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Determination of polyphenols, tannins, flavonoids and antioxidant activity in extracts of two genus *Ircinia* marine sponges of Atlantic Morrocan coast

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Abstract

Marine sponges are multicellular invertebrates that are part of the marine biomass from the Lower Cambrian period. They are present all over the planet and live in many aquatic ecosystems. It is found that the marine sponges are the source of the greatest number of metabolites isolated and characterized. These metabolites can beings present in high concentrations. This study is focused on the quantitative identification of organic substances such as: polyphenols, tannins, and flavonoids found in the extracts of two genus *Ircinia* marine sponges '*spinulosa* & *oros*' of Atlantic Morrocan coast by various methods. The results of this study indicate that the extracts of *Ircinia spinulosa* are rich in flavonoids 51.48 (±2.10) (Eq mg Quercetin/mg extract) and in Tannins 0.205 (±0.001) (mg Cya/g dry matter), and for the polyphenols, the highest was found in the extract of *Ircinia oros* 15.75 (±0.95) (mg GAE/g dry matter).The evaluation of the antioxidant potential with using the radical scavenging capacity DPPH· test show that the extracts of the two sponges of the genus *Ircinia spinulosa* &*Oros* contain antioxidant activity respectively 28.25%, & 9.34%. The antioxidant activity was observed in the crude extract of *Ircinia spinulosa*, which displayed a similar activity to synthetic BHT. Gas chromatography/mass spectroscopy (GC-MS) analysis of the extract revealed that the active compound was identical to synthetic BHT. The extracts sponges give good results, indicating that it possesses a significant amount of polyphenols, flavonoids, tannins, and an antioxidant activity.

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Introduction

The marine environment has proven to be a rich source of chemical and biological diversity and marine organisms are highly potential sources for commercializing interesting compounds for various industrial applications (Valliappan *et al*., 2014). Among the marine organisms, sponges and seaweeds represent one of the richest sources of natural primary metabolites and antioxidants (Ngo *et al*., 2012). These metabolites can beings present in high concentrations. Many of these molecules are responsible for stability and adapt-ability of marine organisms to a changing environment (Harvell *et al*., 2007).

Sponges are considered as a potential gold mine for the isolation of promising bioactive compounds for human welfare as numerous studies report about isolating antibacterial, antiviral, antioxidant and anti-tumour compounds from sponges. As more than a third of all discovered new bioactive products from the sea (Nithyanand *et al*., 2011).

In general, antioxidant compounds scavenge and degrade the free radicals and other reactive oxygen species that induce tissue damage and oxidative stress (Trivedi *et al*., 2001). As recent research suggests that antioxidants can be employed as prophylactic agents against several diseases where oxidative stress plays a critical role in the a etiology of the disease (Freitas *et al*., 2012)several synthetic and natural compounds are being screened for their antioxidant properties.

The marine environment has proved to be a promising source of antioxidant compounds derived from sponges, sea weed and marine microbes exhibited good antioxidant properties (Balakrishnan *et al*., 2015).

This study is focused on the quantitative identification of organic substances such as: polyphenols, tannins, and flavonoids found in by various methods. The aim of this work is to evaluate the antioxidative properties of extracts of two genus i*rcinia* marine sponges '*spinulosa* & *oros*' of Atlantic Morrocan coast the essential by DPPH. Additionally total phenolic, flavonoid and tannins contents.

Materials and methods

Sponge Materials

The marine sponges were collected in winter 2015 at the littoral Atlantic of El-Jadida (Morocco). All the sponges were identified by Dr. Maria-Jesús Uriz, Research Professor at the Centro de Estudios Avanzados de Blanes (CEAB) and Consejo superior de investigaciones científicas (CSIC) Spain. The collected materials were immediately frozen for one night prior to extraction (El-Amraoui *et al*., 2010).

Preparation of the extracts

Each sponge was lyophilized, homogenized with ethanol at 80% (1/50mL), allowed to agitate in a dark chamber for 48 h and the solid-liquid separation is performed with a centrifuge (5000trs/min). The residue on the filter paper was again extracted with ethanol at 80% (1/50ml) for 24h. After extraction, the aqueous ethanol extracts were combined evaporated at reduced pressure.

