World Journal of Innovative Research (WJIR)
ISSN:2456-8236, Volume-1, Issue-2, December 2016 Pages 09-13

Extraction and Recovery of Bioactive Metabolites from Marine Sponge "*Ircinia spinulosa*"

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***Abstract*- Oceans cover more than 75% of our planet. In some ecosystems, such as coral reefs, experts believe that biodiversity is greater than that of tropical forests. Morocco, with its double facade Atlantic and Mediterranean, long of more than 3500 km, is a country deeply influenced by the sea, which implies the existence of thousands of marine species by reports in other countries. Currently, marine organisms are a very important source of new molecules in pharmacology and thus in the development of new bioactive products. Sponges, in particular, given their very primitive origin and persistence during evolution, have developed a chemical defense system. This study aims the biological valorization of crude extract from a marine sponge collected in Atlantic coastal El Jadida: *Ircinia spinulosa*. The chemical study of *Ircinia spinulosa*, marine sponge was realized for the first time in Morocco, and revealed that this sponge contains different levels of primary and secondary metabolites. Analysis of fatty acids in the lipid extract of *Ircinia spinulosa* is performed by gas chromatography coupled with mass spectrometry (GC-MS) was used to determine the predominance of acid Palmitic. This molecular diversity of sponges has a large deposit of biological and biomedical potential. What pushed us to continue the research to isolate new and original molecules.**

Index terms*- *bioactive molecules, fatty acids, marine sponges, Ircinia spinulosa, primary metabolites, secondary metabolites

I.INTRODUCTION

The natural products often confused with the secondary metabolites are derived from natural sources such as plants, animals or microorganisms. These natural products inspired many developments in organic chemistry, in particular leading to advances in synthesis methodologies and the possibility of having similar pharmacological and pharmaceutical properties improved [1]. Marine organisms have often been less studied than terrestrial organisms because of their difficult access and isolation although they appear as single sources of natural bioactive molecules often having new modes of action [2]. But the marine organisms, such as sponges represent one of the richest sources of natural primary and secondary metabolites [3]. Recently, the research focus for natural products has been shifted from terrestrial to marine sources because of the superior chemical and biological novelties of marine compounds [4]. In general, natural molecules isolated from marine environment show higher and more significant bioactivity than those from terrestrial environment [5] . One important group of metabolites that have been much studied is the phospholipid fatty acids from these invertebrates since they are compounds that are always present in the sponges and show a good number of structural variations from species to species [6]. Marine sponges of the genus *Ircinia* (order Dictyoceratida) are known as common sources of furanosesterterpene tetronic acids and of linear C21 furanoterpenes (1,2) [7] .

This study is focused on the identification of organic substances such as: Alkaloids, tannins sterol, saponins, free Quinones, and Flavonoids found in by various methods.

Also we studied the chemical composition of fatty acid in the hexanic extract of marine sponge '*Ircinia Spinulosa*' of Atlantic Morrocan Coast by gas chromatography coupled with mass spectrometry (GC-MS).

II. MATERIALS AND METHODS

A. Sponge Materials

The marine sponge is collected in 2015 at the littoral Atlantic of El-Jadida (Morocco), and 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 material is immediately frozen for one night prior to extraction [8].

The determination of the chemical composition has consisted in dosage of the primary metabolites and to search some secondary metabolites know for their biological activity [9]. All the tests were repeated three times to estimate the statistical quality of the results.

B. Primary metabolism

Dosage of lipids

The dosage of lipids was realized by using a soxhlet. The sample was continuously extracted with hexane which gradually dissolves the fat. The solvent containing the fat returns into the balloon by successive spills. Once the solvent

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was evaporated, nd the fat was weighed and percentage was calculated using following formula [9] :

$$\% \, lipids = \frac{M(lipids) \times 100}{M(sample)}$$

Dosage of total sugars

The total of sugars was performed using phenol/ silfuric acid "Dubois method" [10] . 1ml of phenol at 5% w / v (5 g of phenol in 100 ml of ice-cold distilled water), 1 ml of sample is added, the whole is homogenized by a vortex, then 5 ml of concentrated H_2SO_4. After were incubated in a water bath at 100 ° C for 5 min. On leaving the bath, the sample is stored for 30 minutes in the dark. Reading the sample absorbance at wavelength 490 nm is carried out using a spectrophotometer. To determine the calibration curve ten concentrations of standard solutions ranging from 0 to 70 mg / ml were prepared from a glucose standard solution (w / v) (1 ml for each solution), physiological saline is used as diluent. The quantity of sugars Q (mg) contained in each sample is determined using the following equation:

$$Q = 1 \times 20 \times c$$

C is the concentration of sample sugars, released from the calibration curve.

