International Journal of Innovation and Scientific Research
ISSN 2351-8014 Vol. 26 No. 1 Aug. 2016, pp. 14-22

http://www.ijisr.issr-journals.org/

Chemical characterization of *Cliona viridis*: Sponge of Atlantic Moroccan Coast

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ABSTRACT: The chemical study of *Cliona viridis*, marine sponge recognized by its antibacterial and antifungal activity was investigated for the first time in Morocco. Cliona viridis contains different levels of primary and secondary metabolites. The antioxidant activity was evaluated by the DPPH test and calculated in mg Vit E equivalent/g of dry weight. The fatty acid composition determined by gas chromatography (GC) showed a predominance of palmitic, oleic and linoleic acids. Furthermore, we found the presence of several sterols which cholesterol is the most abundant.

KEYWORDS: Marine sponge, *Cliona viridis*, Chemical composition, Primary metabolites, Secondary metabolites, Antioxydant activity, Fatty acid, Sterols.

1 INTRODUCTION

Marine sponges are the primitive multicellular invertebrates that are part of the marine biomass from the Lower Cambrian period (600,106 years). They are present all over the planet and are living in many ecosystems.

Physical and behavioral defenses are rare among sponges. However, being sessile and soft organisms, they have to face many dangers, such as predation, competition for living space, epibiosis or infection by pathogenic microorganisms. It seems reasonable to study these organisms even if you have to put your head under water [1]. Most of the bioactive compounds from sponges consist of anti-inflammatory, antitumor, immunosuppressive (or) neurosuppressive, antiviral, antibiotics, antifouling and antimalarial properties [2].The aim of the present work was to analyse the chemical composition of the marine sponge Cliona viridis and to investigate the antioxidant activity by evaluating the reduction of 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent. Also we studied the chemical composition of fatty acid and sterols of hexanic extract of the marine sponge *Cliona viridis*.

2 MATERIALS AND METHODS

2.1 BIOLOGICAL MATERIAL AND SAMPLING SITE

The marine sponge was collected during winter season, in January 22, 2010 at low tide to a depth of 3 meters below the waves of breezeblocks in the commercial port of Jorf Lasfar, El Jadida. After harvest, marine sponge was washed, frozen and lyophilized.

The systematic identification of the marine sponge was carried out by Dr. Maria-Jesús Uriz, Professor at the Centro de Estudios Avanzados de Blanes (CEAB) and Consejo Superior de Investigaciones Científicas (CSIC), Spain.

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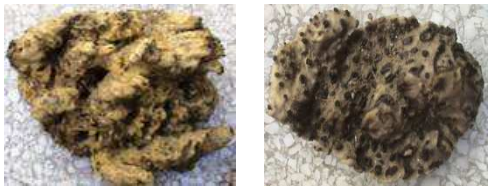

Figure 1: Collected sponge Cliona viridis

2.2 CHEMICAL COMPOSITION OF THE MARINE SPONGE

The determination of the chemical composition has consisted in dosage of the primary metabolites and to search some secondary metabolites known for their biological activity.

2.2.1 COMPOUNDS BELONGING TO THE 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 extraction was complete, the solvent was evaporated, and the fat was weighed and the percentage was calculated using following formula:

$$\%lipides = \frac{M(lipides)\times 100}{M(échantillon)}$$

DOSAGE OF TOTAL SUGARS

The dosage of total sugars was performed using phenol /sulfuric acid method as described previously [3]. Briefly, in the presence of concentrated sulfuric acid, the oses were dehydrated and the products condense with phenol to give yellow-orange complex. Then, we measured the optical density at 490nm. A standard was prepared from a solution of glucose with different dilutions ranging from 0 to 0.4mg /ml.

DOSAGE OF PROTEINS

The dosage of proteins was done by dosing the nitrogen compounds, that runs in three steps: digestion, distillation and titration [4].