Determination of polyphenols contents

The determination of phenolic compounds was performed according to the method of reagent Folin-ciocalteu, 2.5ml of folin (diluted 10 times) was added to 0.5ml of the liquid extract (diluted 100 times). 2mL of Sodium carbonate Na_2CO_3 (75g/L) are added. The mixture was placed in a water bath maintained at a temperature of 50°C for 5 minutes protected from light. Absorbance was measured at 760 nm by a spectrophotometer UV-3100PC VWR. The total polyphenols content is calculated from the calibration curve established with gallic acid (calibration range 0-80µg/ml).The results obtained are equivalent in micrograms/mLgallic acid per gram of the raw material (mg GAE/g) (Singleton *et al*., 1965).

The concentrations of phenolic compounds were calculated according to the following equation that was obtained from the standard gallic acid graph (Fig. 1):

$A = 0.006\ C - 0.007$ ($R^2 = 0.99$).

Determination of condensed tannins (Cyanidin equivalent content)

Proanthocyanidin content was determined with a BuOH/HCl test as described by Rhazi *et al*., 2015: 0.5 mL of aqueous extract was

added to 5mL of an acidic ferrous solution (77mg of $FeSO_4 \cdot 7H_2O$ in 500mL of Hcl/BuOH (2/3)).

The tubes were covered and placed in a water bath at 95°C for 15 min. The absorbance was read at 530 nm and results were expressed as follows: milligram of cyaniding equivalent (Cya) per gram of dry (mg Cya/g).The condensed tannins content was calculated using the formula given below:

$$\text{CyaE/gbark} = \frac{AV \times D \times M \times V2}{l \times £ \times v \times m}$$

Where A is the sample absorbance at 530 nm; V is the total reaction volume (mL); D is the dilution factor; M is the cyanidin molarmass (g mol^{-1}); V_2 is the aqueous volume extract, recovered after extraction (mL); l is the path length (cm^{-1}); £ is the molar extinction coefficient (34.700 L $mol^{-1}cm^{-1}$); v is 0.5 mL; and m is the dry weight mass of sponge (g).

Determination of flavonoids (Quercetin equivalent content)

The flavonoids are quantified by a colorimetric method with aluminum trichloride ($AlCl_3$) and sodium hydroxide (NaOH). Aluminum trichloride forms a yellow complex with flavonoids and soda form a pink complex which absorbs in the visible at 510nm (Benariba *et al*.,2013).(0,5)ml of each extract (or *quercetin*) is mixed with 2ml of distilled water, 0.15 ml of 150g/L solution of sodium nitrite ($NaNO_2$), 0.15mL of a 100g/L solution of aluminum trichloride ($AlCl_3.6HA_2O$). After 6 min incubation at ambient temperature and addition of 2mL NaOH (1mol/L), the volume was brought to 5.0mL distilled water, and after a further incubation of 15 min, the absorbance measured at 510 nm.

A standard range was performed under the same operating conditions using quercetin at different final concentrations (0,1.56, 3.125, 6.25, 12.5, 25, 50, 100µg/ml). The results obtained expressed in µg Eqquercetin/mg of extract is calculated using the following formula:

$$[\text{Flavonoid}] = \frac{a \times f}{c}$$

Where a: flavonoid concentration (ug/ml) determined from the standard, f: dilution factor c: concentration of the extract.

Determination of DPPH, The activity of trapping of the radicals

The extracts were dried after elimination of the solvent by using a rotary evaporator in 60°C under vacuum, then frozen and freeze-dried with a lyophilizator (ALPHA-2 LD) shielded from the light, and obtained a powder dry product. The antioxidant power of extracts was estimated with the method described by Yuanting Zhang (Yuanting *et al*., 2013), Based on the reduction of the free radical 1,1-diphenyl-2-picrylhydrazyl DPPH., who is relatively stable. The extracts which have an antioxidant power changes their tint (coloring) from violet to yellow during the reduction of diphenylpicrylhydrazine DPPH. This essay requires the preparation of a range of concentrations of the samples from 0 to 10000µg/ml. In the assay, 0.5mL of diluted extract was mixed with 1.5mL of 0.1mmol/L solution of DPPH in ethanol. The mixture was incubated in the dark at room temperature for 30 min, and the absorbance at 517 nm was measured. All tests were performed in triplicate. The scavenging capacity was calculated as:

$$\%(AA) = [(Ac - As)/Ac] \times 100$$

Where Ac is the absorbance of the control and As is the absorbance of the tested sample after 30 min. Butylatedhydroxytoluene (BHT) was used as standard. The free radical scavenging capacities of samples were expressed as IC_{50} values (concentration of samples required to scavenge 50% of DPPH· radicals).