C. Secondary metabolites: Preliminary tests

Preparation of extracts

The sponge was lyophilized, homogenized with solvent at (Ethanol-Chloroforme) 50% (1/50 mL), allowed to agitate in a dark chamber for 48 h and the solid-liquid separation is performed with a centrifuge (5000trs / min). The residue was again extracted with (Ethanol-Chloroforme) 50% (1/50 ml) for 24h. After extraction, the extracts were combined evaporated at reduced pressure [8]. The crude extract (ethanol-chloroform) was separated first with a nonpolar solvent which is the petroleum ether until exhaustion (A). The second step is the separation with a moderately polar solvent that is ethyl acetate until exhaustion (B). The last step is the recovery of the aqueous phase with a polar solvent that is methanol (C).

Research of Alkaloids

Two tests based on the ability of alkaloids combine with heavy metals or with iodine were performed [11].

Draggendorf Test

A thin layer chromatography which we will call CCM is performed for a few µl extract. The migration solvent is EtAc / MeOH / 50%. After migration, the spots are sprayed with Dragendorff reagent (tétraiodobismuthate of potassium). The appearance in visible light orange spots shows the presence of alkaloids.

Mayer test

After evaporation to dryness of the extracts, the residue is taken up in a few ml of 50% HCl. The formation of a yellow precipitate, after adding a few drops of reagent Mayer (potassium mercuritétraiodure), testifies to the presence alkaloids.

Research of tannins

1.5 g dry material was placed in 10 ml of EtOH 80%. After 15 minutes of stirring, the extracts are filtered and placed in tubes. The addition of 1% $FeCl_3$ is used to detect the presence or absence of tannins. The color changes to dark blue in the presence of gallic tannins and greenish brown in the presence of catechin tannins [12].

Research of sterol

The research was based on the Liberman-Burchard reaction [13]. To test extract A, equal volume of acetic anhydride was added and gently mixed. Then 1 ml of concentrated H_2SO_4 was added down the side of the tube. The appearance of a brownish-red ring at the contact zone of the two liquids and a greenish colour in the separation layer indicates the presence of sterols and triterpenes.

Research of saponins

Frothing test: The powdered of sponge (0.5 g) was placed in a test tube and 10 ml of distilled water was added and shaken vigorously for 30 s. It was then allowed to stand for 30 min and observed. Formation of honey comb froth indicates the presence of saponins [13].

Research of free Quinones

One gram of the powdered of sponge is placed in a tube with 15 to 30 ml of petroleum ether. After stirring and standing for 24 hours, the extracts are filtered and concentrated in a rotary evaporator. The presence of quinines free is confirmed by the addition of a few drops of NaOH 1/10, when the aqueous phase turns yellow, red or purple [12].

Research of Flavonoids

The alcoholic extract (5 ml, corresponding to 1 g of sponge) was treated with a few drops of concentrated HCl and magnesium turnings (0.5 g).The presence of flavonoids was indicative if pink or magenta-red color developed within 3 min [14].

Determination of polyphenols contents

The determination of phenolic compounds was performed according to the method of reagent Folin-Ciocalteu, 2.5 ml of Folin (diluted 10 times) was added to 0.5 ml of the liquid extract (diluted 100 times). 2mL of sodium carbonate Na_2CO_3 (75 g/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 / mL gallic acid per gram of the raw material (mg GAE / g) [8] .

D. Study of the composition of fatty acids

Preparation of methyl ester of fatty acids (FAME)

1 g of the extract of petroleum ether, is added 100 ml of an ethanolic solution of potassium or sodium hydroxide (KOH / NaOH) 1M. After refluxing for one hour in a mounting reflux, 150 ml of distilled water was added, the cold solution is extracted with 4 times 20 ml of ethyl oxide (ethyl ether). The hydro-alcoholic phase contains fatty acids in the form of salts, and the ethereal phase containing the unsaponifiable fraction.

