Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 5, Suppl 2, 2013

Research Article

IN VITRO ANTIPROLIFERATIVE AND ANTIOXIDANT ACTIVITIES OF THE ORGANIC EXTRACT AND ITS SEMI-PURIFIED FRACTIONS FROM THE MEDITERRANEAN GORGONIAN EUNICELLA SINGULARIS

MONIA DEGHRIGUE[1], AFEF DELLAI[1,2], ABDERRAHMAN BOURAOUI[1]

[1]Unité de Recherche des Substances Actives Marines (URSAM), Laboratoire de Pharmacologie. Faculté de pharmacie, Monastir, Tunisie, [2]Laboratoire de Pharmacologie des Médicament Anticancéreux, Institut Bergonié, Bordeaux, France. Email: moniadeghrigue@yahoo.fr

Received: 14 Mar 2013, Revised and Accepted: 28 Apr 2013

ABSTRACT

Objective: Resistances to current anticancer and antioxidant drugs are growing global concerns. The aim of this study was to identify natural products from marine invertebrates with therapeutic potential.

Methods: The organic extract of the Mediterranean gorgonian, *Eunicella singularis* and its semi-purified fractions were evaluated for in vitro antiproliferative effect in three human cancer cell lines using the MTT colorimetric method and clonogenic assay. The antioxidant activity was determined by two methods, the stable radical 1,1 -diphenyl-2-picrylhydrazyl (DPPH), and the ferric-reducing power (FRAP) assays.

Results: The organic extract and methanol/dichloromethane fraction (F-MeOH/CH_2Cl_2) showed interesting antiproliferative activities in a dose dependant manner against two cancer cell lines (A549 and MCF7). The IC_{50} values of the organic extract were 36 and 52 µg/ml for A549 and MCF7 cancer cell lines, respectively. For the F-MeOH/CH_2Cl_2, the IC_{50} was 31 µg/ ml for both cell lines studied. In addition F-MeOH/CH_2Cl_2 possessed a potent antioxidant activity.

Conclusion: *Eunicella singularis* fraction might be used as a potential source of natural antiproliferative and antioxidant agents.

Keywords: *Eunicella singularis*, Antiproliferative, Antioxidant, Organic extract, Semi-purified fractions.

INTRODUCTION

Marine organisms, which represent approximately one half of the total global biodiversity, are rich reservoirs of biologically active natural products [1, 2]. Among them, Sponges, bryozoans and tunicates, which have furnished a large variety of bioactive metabolites, are considered as marine sources of new active compounds for drugs development [3]. [4] reported that cnidarians have also proven to be a source of biologically active chemical molecules. Approximately 15,000 pharmacologically active compounds have been isolated from marine species, whose the structure of the most was unique and absent in terrestrial organisms [5].

In recent years, anticancer drugs from natural sources such as plants, marine organisms and microorganisms account approximately 60% of all anticancer drugs [6]. However, many anticancer drugs used in chemotherapeutic treatments developed resistance and side effects [7, 8]. That is why the search and isolation of new effective non toxic compounds from natural sources is actual problem [9].

Out of these, reactive oxygen species (ROS) and free radicals attack macromolecules such as DNA, proteins and lipids, leading to many health disorders including cancer [10, 11]. The harmful effect of the free radicals can however, be blocked by synthetic antioxidants, but due to their adverse side effects, search for effective and natural antioxidants has become crucial [12, 13].

Hence, the search is still on to find a natural drug possessing antiproliferative and antioxidant properties. The soft corals and gorgonians of Octocorollia have become a new marine resource for searching novel bioactive marine natural products as lead compounds in drug development [14]. Therefore, in a program to find new natural sources of antiproliferative and antioxidants agents from Tunisian marine invertebrates, we studied the efficiency of organic extract and its semi-purified fractions from the white gorgonian *Eunicella singularis* (Cnidaria: Octocorallia) Esper 1794, characteristic of coralligenous biocenosis, and one of the most abundant and widely distributed specie in the Mediterranean Sea [15,16,17].

MATERIAL AND METHODS

Sample collection

Eunicella singularis was collected from the Mediterranean Sea in various areas of the coastal region of Tabarka (Tunisia), in June 2010, at a depth between 20 and 30 m. The collected samples were cleaned by rinsing with seawater and distilled water and transported in cool box to the laboratory. The cleaned material was then air dried to dryness in the shade at 30°C. The dried samples were finely powdered and stored at -20°C until use. Identification of specimens was carried out in the National Institute of Marine Sciences and Technologies (Salamboo, Tunisia).

Preparation of the organic extract and its semi-purified fractions

The organic extract of *Eunicella singularis* was prepared by maceration of finely powdered material packed in small bags (5 x 10 cm) of Whitman filter paper no.1 with methanol and dichloromethane (1:1, v/v) for 48h three times. The organic extract was concentrated to solvent free by evaporation in a rotating evaporator (Buchi, B- 480) at 40°C.

In order to localize the active fractions, the organic extract was purified, using C18 cartridges (Sep-pack, Supelco), by gradient elution with different organic solvents in the order of decrease polarity: ethanol and methanol/ dichloromethane (1:1) to give ethanol (F- EtOH), and methanol-dichloromethane (F-MeOH/CH_2Cl_2) fractions. Organic solvents were removed from recuperated fractions using rotating evaporator at 40 °C.

Qualitative chemical screening

The organic extract and its semi-purified fractions (F-EtOH, F-MeOH/CH_2Cl_2) of *Eunicella singularis* were characterized for the presence of alkaloids (Mayer's test), glycosides (Keller-Killiani test), terpenoids (Libermann-Burchard's test), steroids, and saponins [18, 19].