2.2.2 STUDY OF THE CHEMICAL COMPOSITION OF THE MARINE SPONGE: PRELIMINARY TESTS

The tests were conducted according to the standard techniques described by Paris and Moyse [5], Bouquet [6], and Debray et al [7]. These tests consist to highlight the presence of a certain number of chemical groups known to their biological activities [8].

RESEARCH OF ALKALOIDS

The test based on the ability of alkaloids to combine with the heavy metal or with iodine was performed.

Mayer Test: 0.5 g of powder material was added to 15 ml of 70% ethanol. After a long agitation, the extract was allowed to stand until complete decantation, followed by filtration and evaporation. The residue was taken up in a few ml of 50% HCl. After adding a few drops of Mayer's reagent (mercuritetraiodure potassium), the formation of a yellow precipitate, indicated the presence of alkaloids.

RESEARCH OF STEROLS

The research was based on the Lieberman-Burchard reaction [9]. Three grams of dry ground material were macerated in 15 ml of chloroform for 20 minutes. Then, the mixture is filtered and concentrated to 2 ml. One milliliter of acetic anhydride and 1 ml of concentrated sulfuric acid were successively added. The presence of sterol compounds gives a red-brown color veering to purplish-brown.

RESEARCH OF SAPONOSIDES

It is based on the determination of the index of moss. In a 500 ml conical flask, 100 ml of boiling water and 2 g of ground material were introduced. Boiling was maintained for 30 minutes. The mixture was filtered, cooled and adjusted to 100 ml.

In a series of ten test tubes were successively introduced 1, 2, 3, 4, ... 10 ml of decocted, all tubes were filled to 10 ml with distilled water and agitated vigorously for 15 seconds. After standing for 15 minutes, the height of moss was measured. If the latter was equal to 1 cm, the dilution of the substance in the tube corresponds to the index of moss searched. An index greater than 100 was considered as a positive reaction indicating a wealth of material in saponosides.

RESEARCH OF TANNINS

1.5 g of dry ground material was placed in 10 ml of 80% methanol. After 15 minutes of agitation, the extracts were filtered and placed in tubes. The addition of 1% $FeCl_3$ can detect the presence or not of tannins. The color turns to blue-black in the presence of gallic tannins and to greenish brown in the presence of catechin tannins.

RESEARCH OF FREE QUINONES

One gram of dry ground material was placed in a tube with 15-30 ml of petroleum ether. After agitation and standing for 24 h, the extract was filtered and concentrated on a rotary evaporator. The presence of free quinones was confirmed by adding a few drops of NaOH 1/10, when the aqueous phase turns yellow, red or purple.

RESEARCH OF ANTHRAQUINONES

At the chloroform extract was added aqueous KOH 10% (v/v). After agitation, the presence of anthraquinones is confirmed by a turn of the aqueous phase to red.

RESEARCH OF COUMARINS

An infused at 10% or an alcoholic extract was examined under UV light. A bluish fluorescence indicates a positive reaction.

Note: Other compounds also show a marked fluorescence to UV. This revelation is therefore only an indication.

RESEARCH OF FLAVONOIDS (REACTION TO CYANIDIN)

At 2 ml of infused, 2 ml of hydrochloric alcohol and some magnesium turnings were added. An orange to red color appears in the presence of flavonoids.

2.2.3 DOSAGE OF THE POLYPHENOLS

EXTRACTION OF PHENOLIC COMPOUNDS

The polyphenols were extracted by maceration of 50 mg of powder in 2 ml of organic solvent (acetone 80%), for 1 hour and at 4 ° C to stop the action of polyphenoloxidases which degrade phenolic compounds. After centrifugation, the supernatant containing the polyphenols was recovered. Therefore, we proceed to a second extraction overnight on the base to extract supplementary polyphenols to obtain a dosage more comprehensive. The two supernatants were added to the dosage [10].

DOSAGE OF THE POLYPHENOLS USING THE FOLIN-CIOCALTEU REAGENT

To 100 µl of extract, 500 µl of Folin reagent diluted 10 times and 400 µl of sodium carbonate (75g / l) were added to the tubes. After agitation, the tubes were incubated 5 min at 40 ° C and the absorbance was read at 735 nm after 60 min. A standard range was prepared by gallic acid in concentrations ranging from 0 to 500 ppm [10].

