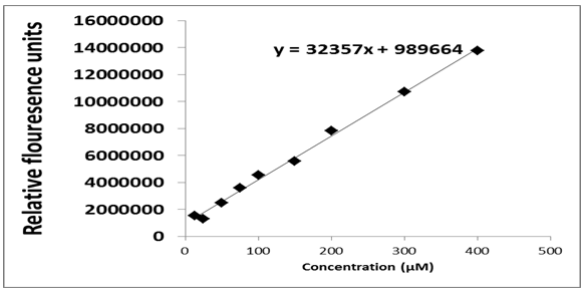

Figure 3: Trolox standard curve

2.4.2. Statistical Analyses.

Data was analyzed using SPSS programme (version 20) through one-way analysis of variance (ANOVA). Data were expressed as mean ± standard deviation (SD). P values < 0.05 is significant.

3. Results and discussion

Essential source of new chemical substances with potential therapeutic effects is thought to be obtained from medicinal plants. The antioxidant contents of medicinal plants may contribute to protection against diseases [30]. Natural antioxidants have attracted a great deal of public and scientific attention because of their health-promoting effects [31]. An imbalance between the production of reactive oxygen species (ROS) and the activity of the antioxidant defences leads to oxidative stress. In the pathology of several human diseases such as atherosclerosis, inflammation, cancer, rheumatoid arthritis, and neurodegenerative diseases like Alzheimer's disease and multiple sclerosis, ROS has been implicated [32]. Attention has been gained on antioxidant agents of natural origin due to their abilities to scavenge free radicals [33]. Antioxidant capacity is associated with compounds that can protect a biological system against the damaging effect of ROS and reactive nitrogen species (RNS) [34].

3.1 GC-MS analysis results

GC-MS analysis revealed that, 26 majors different phyto-components were recognized in the water extract of *A. coffeaeformis* along with their retention time (RT), molecular formula, and peak area (figure 3). The major identified bioactive constituents (table 1) are characterized by the antimicrobial, anticancer, antioxidant and anti-inflammatory.

From GC-MS spectra results, some compounds have been formerly stated to possess anticancer activity, including squalene [35] and 3',4',7-trimethylquercetin [36] which was found to exhibit cytotoxic effect against cancer cells; docosan [37] and heneicosane [38] which own antimicrobial activities. In addition, other components in A. coffeaeformis extract have been shown to display antioxidant activity such as the phytol [39], squalene [35] and hesperetin 7-neohesperidoside [40], while others show anti-inflammatory activities as nerolidol [41] and phytol [39].

Table 1: Gas Chromatography Mass-Spectrometry Activity of A *coffaeiformis extract*

No.	Retention Time	Compound Name	Peak area (%)
1	6.404	1-Pyrroline, 2-phenyl-	3.1
2	10.341	Heptacosane	3.98
3	10.726	Docosane	2.12
4	11.407	3',4',7-Trimethylquercetin	2.63
5	11.932	Heptacosane	5.1
6	12.592	Norphytan	3.94
7	12.826	Phytol	6.85
8	12.957	Geranyl isovalerate	5.8
9	13.088	(S)-(-)-Citronellic acid	4.63
10	13.351	Squalane	3.73
11	13.613	Hexadecane	1.63
12	13.863	(+)-Nerolidol	4.34
13	14.417	Eicosanoic acid	2.83
14	14.667	Pentacosane	2.53
15	14.876	Dotriacontane	2.37
16	15.024	Octacosanol	3.02
17	15.803	Nonacosane	4.72
18	16.529	Eicosane	2.51
19	17.48	Octadecanoic Acid	1.71
20	17.907	Heneicosane	18.14
21	18.776	Salicylic acid β-D-O-glucuronide	3.06
22	19.887	Hesperetin 7-neohesperidoside	2.35
23	20.178	1-Tricosanol	3.55
24	21.117	Hexa-hydro-farnesol	0.19
25	21.437	2-Hexadecanol	2.05
26	22.549	3,2',4',5'-Tetramethoxyflavone	3.11