Antiproliferative activity

Cell culture

The human cancer cell lines A549 (lung cell carcinoma), HCT15 (colon cell carcinoma) and MCF7 (breast adenocarcinoma) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were routinely grown with DMEM supplemented with 10% fetal calf serum and 1% penicillin/streptomycin, obtained from Biochrom AG (Berlin,

Germany). They were grown on Flasks (Nunc, Denmark) at 37˚C in a humidified atmosphere containing 5% CO_2. Cells were replicated every 4-5 days and the medium changed once in-between.

Viability assay

The potential effects on cell viability were investigated according to previously reported conditions using the MTT assay [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, Sigma-Aldrich Chimie, Saint-Quentin-Fallavier, France] as an indicator of metabolically active cells [20, 21, 22].

Results were evaluated by comparing the absorbance of the treated cells with the absorbance of wells containing cell treated by the solvent control. Conventionally, cell viability was estimated to be 100% in the solvent control. The concentration of substance required for 50% growth inhibition (IC_{50}) was estimated as that giving a 50% decrease in absorbance as compared to controls incubated simultaneously without substances.

Clonogenic Inhibition Assay

The clonogenic inhibition assay was performed as described previously by [23] with some modifications. Known number of A549, HCT15 or MCF7 cells (2.10^4) were transferred into six-well plates (Becton Dickinson Lab ware, USA) in a volume of 2 ml of culture medium and incubated for 24h before addition of test compounds.

Cells were then exposed for 24h at 37˚C to known concentrations of the compound to be tested. After drug exposure, the cells were washed with phosphate-buffered saline and subsequently re-plated in appropriate dilution in triplicate to assess clonogenic ability. After incubation for 14 days, each plate was stained with crystal violet and colonies were counted with a "colony counter pen". The surviving fraction was calculated as the ratio of the number of colonies formed after treatment to the product of the number of cells plated and the plating efficiency. The IC_{50} value is the concentration of the extract or fraction which is capable of bringing about 50% inhibition of colony formation.

Antioxidant activity

DPPH radical-scavenging activity

The free radical-scavenging activity of the organic extract and its semi-purified fractions from *Eunicella singularis* was evaluated using the stable radical DPPH, according to the method of Kim et al [24]. The radical-scavenging activity of test samples, expressed as percentage inhibition of DPPH, was calculated according to the formula:

% inhibition = $[(A_B \_ A_A)/A_B] \times 100$, where A_B and A_A are the absorbance values of the control and of the test sample, respectively. The fraction concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage plotted against test samples concentration.

DPPH radical-scavenging activity of the organic extract and its semi-purified fractions of *Eunicella singularis* were compared with ascorbic acid used as standard.

Ferric reducing antioxidant power

The ferric reducing antioxidant power (FRAP) of *Eunicella singularis* organic extract and its semi-purified fractions was evaluated using the method described by Oyaizu [25]. The absorbance of all sample solutions was measured at 700 nm and compared with ascorbic acid used as standard.

Statistical analysis

Data are presented as the mean ± standard error of the mean (s. e. m). Statistical analysis was performed using Student's *t*-test. The significance of difference was considered to include values of $P<0.05$.

RESULTS AND DISCUSSION

Evaluation of cytotoxicity against cancer cell lines

The organic extract and its semi-purified fractions of *Eunicella singularis* were tested for their effect on inhibition of growth in three human tumor cell lines (A549, HCT15, and MCF7) over a concentration range of 12.5 – 1000 µg/ml to determine their potency. Assays were performed on exponentially growing cells and activity evaluated by measuring levels of surviving cells after incubation for 24h with the test samples using the MTT colorimetric assay [22, 26]. This is the first step in our anticancer drug development program and is designed to identify fractions with cytotoxic activity. The results of this primary screening are shown in Table 1 and in figures 1-3.

At concentrations of 12.5 – 1000 µg/ml, organic extract and its semi-purified fractions of *Eunicella singularis* suppressed dose-dependently the proliferation of the three cell lines. The organic extract produced significant cell growth inhibition in A549, HCT15 and MCF7 cells, from 38 to 85%, 11 to 84% and 39 to 88%, respectively (figure 1).

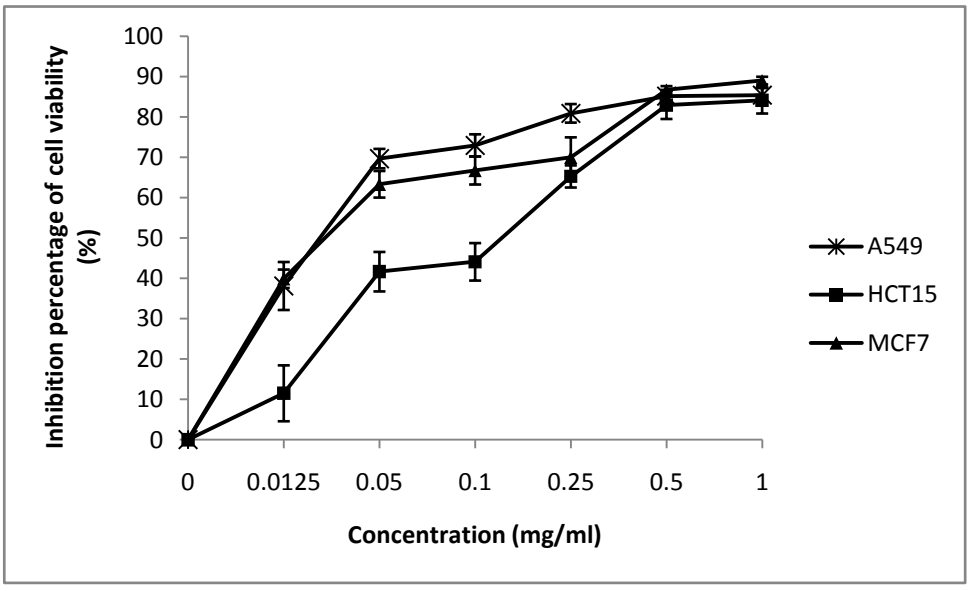

Fig. 1: Effect of the organic extract of *Eunicella singularis* on cellular growth against three human tumor cell lines (A549, lung cell carcinoma; HCT15, colon cell carcinoma; and MCF7, breast adenocarcinoma).