2.2.4 EVALUATION OF THE ANTIOXIDANT ACTIVITY

DPPH TEST

The antioxidant activity was measured by the DPPH (1,1-diphenyl-2-picrylhydrazyl) method , according to the protocol described previously [11]. In this test the antioxidants reduce the DPPH from a violet color to a yellow compound, the diphenyl picryl hydrazine, the intensity of the color is inversely proportional to the ability of the antioxidants present in the medium to give protons. The solution of DPPH was prepared by dissolving 4 mg of DPPH in 100 ml of methanol. 0.5 ml of the extract to be tested was added to 125 µl of the DPPH solution and 0.5 ml of the methanol. After agitation by a vortex, the mixture was left in the dark for 30 min and discoloration relative to the negative control containing only DPPH solution was measured at 517 nm by a spectrophotometer. The positive control was represented by a standard solution of an antioxidant, BHT (butylhydroxytoluene) and the absorbance was measured in the same conditions as the test sample. The antioxidant activity, that expresses the capacity of trapping the free radical, was estimated by the percentage of discoloration of the DPPH dissolved in methanol.

PPM TEST

PPM test (phosphomolybdate) was based on the reduction of molybdenum in the oxidation state (VI) to the oxidation state (V). This reduction was materialized by forming a greenish complex (phosphate / Mo (V)) at an acidic pH. The method consisted in introducing into an Eppendorf tube 100 µL of the mixed extract with 900 µl of a reagent composed of (0.6 M) sulfuric acid, (28 mM) sodium phosphate and (4 mM) ammonium molybdate [12]. The tube was capped and incubated at 95°C for 90 min. After the sample had cooled to room temperature, the absorbance was measured at 695 nm. The control is constituted of 100 µL of methanol mixed with 900 µl of the reagent mentioned above. The calibrators, the controls and the samples were incubated under the same conditions. The antioxidant capacity was expressed as mg equivalent vitamin E per gram of dry matter (mg EVE / GMS).

2.2.5 STUDY OF THE COMPOSITION OF FATTY ACIDS AND STEROLS

PREPARATION OF METHYL ESTERS OF FATTY ACIDS (FAME)

One g of the hexane extract obtained by Soxhlet was submitted to the action of methanolic potassium hydroxide (2N) with a volume of isooctane. After vigorous stirring the mixture for 30 seconde the sodium hydrogensulfate monohydrate was added and stirred vigorously. The upper layer containing the methyl esters (FAME) was allowed decant [13].

The FAME was analyzed by Gas Chromatography (GC).

(Compound chromatographic system: flame ionization detector, temperature of the detector and the injection chamber is 200 °C, helium gas)

PREPARATION OF STEROLS

The sterols were analyzed according to the Norm ISO 12228 [14]. After saponification of a test of 250 mg taken by an ethanolic KOH solution under reflux, then the unsaponifiables was extracted with ethyl ether. The sterol fraction was separated by chromatography on a silica gel plate; the sterols recovered from the silica gel were transformed into trimethylsilyl ethers and analyzed by gas chromatography.

3 RESULTS

3.1 COMPOUNDS BELONGING TO THE PRIMARY METABOLISM

The averages levels in lipids, carbohydrates and proteins of *Cliona viridis* were shown in Table 1:

Table 1: Averages levels of lipids, carbohydrates and proteins expressed in percentage relative to the dry weight

Lipids	Carbohydrates	Proteins
2.9% (+/-1.46)	15.35% (+/-0.03)	2.67% (+/-0.02)

The sugar content was calculated from a calibration curve of a range of glucose (Figure 2).