GC-MS Conditions

Gas chromatography (GC) coupled with mass spectrometry (MS) was used for the trace analysis of BHT in the samples. The GC/MS analyses of the extracts were carried out by injection of a 1ml aliquot of each extract into the injector using the split less mode. The injector temperature was kept at 180°C. Separation was performed by gas chromatography using a capillary column with a film thickness of 0.25 mm. Helium served as the carrier gas with a back pressure of 70kPa. The GC oven temperature was first held at 60°C for 0 min, then increased to 120°C at a rate of 15°C/ min, then to 260 at a rate of 20°C/ min,

and kept at the final temperature for 15 min. Mass spectrometric analysis with the Electron impact (EI) mass spectra were obtained at 70eV electron energy and monitored from 50 to 600 m/z.

Results and discussion

Dosage of polyphenols, tannins and total flavonoids

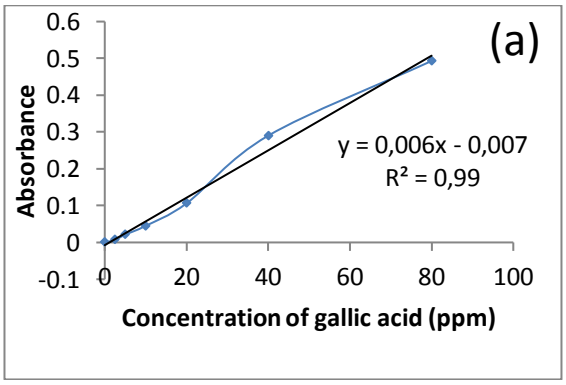
The tests performed on crude extracts were used to determine the polyphenols contents, tannins and the flavonoids were quantified and presented in (Table 1) content of two genus *Ircinia* marine sponges *spinulosa* & *oros*.

The polyphenols and the flavonoid content were calculated relative to a reference gallic acid and Quercetin after plotting a calibration curves (Fig. 1), the polyphenols content were 15.75±0.95mg GAE/g dry matter for *Ircinia spinulosa and* 25.20±0.78mg GAE/g dry matter for *Ircinia oros*.

The condensed tannins content determined with a Bu OH/HCl test assay is close to that of *Ircinia spinulosa* (0.25±0.001mg Cya/gdry matter) and higher than that of *Ircinia oros* (0.023±0.001mg Cya/g dry matter) (Sub *et al*., 2007). The quantitative analysis (Table 1) show that *Ircinia spinulosa* is richer in total flavonoids (51.28±2.10µg Eqquercetin/mg of extract) that *Ircinia oros* (10.83±1.76µg Eqquercetin/mg of extract).

Table 1. The yields in polyphenols, Tannins, and Flavonoids of the extracts.

Marine sponges	Polyphenols (mg GAE/gdry matter)	Tannins (mg Cya/gdry matter)	Flavoloids (µg Eqquercetin/ mg of extract)
Ircinia spinulosa	15.75±0.95	0.25±0.001	51.28±2.10
Ircinia oros	25.20±0.78	0.023±0.001	10.83±1.76



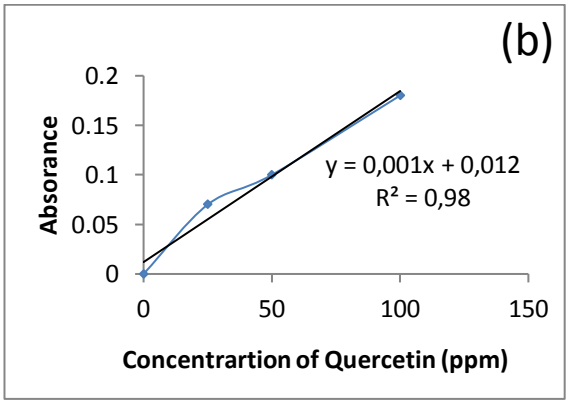


Fig. 1. The calibration curves of gallic acid (a), and Quercitin (b).

Radical scavenging (antioxidant) activity

The graph illustrated in (Fig. 2), we show that all the extracts tested are capable of neutralizing the radical DPPH• over time and depending on the concentration of antioxidants, this results in the decrease of the initial concentration of the radical DPPH• (Benhammou *et al*., 2011).