The ethanolic fraction produced important cell growth inhibition in the three cell lines, from 28 to 79%, 14 to 86%, and 13 to 84%, respectively (figure 2).

The methanol/dichloromethane fraction produced also significant cell growth inhibition in the three cell lines, from 32 to 79% in A549 cells, 10 to 67% in HCT15 cells, and 36 to 72% in MCF cells (figure 3).

The concentration of fractions required to reduce growth of cancer cell lines by 50% (IC_{50}) after 24h of incubation are presented in Table 1. The organic extract produced significant cytotoxicity with IC_{50} values of 36, 175 and 52 µg/ml, respectively, against A549, HCT15, and MCF7 cells. The methanol/dichloromethane fraction exhibited significant cytotoxicity against A549, HCT15, and MCF7 cell lines with IC_{50} values of 31, 175, and 31µg/ml, respectively (Table 1). The ethanolic fraction produced also significant cytotoxicity with IC_{50} values of 200, 174, and 375µg/ml, respectively, against A549, HCT15, and MCF7 cells (Table 1).

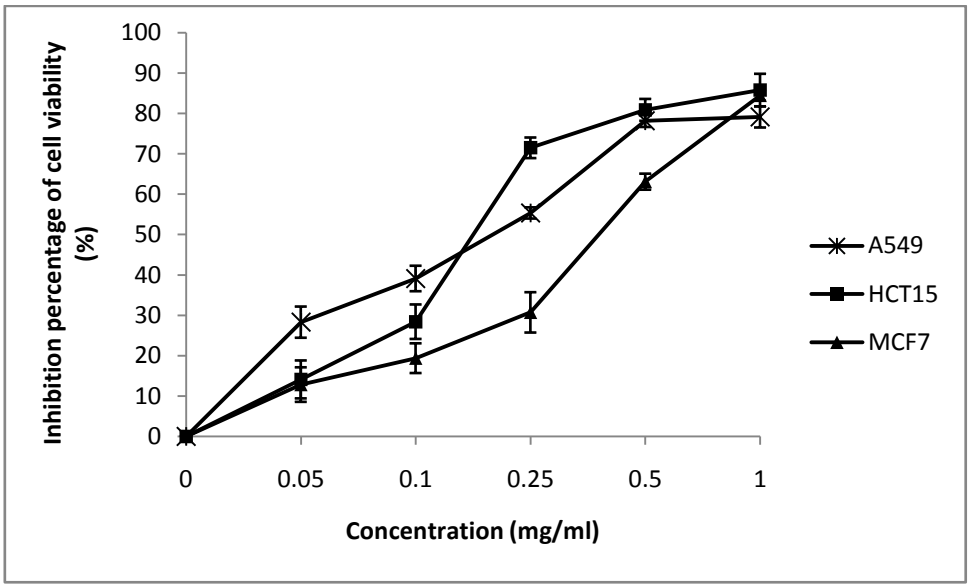

Fig. 2: Effect of the ethanolic fraction (F-EtOH) of *Eunicella singularis* on cellular growth against three human tumor cell lines (A549, lung cell carcinoma; HCT15, colon cell carcinoma; and MCF7, breast adenocarcinoma).

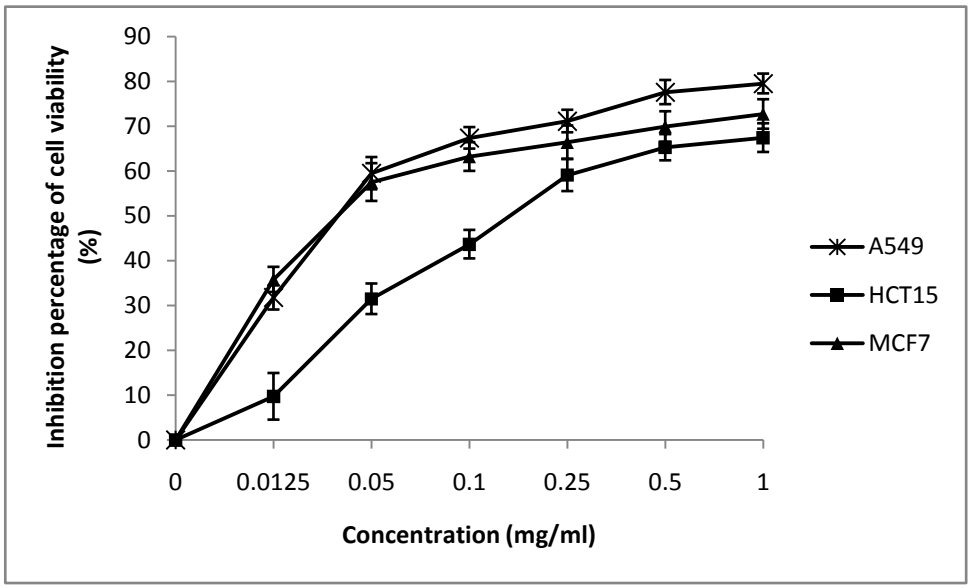

Fig.3: Effect of the methanol/dichloromethane fraction (F-MeOH/CH_2Cl_2) of *Eunicella singularis* on cellular growth against three human tumor cell lines (A549, lung cell carcinoma; HCT15, colon cell carcinoma; and MCF7, breast adenocarcinoma).