The basic aqueous-alcoholic phase is acidified to pH = 1 by addition of an aqueous solution of acid Chloride 50%. The thus regenerated fatty acids are extracted with 4 times 100 ml of diethyl oxide, the ether phase is washed with fractions of 100ml of distilled water until neutralization, dried over anhydrous sodium sulfate, filtered and evaporated the solvent under reduced pressure, the total fatty acids is obtained. After separation of the total fatty acids, they are methylated with 10% BF_3 in MeOH (3mL) for 15 minutes at 70 ° C. The resulting methyl esters of fatty acids (FAME) are extracted from the reaction mixture with hexane [15] .

The FAME was analyzed by Gas Chromatography coupled with mass spectrometry (GC-MS). (Capillary column 30 m, 0.25 mm in diameter. Injection temperature 260 °C. The carrier gas: helium. The ion source was 200 ° C, and the temperature of the GC-MS interface was 260° C. Programming the oven temperature was as follows: 170 ° C for 3 min, followed by an increase of 3 ° C / min to 220 ° C (10min), then another elevation of 15 ° C / min to at 280 ° C (4min).

III. RESULTS AND DISCUSSION

A. Compounds belonging to the primary metabolism

Table I: Averages levels of lipids and sugars

LIPIDS%	SUGARS (MG)
7.15±1.93	17.84±0.25

The results in Table 1 show that the extract of the sponge *Ircinia spinulosa* has a good levels of primary metabolites, an interesting lipid content (7.15 ± 1.93.), Compared with other marine sponges [16]. And the sponge has a significant proportion of sugar. So it is found that *Ircinia Spinulosa* contains a high content of primary metabolites principally; the lipids and sugars. The latter are recognized by their role in the mechanism of recognition and cellular interactions of the sponge. Some sugars may be related to proteins or lipids, thus forming glycolipids or glycoproteins, molecules implicated in many physiological processes and showed a proven pharmacological activity [17].

Table II: Determination of chemical composition of the crushed sponge

CHEMICAL SUBSTANCES	RESULTS OF TESTS
ALKALOIDS	++
TANNINS	++
STEROLS	+++
SAPONOSIDS	++
FREE QUIONONES	-
FLAVONOIDS	++

(+++: Presence in abundance, +: Presence in low quantity, -: Absence)

B. Compounds belonging to the secondary metabolism

The screening performed on the sponge *Ircinia* spinulosa yielded the following results:

As we can see in Table 2, various secondary metabolites have been highlighted in the sponge *Ircinia Spinulosa* The screening revealed the presence of Alkaloids in the extract A and B , Catechic Tannins, Sterols, Saponosids, , and the Flavonoids. Whereas, Free Quinones was absent. The screening has been performed the first time in Morocco, also *Ircinia spinulosa* has proved rich in secondary metabolites, some of which are present at low quantity, however, they should not be neglected, as its richness in flavonoids and tannins, natural antioxidants products[8], it has different pharmacological properties.

C. Determination of polyphenols

The polyphenols content were calculated relative to a reference gallic acid after plotting a calibration curves Fig 1, the polyphenols content were 4.46 ±0.5 mg GAE/g dry matter.

Phenolic compounds, or polyphenols, can range from simple molecules, such as phenolic acids, to highly polymerized compounds, such as tannins. Flavonoids are reported to be the most abundant polyphenols in human diets. The reasons for recent renewed interest in phenolics are that most phenolics possess strong antioxidant capacity in vitro and some of them have been demonstrated to be significantly bioavailable in vivo reported that the content of total phenolics was well correlated with the antioxidant capacity of dry sponge [8], [18] .