The results were expressed in percentage of inhibition of DPPH• the capacity of radical scavenging of the crude extracts of the marine sponge *Ircinia spinulosa* and *Ircinia oros*.

The IC_{50} of 27.10 ppm was determined for the BHT positive control. (Fig. 3.) shows that the extracts have different antioxidant activities.

The extract of the sponge *Ircinia spinulosa* has the best total antioxidant capacity of about 28.25 %, while the extract of the sponge *Ircinia oros* reveal a reducing activity of the order of 9.34 %.

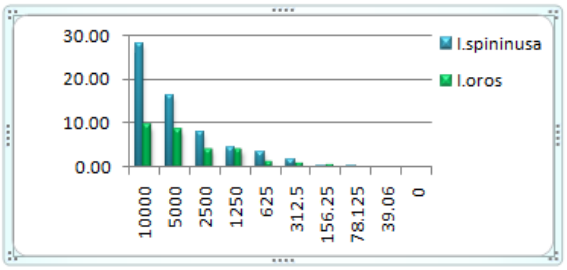


Fig. 2. Reducing power of the extracts studied.

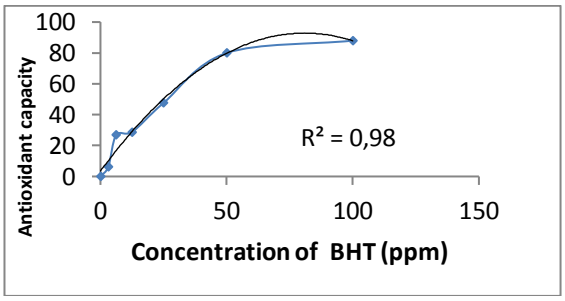


Fig. 3. Reducing power of the positive control BHT.

GC/MS analysis

The gas chromatogram of the extract of *Ircinia spinulosa* exhibited a distinct peak with a retention time identical to that of the synthetic BHT compound (Fig. 4). And the mass spectrum of the fragmentation pattern of the extract of *Ircinia spinulosa* exhibited a molecular ion [M] + at m⁄z = 220 (Fig. 5).

The base peak was observed at m⁄z = 205 mainly due to loss of a methyl group corresponding with [M-CH3]+ and a second fragment at m⁄ z = 177 corresponding with [M-C2H3O]+ (Bakthavachalam *et al*., 2008). The fragmentation pattern of this extract was identical to the mass spectrum of a synthetic BHT compound (Fig. 6.) and the data published in the NIST library for the BHT compound.

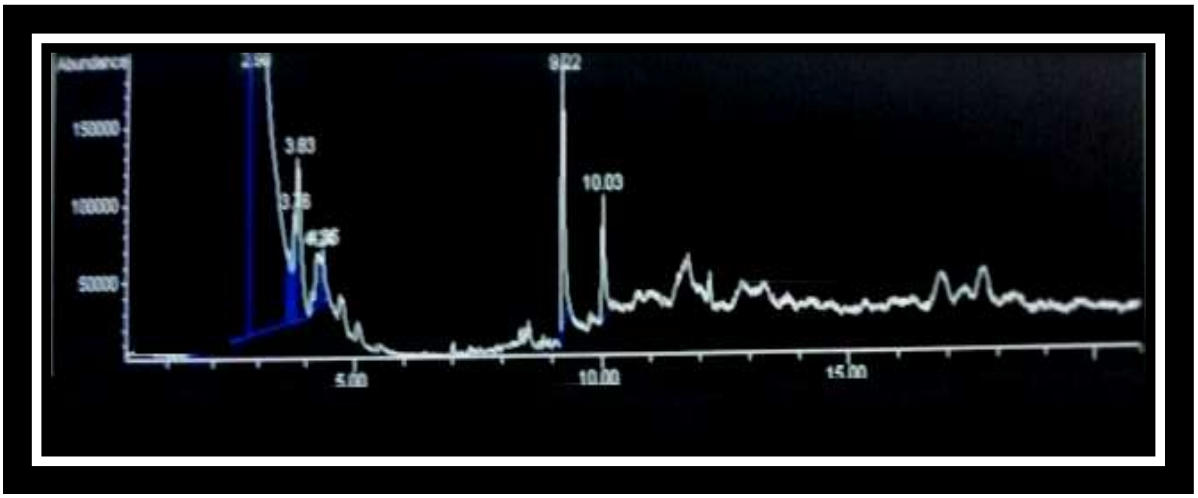


Fig. 4. Total ion chromatogram analysis of the extract of *Ircinia spinulosa*.