Table 1: *In vitro* growth inhibitory activity (expressed by the IC_{50}) of the organic extract and its semi-purified fractions (F-EtOH, F-MeOH/CH_2Cl_2) of *Eunicella singularis* against the three human tumor cell lines A549 (Lung Cell Carcinoma), HCT15 (Colon Cell Carcinoma) and MCF7 (Breast Adenocarcinoma)

Sample	IC_{50} (µg/ml)		
	A549	HCT15	MCF7
Organic extract	36±0.6	175±0.9	52±0.5
F-EtOH	200±0.8	174±0.4	375±0.4
F-MeOH/CH_2Cl_2	31±0.5	175±0.4	31±0.5

Note: IC_{50}: 50% inhibitions of cell growth, values are expressed as mean ± SEM.

Once the inhibitory effects of the organic extract and its semi-purified fractions of *Eunicella singularis* on cell growth inhibition (MTT colorimetric assay) were established, their effects on cell viability were assessed using the clonogenic inhibition assay and the same three human tumor cell lines over the same concentration range [27]. The antiproliferative effects of organic extract and its semi-purified fractions on A549, HCT15, and MCF7 cells showed a significant clonogenic concentration-related inhibition (figure 4-6). The organic extract produced significant clonogenic inhibition against A549, HCT15, and MCF7 cells, from 48 to 95%, 21 to 95% and 51 to 98%, respectively (figure 4) with IC_{50} values of 26 µg/ ml against A549 cells, 48 µg/ ml against HCT15 cells and 17 µg/ml against MCF cells (Table 2).

Ethanolic fraction also produced significant clonogenic inhibition (figure 5) with IC_{50} values of 98, 152, and 333 µg/ ml, respectively against A549, HCT15, and MCF7 cells (Table 2).

Methanol/dichloromethane fraction produced also significant clonogenic inhibition against A549, HCT15, and MCF7 cells, from 39 to 90%, 15 to 92%, and 43 to 87%, respectively (figure 6) with IC_{50} values of 30 µg/ml against A549 cells, 150 µg/ ml against HCT15 cells and 30 µg/ ml against MCF7 cells (Table 2).

The preliminary chemical screening of the organic extract and the tested fractions of *Eunicella singularis* revealed the presence of alkaloids, glycosides, terpenoids and saponins as showed in Table 3. However, only F-EtOH contains steroids. So, the antiproliferative activity of organic extract, F-EtOH and F-MeOH/CH_2Cl_2 fractions could be attributed to the presence of these compounds (alkaloids, glycosides, terpenoids and saponins) found in these samples. The strong antiproliferative activity of the F-EtOH could be also presumed to emerge from its steroids content; however, it still needs further investigations.

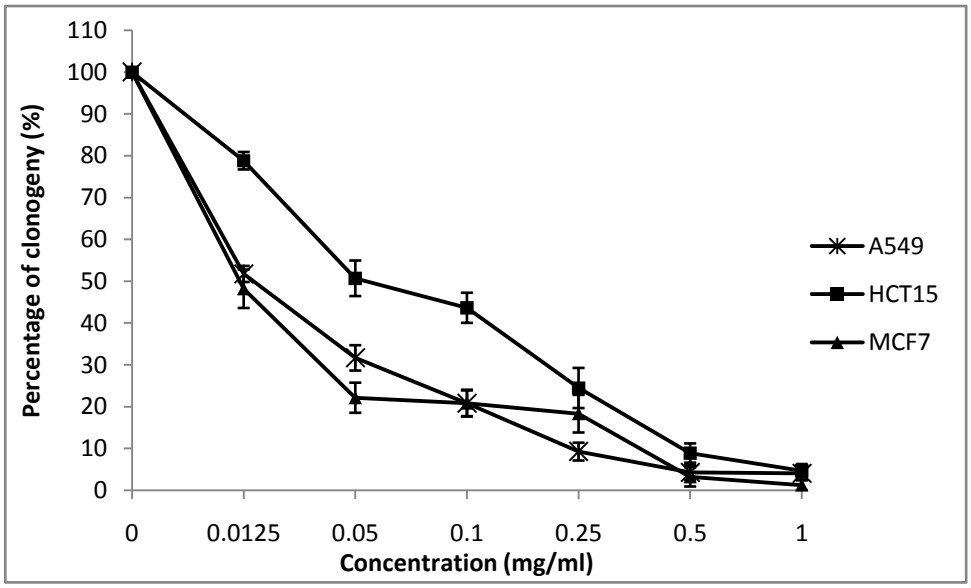

Fig. 4: Organic extract of *Eunicella singularis* induced clonogenic inhibition in human tumor cell lines (A549, lung cell carcinoma; HCT15, colon cell carcinoma; and MCF7, breast adenocarcinoma).