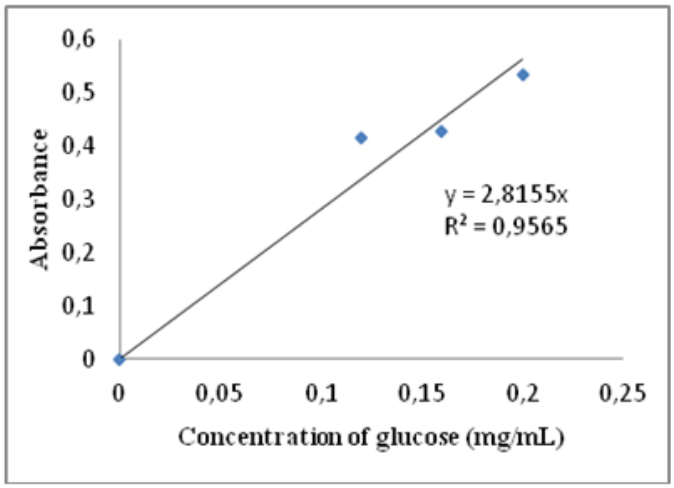

Figure 2: Calibration curve of glucose

From the Table 1, *Cliona viridis* represents a source of primary metabolites with a significant proportion of carbohydrates compared to lipids and nitrogen compounds.

3.2 COMPOUNDS BELONGING TO THE SECONDARY METABOLISM

These substances are known to be responsible for the pharmacological activity. The results of tests of the chemical composition are presented in the table 2.

Table 2: Determination of chemical composition of the crushed sponge

Chemical substances	Results of the tests
Sterol substances	+++
Saponins	+++
Tannins	+
free quinones	+
Anthraquinones	-
Flavonoids	+
Alkaloids	+
Coumarins	-

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

According to table 2, we observed the presence of different chemical compounds recognized by their biological activity with high concentration of sterol and saponins substances.

Therefore according to our preliminary tests, we found that *cliona viridis* produced molecules with potential biological interest. In the case of particular species of sponges, it seems that the production of these active metabolites does either not directly related to the organisms themselves but rather to heterotrophic endosymbionts (bacteria) or autotrophic (microalgae and cyanobacteria). The ability to produce such metabolites is of obvious interest in the context of the search for new molecules presenting interesting therapeutic potential [15].

3.3 DETERMINATION OF POLYPHENOLS

The polyphenols content was calculated relative to a reference antioxidant; gallic acid after plotting a calibration curve (Figure 3). The polyphenol content was 3.67 (+ / -0.01) mg EGA/g DW.

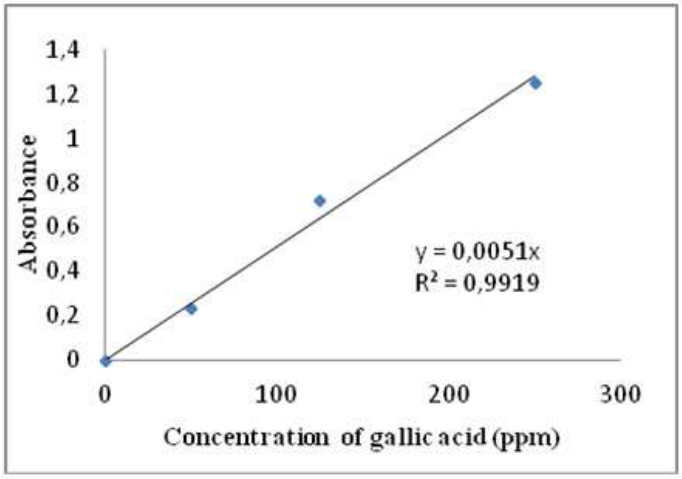

Figure 3: Calibration curve of gallic acid (ppm)

3.4 EVALUATION OF THE ANTIOXIDANT ACTIVITY

3.4.1 DPPH TEST

The result is expressed as percentage of the anti-radical activity according to the following equation:

$$\% \text{ Antioxydant Activity} = \frac{Abs\ control - Abs\ test}{Abs\ control} \times 100$$

Where Abs is the absorbance at the wavelength of 517 nm.

The result was expressed by the average of three measurements ± standard deviation, and was expressed relative to those obtained for the BHT as a reference.