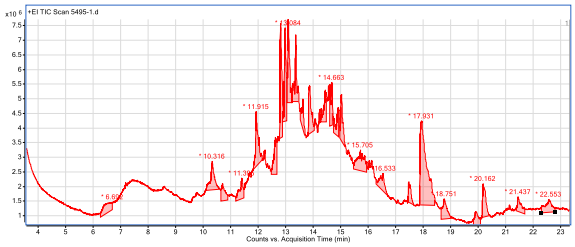

Figure 3: GC-MS chromatogram represents the separated bioactive constitutes of *A coffaeiformis* extract

3.1. Antioxidant Constituents (Phytochemical Composition):

The total polyphenolic contents of the ethanolic extracts of A. coffeaeformis were determined using the diluted Folin-Ciocalteu reagent. Results from our study show that the total polyphenol, and flavonoids, were 37.73±1.83 mg/g (gallic acid equivalent) and 12.69 ± 2.10 mg/g (rutin equivalents) (Table 1 and Table 2).

Table 1: Total polyphenolic content of aquatic extracts of *A. coffeaeformis*, data presented as mean ± SD.

	Average reading at 630 nm	Concentration (µg/mL)	Total phenolic content (mg/g extract)
A coffaeiformis	0.18	75.45±1.83	37.73

Table 2: Total flavonoids content of aquatic extracts of *A. coffeaeformis*, data presented as mean ± SD.

	Average reading at 360 nm	Concentration (µg/mL)	Total flavonoids content (mg/g extract)
A coffaeiformis	0.12	25.38±2.10	12.69

3.2. Free radicals and antioxidant activity assays:

3.2.1. *Oxygen Radical Absorbance Capacity in the water Extracts of the A. coffeaeformis*:

Reactive oxygen species (ROS) are free radicals that contain unpaired electrons which make them extremely unstable, even though they are byproducts of normal aerobic metabolism [35]. ROS include superoxide anion, perhydroxyl radical, hydrogen peroxide and hydroxyl radical. Another type of free radical is reactive nitrogen species (RNS) derived from nitrogen, such as nitric oxide, nitrogen dioxide, and peroxynitrite radicals [36]. The imbalance between ROS effects and the detoxification capacity of a biological system is the predominant factor of oxidative stress[37]. Oxidative stress is involved in many human diseases such as Asperger syndrome, Alzheimer's disease, aging, and age-related diseases. Moreover, it is confirmed that oxidative stress plays a crucial role in the pathology of cancer, neurodegenerative diseases, and inflammatory diseases. Thus, ROS, oxidative stress and antioxidants are of great interest to many chemists and biochemists. The ORAC assay is used to assess the antioxidant activity of biological substrates which range from pure compounds, that is, melatonin and flavonoids, to complex matrices such as vegetables and animal tissues [38]. It measures antioxidant inhibition of peroxyl-radical-induced oxidations and shows the radical chain-breaking antioxidant activity by H-atom transfer [39,40]. The ORAC assay uses 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) for a free radical generation. AAPH which is water-soluble has been widely used as a free radical initiator for biological studies and the hemolysis caused by AAPH allows studies involving membrane damage induced by free radicals [41].

The ORAC results (Table 3) also showed the potency of the aqueous plant extracts to protect against oxidative damage. Oxygen radical absorbance capacity (ORAC) assay has been widely accepted as a tool to test the antioxidant activity where reactive oxygen species, A. coffeaeformis extracts were with a value of 407.20 ± 10.16 µg/mL.

Table 3: Oxygen radical absorbance capacity of aquatic extracts of *A. coffeaeformis*, data presented as mean ± SD.

A coffaeiformis	Average Relative Fluoresce Unite (RFU)	Concentration (µM)	Antioxidant activity (*µ*M TE/equivalent)
	13175775	**407.20±10.16**	**407.20**

4. Conclusions

In conclusion, it can be stated that the tested Amphora sp. extract had a certain level of antioxidant activity in vitro, which suggest that Amphora sp., could be a natural antioxidant agent. Further studies should be done on the isolation and characterization of the pure compounds from this alga, which are responsible for its antioxidant activity.

5. Conflicts of interest

"There are no conflicts to declare".

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