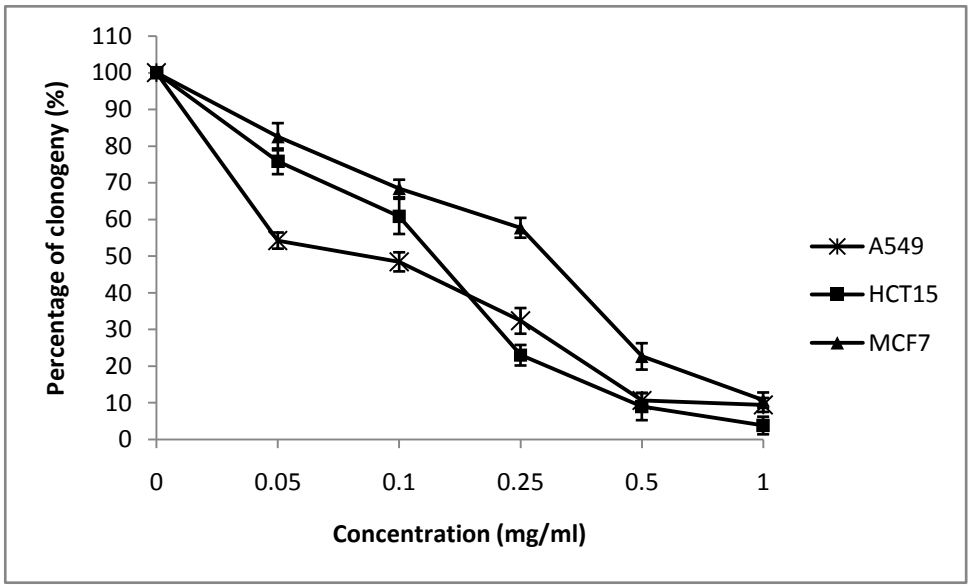

Fig. 5: Ethanolic fraction (F-EtOH) induced clonogenic inhibition in human tumor cell lines (A549, lung cell carcinoma; HCT15, colon cell carcinoma; and MCF7, breast adenocarcinoma).

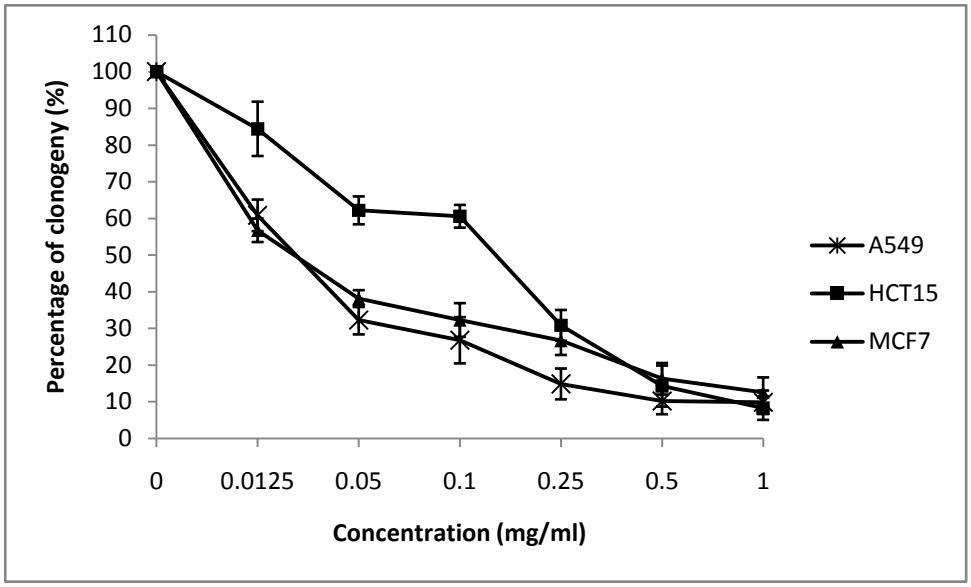

Fig. 6: Methanol/ dichloromethane fraction (F-MeOH/CH_2Cl_2) induced clonogenic inhibition in human tumor cell lines (A549, lung cell carcinoma; HCT15, colon cell carcinoma; and MCF7, breast adenocarcinoma).

Table 2: In vitro colony inhibitory activity (expressed by the IC_{50}) of the organic extract and its semi-purified fractions (F-EtOH, F-MeOH/CH_2Cl_2) of *Eunicella singularis* against the three human tumor cell lines A549 (Lung Cell Carcinoma), HCT15 (Colon Cell Carcinoma), and MCF7 (Breast Adenocarcinoma)

Sample	IC_{50} (µg/ml)		
	A549	**HCT15**	**MCF7**
Organic extract	26±0.4	48±0.6	17±0.8
F-EtOH	98±0.6	152±0.5	333±0.2
F-MeOH/CH_2Cl_2	30±0.2	150±0.2	30±0.2

Note: IC_{50}: 50% inhibition of cell growth, values are expressed as mean ± SEM

Table 3: Qualitative chemical screening of the organic extract and its semi-purified fractions from *Eunicella singularis*

	Organic extract	**F- EtOH**	**F- MeOH/CH_2Cl_2**
Alkaloids	+	+	+
Glycosides	+	+	+
Terpenoids	+	+	+
Steroids	-	+	-
Saponins	+	+	+

Note: '+'indicates the present compound; '–'indicates the absent compound

These data are in agreement with the results of others. Several studies reported that steroids, tryptamine and diterpenoids isolated from other species of the genus *Eunicella* have antiproliferative properties. In addition, Sharma and Paliwal [28] demonstrated that saponins may provide a new line of anticancer agents due to their multiple apoptotic actions on cancer cells. Ioannou et al [29] reported that pregnanes, class of steroids isolated from the organic extract of the gorgonian *Eunicella cavolini*, exerted antiproliferative activity in MCF7 human breast adenocarcinoma cells. Metabolites (9, 11- secosterols) from the gorgonian *Eunicella cavolini* strongly inhibit the growth of LNCaP human prostate adenocarcinoma cells and the estrogen-dependent growth of MCF7 human breast adenocarcinoma cells [30]. Granulata-mides A and B, tryptamine derivatives from the soft coral *Eunicella granulata* have antitumor activity against 16 human tumor cell lines [31].