The percentage of the extract of the sponge is 0.11% (+ / - 0.06), this value remains low compared to BHT; and used as a reference is 90% and thus indicates that this sponge contains the modest antioxidants, which is consistent with the result obtained for the low content of polyphenols in the sponge studied.

3.4.2 PPM TEST (PHOSPHOMOLYBDATE)

The antioxidant capacity was expressed as mg equivalent vitamin E per gram of dry weight (mg EVE / g DW). The figure 4 corresponds to the calibration curve of vitamin E:

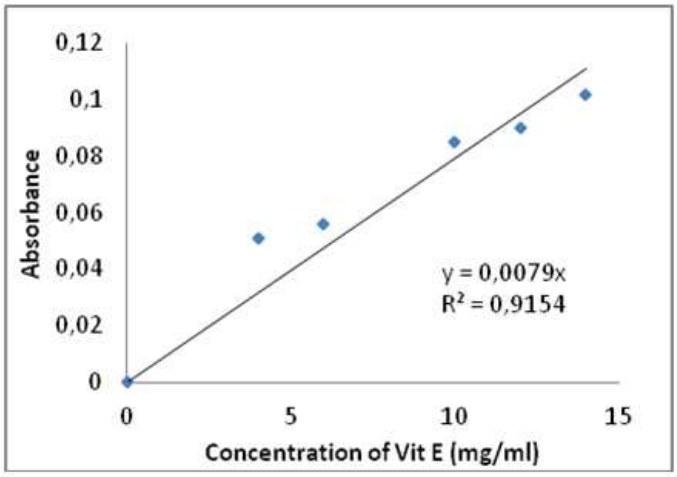

Figure 4: Calibration curve of the Vitamin E

From equation of the curve, it was found that the antioxidant capacity was 461.5 (+ / -0.01) mg Vit E / g of dry weight (461.5 + / -0.01 mg EVE / g DW).

3.5 STUDY OF THE COMPOSITION OF FATTY ACIDS AND STEROLS

3.5.1 THE METHYL ESTERS OF FATTY ACIDS (FAME)

The methyl esters of FA of *Cliona viridis* were identified by comparing their retention times with those of standards. The results are presented in the table 3.

Table 3: Profile of Fatty Acids of the sponge studied

Peak	Ret Time (min)	Area%	Component Name
1	9.096	9.969	-
2	10.916	2.082	-
3	13.576	28.688	Palmitic acid (C16: 0)
4	14.433	7.112	Palmitoleic acid (C16: 1)
5	22.331	6.955	Stearic acid (C18: 0)
6	23.539	28.530	Oleic acid (C18: 1) ω9
7	25.720	16.665	Linoleic acid (C18: 2) ω6

No study has been performed in Morocco on the fatty acid composition of *Cliona viridis*. This study allowed us to identify the variety of fatty acids contained in *C.viridis* with predominance of palmitic, oleic and linoleic acids and as percentages 28.68%, 28.52% and 16.66%, respectively. These two latter acids ω9 and ω6, are part of the family of omega recognized by their cardioprotective effect [16]. Noting also the presence of minority acids which are palmitoleic and stearic acids.

3.5.2 STEROLS ANALYSIS

Silylated sterols of *Cliona viridis* were identified by comparing their retention times with those of standards. The results are presented in the table 4.

Table 4: Profile of Sterols of the sponge studied

Peak	Ret Time (min)	Area%	Component Name
1	25.837	5.909	-
2	27.516	30.804	Cholesterol
3	28.098	1.703	-
4	28.977	16.616	Brassicasterol
5	30.929	7.9344	Campesterol
6	31.201	4.0893	-
7	32.447	3.466	Stigmasterol
8	34.262	1.115	-
9	35.652	25.197	β-Sitosterol
10	36.112	2.337	Δ 5 - Stigmasterol
11	36.598	0.269	-
12	37.217	0.129	-
13	37.735	0.067	Δ 7 - Stigmasterol
14	39.184	0.364	Δ 7- Avenasterol

The identification of the hexane extract of sterols was performed by a methodology which combines by soxhlet extraction, the bypass and the gas chromatography whose conditions are: Capillary Column 25/0, 32/0, 25µm of a nonpolar phase; Injector temperature 300 °C; Detector temperature 300 °C; Oven temperature 200 to 280 ° C with 10 °C / min; 1ml/mn flow, carrier gas is helium at 1 ml / min.