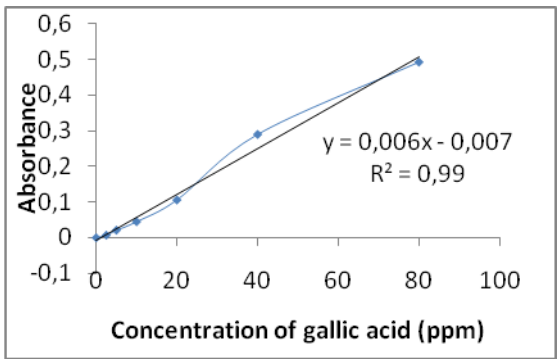

Fig 1: The calibration curve of gallic acid

D. Study of the composition of fatty acids

Table III: Profile of Fatty acids of *Ircinia spinulosa* sponge

PIC	RETENTION TIME	AREA%	COMPOUND NAMES
1	4.934	1,3	PENTACOSANOIC ACID C25:0 (HYENIC ACID)
2	4 .90	0,007	6-OCTADECENOIC ACID C18 :1 (Ω-12) (PETROSELINIC ACID)
3	8.300	51,9	HEXADECANOIC ACID C16:0 (PALMITIQUE ACID)
4	8.900	11,7	EICOSANIOC ACID C20 :0 (ARACHIDIQUE ACID)
5	9.167	3,7	DOCOSANOIC ACID C22:0 (BEHENIC ACID)
6	12.600	31,4	HEPTADECANOIC ACID C17:0 (MARGARIC ACID)

Identification of Fatty acid (FAs) was achieved by comparing molecular mass, ion fragments and abundance ratios in the NIST spectral library.

A typical total ion chromatogram obtained for samples is shown in Fig 2. The analysis led to the identification of 6 different FAs Table 3: including saturated FAs: Hexadecanoic Acid C16:0 (Palmitic Acid), Heptadecanoic Acid C17:0 (Margaric Acid), Eicosanioc Acid C20:0 (Arachidic Acid), docosanoic Acid C22 :0 (Behenic Acid), and Pentacosanoic Acid C25:0 (Hyenic Acid), and one unsaturated FA 6-Octadecenoic Acid C18:1 (ω-12) Petroselinic Acid.

Hexadecanoic Acid, Heptadecanoic Acid and (Eicosanoic Acid were the three most abundant and occupied 51.9 %, 31.4 % and 11.7 % of the total peak area, respectively (Table 3). These results agreed with the literature review on the sponge genus *Ircinia* which showed these chemical constituents to be present [6],[19],[20] .

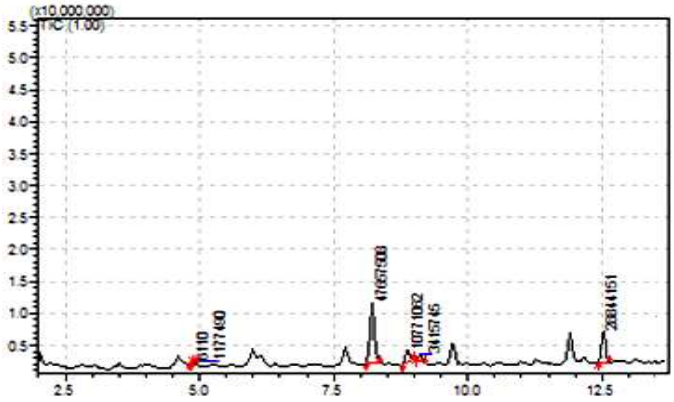

Fig 2: GC-MS chromatogram of methyl ester of fatty acids (GAME) of Ircinia spinulosa marine sponge

IV. CONCLUSION

It is found that *Ircinia spinulosa* marine sponge contains a high content of primary metabolites principally the lipids and sugars. also the sponge has proved rich in secondary metabolites, some which are present at an important quantity Alkaloids Tannins Sterols Saponosids Flavonoids, and phenolic compounds, they shoud not be neglected, because in the marine area, the proportion of new substances with potentially useful biological activity is at least 100 times higher than that encountered in terrestrial plants. Thus, these marine metabolites with therapeutic potential (antitumour molecules,antioxydant, antibiotics, antifungal, antiviral, anti-inflammatory, immunomodulatory, ..) are growing. This study allowed us to identify the variety of fatty acids in Ircinia spinulosa with a predominance of Palmitic acid, Margaric acid and Arachidonic acid. Fats potential interest in all these areas and in particular in the field of biological activity. Thus, the activity of lipids and fatty acids has a very interesting role in cell biology and bone functions. The marine environment is a valuable source of bioactive lipids.

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