In addition to the steroids and tryptamine, the diterpenoids from the genus *Eunicella* have antiproliferative potential: Labiatin B, diterpenoid of the eunicellan class and isolated from the organic extract of the Senegalese gorgonian *Eunicella labiata* exhibited cytotoxic activity against human colon cancer cells HCT 116 [32], while the eunicellan diterpene, massileuni-cellin A from *Eunicella cavolini* have antitumor properties [33].

Also, indole alkaloids isolated from other marine invertebrates showed cytotoxicity against a number of experimental tumor cell lines. Dragmacidin, is a bisindole alkaloid isolated from a deep water marine sponge *Dragmacidin* sp. and showed cytotoxicity against P-388 cell lines and A-549 (human lung), HCT-8 (human colon), and MDAMB (human mammary) cancer cell lines in vitro [34].

The observed antiproliferative effect of the organic extract and the different tested fractions may be attributed to alkaloids, steroids and terpenoids detected in these fractions and to a synergic participation of other compounds.

Evaluation of the antioxidant activity

The role of oxidative stress in the pathogenesis of diseases such as macular degeneration, certain types of cancer, and Alzheimer's disease has received substantial attention. For that reason, we also aimed to look into antioxidant capacities of *Eunicella singularis* organic extract and its semi-purified fractions. Two methods were used to evaluate the antioxidant activity of the organic extract and its semi-purified fractions from *Eunicella singularis*: the FRAP assays and DPPH radicals. The superoxide anion and other ROS contribute to oxidative stress, and are known contributors to genetic damage,

as well as degenerative diseases such as cancer [35]. Since, the FRAP and DPPH radicals are not biologically relevant, the DPPH and FRAP assays were performed as a preliminary study to estimate the direct free-radical scavenging abilities of the test samples.

The radical-scavenging activity of organic extract and its semi-purified fractions of *Eunicella singularis*, measured as decolorizing effect following the trapping of the unpaired electron of DPPH, are shown in figure 7.

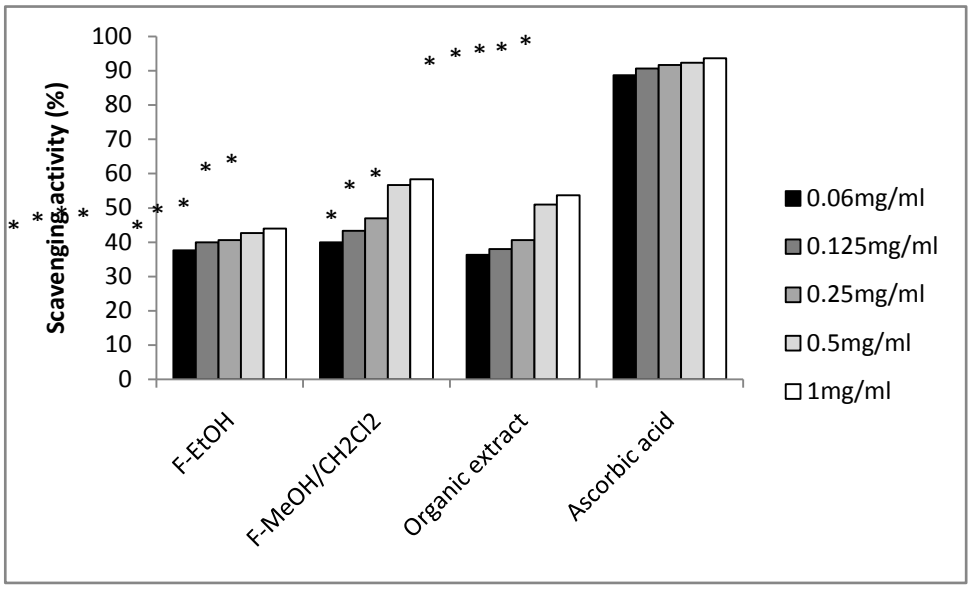

Fig. 7: DPPH free-radical scavenging activity of organic extract and its semi-purified fractions from *Eunicella singularis* and ascorbic acid. Significant difference obtained with: *P< 0.5

The methanol/dichloromethane fraction is the most potent radical scavenger with a percentage decrease versus the absorbance of DPPH standard solution of 58.33% at the concentration of 1mg/ml and an IC_{50} value of 0.36 mg/ml. The organic extract and ethanol fraction showed scavenging activity with a percentage decrease, versus the absorbance of DPPH standard solution of respectively 53% and 44% at a concentration of 1mg/ml, and IC_{50} values of respectively 0.46 and 0.99 mg/ml.

It can be noticed that methanol/dichloromethane fraction showed significant anti-radical activity, as measured by their capacity to scavenge the stable free radical DPPH, while ethanol fraction do not reduce the absorbance at 517 nm except at high concentration.

In addition, FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex and producing a colored ferrous tripyridyltri-azine (Fe^{2+}-TPTZ) [36]. Generally, the reducing properties are associated with the presence of compounds which exert their action by breaking the free-radical chain by donating a hydrogen atom [37].

(Figure 8) depicts the Fe^{3+}-Fe^{2+} transformation investigated in the presence of organic extract, ethanol, and methanol/dichloromethane fractions using the potassium ferricyanide reduction method. The reducing activity of the different fractions mentioned above, increased with increasing sample concentration.