Cliona viridis contains therefore principally the unsaturated sterols C27-C29, the same result found in previous work done by DONATO SICA et al., 1978 in the same species of Italy always with a predominance of cholesterol [17].

The sponges contain a wide variety of fats (sterols) compared to other animals. Some sterols were found only in sponges such as clionasterol and poriferasterol, others like cholesterol are common with other animals.

4 CONCLUSION AND DISCUSSION

Cliona viridis, belongs to demosponges and is the most important class of the phylum Porifera whose skeleton is formed by siliceous spicules or spongin (famous fibrous protein forming a rigid network), sometimes in association of two. It includes the species of the genus *Suberiles*, covering some crabs, the sponges of the genus Cliona, which perforate the pebbles and shells, and those of the genus *Spongilla*, enfeoffed to fresh water. The sponges of the genus *Hippospongia* and *Euspongia* with which we do the spongesof toilet, also belong to this group [18-19].

Sedentaries at maturity, sponges have a priori no defense against their predators expect the metabolites that they secrete into their environment (Genin, 2002) [20].

In this preliminary study, we obtained leant the value of primary and secondary metabolites as a new source that can be exploited in the pharmaceutical scale. However, it remains unclear whether the sponges produce themselves all of these metabolites or the latter are synthesized by the microorganisms living in symbiosis with them.

So, it is found that *Cliona viridis* contains a high content of primary metabolites principally the 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 [21-22].

As components of cellular membranes, they organize the cell surface characteristic and modify the process of interaction, communication and differentiation.

Also, *Cliona viridis* has proved rich in secondary metabolites, some of which are present at low quantity, however, they should not be neglected, because the synergy between the different chemicals should be considered in the biological activity [23].

The antioxidant activity evaluated is due to the presence of polyphenols. The search of new natural antioxidants of marine origin, especially from sponges, has recently been the subject of several studies [24-25].

On the other hand, the GC analysis of the hexane extract of *Cliona viridis* has allowed the identification of fatty acids and sterols with interesting biological activity.

Indeed, for the study of fatty acids, the basic analysis is addressed to methyl esters, volatile derivatives producing many diagnostic fragment ions after electron impact. However, the methyl esters do not permit most often to precisely determine the position of a double or triple bond, a ramification, an additional function. It is then necessary to use another type of derivative containing a nitrogen atom [26], especially N-acyl pyrrolidide [27] dimethyloxazoline [28] picolynique ester [29]. Moreover, screening of chemical compounds in *C. viridis* revealed the presence of tannins, alkaloids, sterols, saponins, flavonoides, free quinones and polyphenols. The antioxidant activity of *C. viridis* extract is due to the presence of these chemicals compounds and thus the sponge could serve as potential source in medicine drugs. In the current investigation, the extracts from *C. viridis* gave good results indicating that it possesses significant amount of chemicals compounds and in vitro antioxidant activity. Phenolic compounds and other chemicals compounds appear to be responsible for the in vitro antioxidant activity of the extracts and may contribute to the therapeutic activity observed. On the basis of the results obtained, *C. viridis* extracts are rich sources of natural antioxidants appears to be an alternative to synthetic antioxidants. Further investigation to determine antioxidant activity by in vivo methods could be considered.

REFERENCES

[1] E.J. Dumdey, J.W. Blunt, M.H.G. Munro, C.N. Battershill, M.J.In. Page: *Sponge Sciences; Multidisciplinary Perspectives*, Y. Watanabe, N. Fusetani, eds., Tokyo, Springer-Verlag, pp 353.

[2] E. Fattorusso, O. Taglialatela-Scafati, Mar Drugs. , 7(2) (2009)130-152.