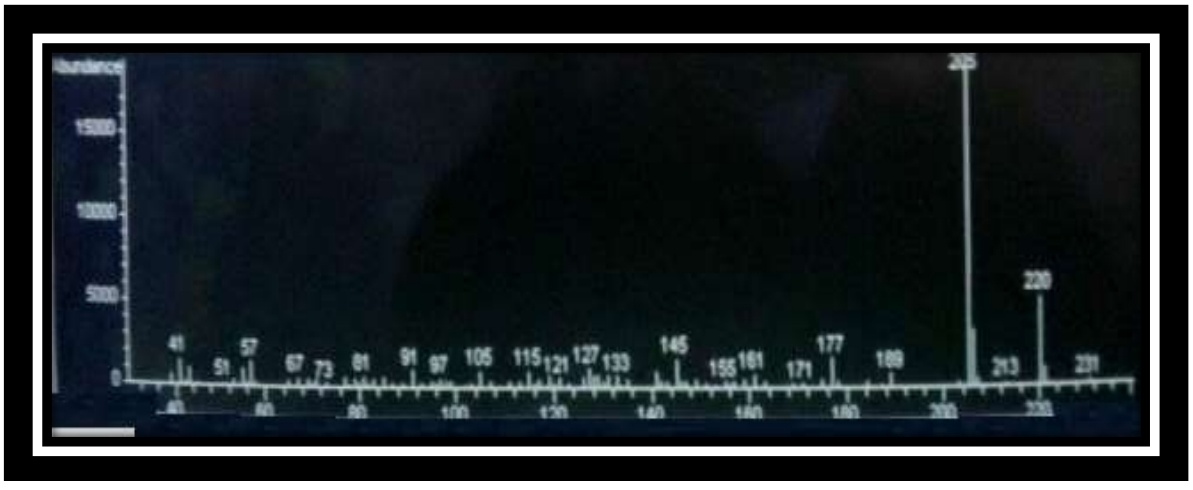


Fig. 5. Mass spectrum showing the fragmentation pattern of the extract of *Ircinia spinulosa*.

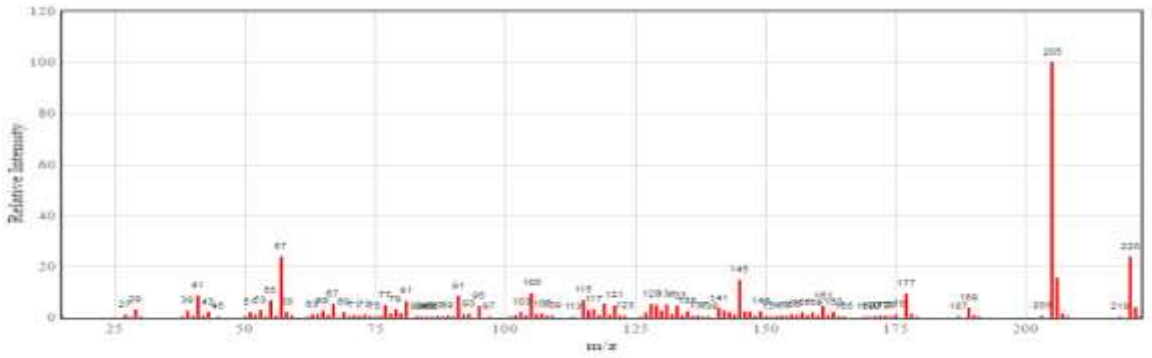


Fig. 6. Mass spectrum showing the fragmentation pattern of the BHT (NIST).

The results of this study indicate that the extracts of *Ircinia spinulosa* is rich in flavonoids 51.48 (Eq mg Quercetin/mg extract) and in Tannins 0.205mg Cya/g dry matter), and for the polyphenols, the highest was found in the extract of *Ircinia oros* 15.75mg GAE/g dry matter. the extract of *Ircinia spinulosa* produce a natural BHT that exhibits antioxidant activity similar to that of synthetic BHT. Thus, this sponge species has the potential to be used as an alternative commercial source for BHT production.

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