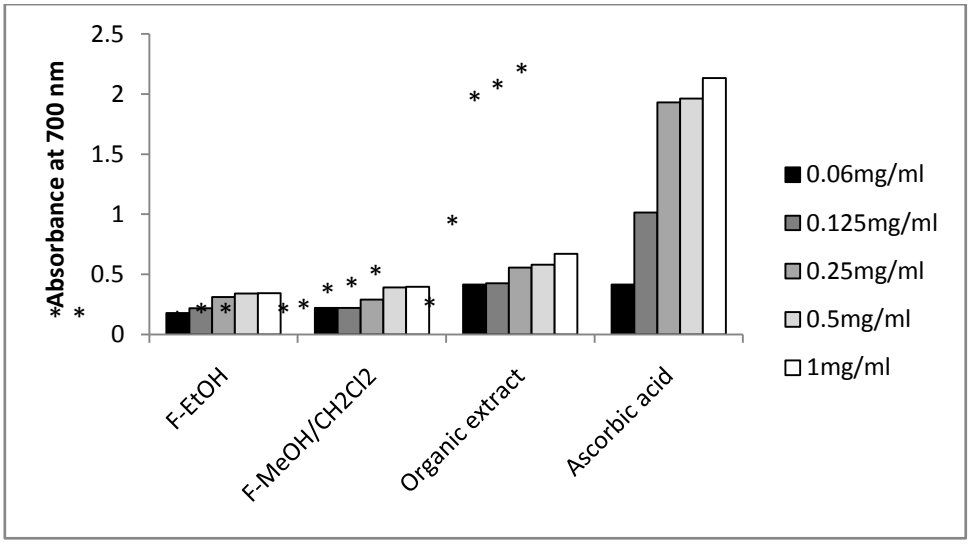

Fig. 8: The Fe^{3+} -Fe^{2+} reducing activity of organic extract and its semi-purified fractions from *Eunicella singularis* and ascorbic acid at different concentrations tested (0.06-1mg/ml) using reducing power assay. Significant difference obtained with: *P< 0.5

Therefore, at the same concentration of 1mg/ml, organic extract and methanol/dichlorometh-ane fraction reduce a maximum of Fe^{3+} ion by 0.66 and 0.39 mg/ml respectively; whereas ethanol fraction presents 0.34 mg/ml.

As can be seen in (figure 8, table 4), organic extract showed greater effective reducing activity than methanol/dichloromethane and ethanol fractions, at different concentrations (0.06, 0.125, 0.25, 0.5, and 1mg/ml).

Table 4: IC_{50} values of DPPH radical-scavenging activity and reducing power of organic extract and its semi-purified fractions of *Eunicella singularis*

Sample	IC_{50}[a] of DPPH radical-scavenging activity (mg/ml)	IC_{50}[b] of reducing power (mg/ml)
Organic extract	0.46±0.02	0.20±0.03
F- EtOH	0.99±0.05	1.35±0.04
F- MeOH/CH_2Cl_2	0.36±0.02	1.14±0.08
Ascorbic acid	0.05±0.01	0.07±0.02

Note: Values are expressed as mean ± SEM of triplicate measurement.

[a] IC_{50} means the concentration of sample that can decrease DPPH concentration by 50%.

[b] IC_{50} is the concentration for which the absorbance at 700nm is 0.5.

Results show that, organic extract, F-EtOH, and F-CH_2Cl_2 fractions revealed relatively strong antiradical activity towards the FRAP and DPPH free radicals. Thus, we can suggest that the antiproliferative activity of the organic extract, F-EtOH and F-CH_2Cl_2 fractions occurs through their antioxidant potential. This experiment suggests that the inhibition of tumor cell proliferation in vitro by the organic extract and its semi-purified fractions of *Eunicella singularis* cannot be solely explained by the presence of alkaloids, glycosides, terpenoids, steroids and saponins. The inhibition of cancer cell proliferation may be attributed to some unknown compound(s) present in the organic extract and its semi-purified fractions of *Eunicella singularis*. Other compounds may play a major role in the antiproliferative activity.

CONCLUSION

The organic extract and its semi-purified fractions of *Eunicella singularis* appear to contain compounds with antiproliferative and antioxidant properties. However, further studies should be performed to isolate and identify the antiproliferative or antioxidant components.