[3] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F.Smith. Analytical Chemistry. , 28 (3) (1956) 350-356.

[4] R. Thibault, M. Tremblay, "the dosage of proteins", Expo-Journal, Internal Report, Department of Natural Sciences, Cegep of Saint-Félicien, (2002) 8 pages.

[5] R. Paris, H. Moses, "Précis of medicinal material", Paris, Masson (1969).

[6] M. Bouquet, "Works and documents of Orstom", (1971) (Paris, No. 13).

[7] M. Debray, H. Jacquemin, R. Razafindrambo, "Works and documents of the Orstom" (1971) (Paris, No. 8).

[8] N. Dohou, K. Yamni, S. Tahrouch, L.M. Idrissi Hassani, A. Badoc, N. Gmira, Bull. Soc. Pharm. Bordeaux. , 142 (2003) 61-78

[9] R.P. Cook, "Reactions of Steroids with Acetic anhydride and Sulphuric Acid (the Liebermann-Burchard test)", analyst, 86 (1961) 373-381.

[10] B. Nathalie, J.P. Charpentier, "Rapid method of evaluation of the content of phenolic compounds of organs of a forest tree", Special Issue (2006).

[11] C. Popovici, I. Saykova, B. Tylkowski, Revue de génie industriel. , 4 (2009) 25-39.

[12] P. Prieto, M. Pineda, M. Aguilar, Anal. Biochem. , 269 (1999) 337-341.

[13] International Olive Council. Preparation of methyl esters of fatty acids of olive oil and olive pomace oil. COI/T.20/Doc. No. 24-(2001).

[14] Norm ISO 12228, Fractionation of unsaponifiable: Determination of content of individual and total sterols.

[15] A. Bendaoud, Photosynthetic symbionts of sponges: Isolation, cultivation and production of active metabolites, Thesis (2007).

[16] PM. Kris-Etherton, AHA Science Advisory. Monounsaturated fatty acids and risk of cardiovascular disease. American Heart Association. Nutrition Committee.Circulation. (1999)

[17] D. SICA, F. SIMONE, E. RAMUNDO, F. ZOLLO, Biochem. Syst. Ecol. , 6 (1978) 77-79.

[18] C. LEVI, P. LABOUTE, G. BARGIBANT, J.L. MENOU, "Sponge of the New Caledonian Lagoon", Edition de l'ORSTOM, collection Faune tropicale N°XXX, Paris (1991).

[19] J.N.A. HOOPER, F. WlEDENMAYER, "Zoological catalogue of Australia : Porifera", Edition CSIRO Australia, 12 (1994) 187-190, 225-247.

[20] B. Aurélien "Isolation of active products from extracts of marine organisms. Internship report -september 2009.

[21] E. Fattorusso, A. Mangoni. , Marine Glycolipide. *Progress in the Chemistry of Organic Natural Products* 72 (1997) 215-301.

[22] V. Costantino, E. Fattorusso, A. Mangoni , M. Di Rosa , A. Ianaro , Bioorg. Med. Chem. Letters 9. , (1999) 2-276.

[23] S. Bourgou, R. Ksouri, A. Bellila, I. Skandrani, H. Falleh , B. Marzouk, C R Biol. , 331 (2008) 48–55.

[24] A. Longeon, B.R. Copp, E. Quévrain, M. Roué, B. Kientz1, T. Cresteil, S. Petek, C. Debitus, M.L.B. Kondracki, Mar. Drugs., 9 (2011) 879-888.

[25] N.K. Utkina, Chem. Nat. Compd. , 45(6) (2009) 849-853.

[26] Christie WW. The Lipid Library. www.lipidlibrary.co.uk (Update: 9 juillet 2010).

[27] BA. Andersson, Prog Chem Fats Other Lipids., 16 (1978) 279-308.

[28] G. Dobson , WW. Christie, Eur. J. Lipid Sci. Technol., 104 (2002) 36-43.

[29] Christie WW (ed) Advances in Lipid Methodology. Vol. 1. Royaume-Uni: The Oily Press, Dundee, 1992.