REFERENCES

1. Kim SK, Wijesekara I. Development and biological activities of marine-derived bioactive peptides: A review. Journal of Functional Foods 2010; 2: 1–9.
2. Mhadhebi L, Chaieb K, Bouraoui A. Evaluation of antimicrobial activity of organic fractions of six marine algae from Tunisian Mediterranean coast. International Journal of Pharmacy and Pharmaceutical Sciences 2012; 4 (1): 534-537.
3. Faulkner DJ. Marine natural products. Nat Prod Rep 2001; 18: 1- 49.
4. Blunt JW, Copp BR, Hu WP, Munro MHG, Northcotec PT, Prinsep MR. Marine natural products. Natural Products Reports 2008; 25: 35–94.
5. Newman DJ, Cragg GM. Marine natural products and related compounds in clinical and advanced preclinical trials. Journal of Natural Products 2004; 67: 1216–1238.
6. Cragg GM, Newman DJ. Plants as a source of anticancer agents. J Ethnopharmacol 2005; 100:72-79.
7. Panchal RG. Novel therapeutic strategies to selectively kill cancer cells. Biochem Pharmacol 1998; 55: 247–252.
8. Yang LL, Lee CY, Yen KY. Induction of apoptosis by hydrolysable tannins from Eugenia jambos L on human leukaemia cells. Cancer Lett 2000; 157: 65–75.
9. Anastyuk SD, Shevchenko NM, Ermakova SP, Vishchuk OS, Nazarenko EL, Dmitrenok PS et al. Anticancer activity *in vitro* of a fucoidan from the brown alga *Fucus evanescens* and its lowmolecular fragments, structurally characterized by tandem massspectrometry. Carbohydrate Polymers 2012; 87: 186 – 194.
10. Butterfield DA, Abdul HM, Opii W, Newman SF, Joshi G, Ansari MA et al. Review: Pin1 in Alzheimer's disease. Journal of Neurochemistry 2006; 98: 1697–1706.
11. Ngo DH, Wijesekara I, Vo TS, Ta QV, Kim SV. Marine food-derived functional ingredients as potential antioxidants in the food industry: an overview. Food Research International 2011; 44: 523–529.
12. Choi Y, Jeong HS, Lee J. Antioxidant activity of methanolic extracts from some grains consumed in Korea. Food Chem 2007; 103:130-138.
13. Adeolu AA, Florence OJ, Srinivas K, Masika P, Anthony JA. Asessment of the medicinal potential of the methanol extracts of the leaves and stems of Buddleja saligna. Complem Altern Med 2009; 9: 21-28.
14. Wang C, Liu H, Shao C, Wang Y, Li L, Guan H. Chemical defensive substances of soft corals and gorgonians. Acta Ecologica Sinica 2008; 28 (5): 2320-2328.
15. Carpine C, Grasshoff M. Les gorgonaires de la Méditerranée. Bull Inst Océanogr (Monaco) 1975; 71: 1-140.
16. Weinberg S. Mediterranean Octocorallian Communities and the abiotic environment. Mar Biol 1970a; 49: 41-57.
17. Weinberg S. Autoecology of shallow-water octocorallia from Mediterranean rocky substrata. II. Marseille, cote d'Azur and Corsica. Bijdr Dierkd 1980 ; 50: 73-86.
18. Randerath K. Chromatographie sur couches minces. Paris : Édition Gauthier-Villars 1971 ; 337-339.
19. Edeoga HO, Okwu DE, Mbaebie BO. Phytochemical constituents of some Nigerian medicinal plants. African Journal of Biotechnology 2005; 4(7): 685-688.
20. Hu YP, Robert J. Azelastine and Flezelastine as reversing agents of multidrug resistance: Pharmacological and molecular studies. Biochem Pharmacol 1995; 50: 169-175.
21. Mossmann T. Rapid calorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Met 1983; 65:55-63.
22. Martine VL, Stephane M, Stephane L, Daniele M, Jacque R, Alain N. Synthesis and antiproliferative activity of aryl- and heteroaryl-hydrazones derived from xanthone carbaldehydes. Europ J Med Chem 2008; 43:1336-1343.
23. Nicolaas APF, Hans MR, Jan S, Jaap H, Chris VB. Clonogenic assay of cells *in vitro*. Nat Prot 2006; 1:2315-2319.
24. Kim JK, Noh JH, Lee S, Choi JS, Suh H,Chung HY et al. The first total synthesis of 2, 3, 6-tribromo-4, 5- dihydroxybenzyl methyl ether (TDB) and its anti-oxidant activity. Bull Korean Chem Soc 2002; 23(5): 661-662.
25. Oyaizu M. Studies on product of browning reaction prepared from glucose amine. Japan J Nutr 1989; 44: 307-315.
26. Suganumak KT, Saikawa Y. Possible chemoresistance-related genes for gastric cancer detected by C DNA microarray. Cancer Sci 2003; 94:355-359.
27. Fransis L, Fransis B, Jean Francois V, Christophe G, Philippe B, Josy R. Drug resistance in acute myeloid leukemias. Hematologie 1996; 2: 417-425.
28. Sharma V, Paliwal R. Isolation and characterization of saponins from Moringa oleifera (Moringaeceae) pods. International Journal of Pharmacy and Pharmaceutical Sciences 2013; 5 (1): 179-183.
29. Ioannou E, Abdel-Razik AF, Alexi X, Vagias C, Alexis MN, Roussis V. Pregnanes with antiproliferative activity from the gorgonian *Eunicella cavolini*. Tetrahedron 2008; 64: 11797-11801.
30. Ioannou E, Abdel-Razik AF, Alexi X, Vagias C, Alexis MN, Roussis V. 9,11-Secosterols with antiproliferative activity from the gorgonian *Eunicella cavolini*. Bioorganic and Medicinal Chemistry 2009; 17: 4537-4541.
31. Reyes CP, Núñez MJ, Jiménez IA, Busserolles J, Alcaraz MJ, Bazzocchi IL. Activity of lupine triterpenoids from *Maytenus* species as inhibitors of nitric oxide and prostaglandin E2. Bioorg Med Chem 2006; 14: 1573-1579.
32. Roussis V, Fenical W, Vagias C, Kornprobst JM, Miralles J. Labiatamides A,B and other Eunicellan Diterpenoids from the Senegalese gorgonian Eunicella labiata. Tetrahedron 1996; 52 (8): 2735-2742.
33. Hanson JR. Diterpenoids. Nat Prod Rep 2001; 18: 88–94.

34. Kohmoto S, Kashman Y, McConnell OJ, Rinehart KL, Wright A, Koehn F. Dragmacidin, a new cytotoxic bis (indole) Alkaloid from a deep water marine sponge, *Dragmacidon* sp. Journal of Organic Chemistry 1988; 53: 3116– 3118.
35. Sander CS, Chang H, Hamm F, Elsner P, Thiele JJ. Role of oxidative stress and the antioxidant network in cutaneous carcinogenesis. Int J Dermatol 2004; 43: 326-335.
36. Benzie I, Strain J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal Biochem 1996; 23: 270–276.
37. Duh PD, Tu YY, Yen GC. Antioxidant activity of the aqueous extract of harn jyur (Chrysanthemum morifolium Ramat). Lebensmittel Wissenschaft Technologie 1999; 32: 269–277.