

EVALUATION OF THE ANTIPROLIFERATIVE ACTIVITY OF METHANOL EXTRACT AND ITS FRACTIONS FROM THE MEDITERRANEAN SEAWEED, *HALURUS EQUISETIFOLIUS*

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ABSTRACT

Objective: Marine macrophytes contain a variety of biologically active compounds, some reported to have anticancer activity in vitro. Thus in the present study we investigated in vitro the efficacy of methanol extract and its semi-purified fractions (F2-F3) of *Halurus equisetifolius* for their antiproliferative effects.

Methods: to study their potential cytotoxic activity we used the MTT colorimetric method and clonogenic inhibition against three human cancer cell lines (A549, lung cell carcinoma, HCT15, colon cell carcinoma and MCF7, breast adenocarcinoma).

Results: Among the series F3 exhibited interesting growth and colony inhibitory effects against the three cell lines in a concentration-related manner which was correlated with its total phenolic content.

Conclusion: These findings suggest that the polar active fraction F3 could contain a new antiproliferative compound(s). The purification and the determination of chemical structure of compound(s) of this active fraction are under investigation.

Keywords: *Halurus equisetifolius*; Cytotoxic activity; MTT colorimetric method; Clonogenic inhibition assay; Lung cell carcinoma; Colon cell carcinoma; Breast adenocarcinoma

INTRODUCTION

Cancer is a dreadful human disease, increasing with changing life style, nutrition, and global warming. Cancer treatments do not have potent medicine as the currently available drugs are causing side effects in some instances. In this context, a variety of ingredients of traditional medicines and herbs are being widely investigated in several parts of the world to analyze their potential as therapeutic agents [1-3]. Seaweeds are important sources of protein, iodine, vitamins, and minerals and hence, their metabolites have shown promising activities against cancer incidences [4]. The seaweeds also contain high amounts of polyphenols such as catechin, epicatechin, epigallocatechin gallate, and gallic acid [5]. These algal natural products demonstrated a broad range of biological activities, such as, anti-inflammatory, antibiotics, antiviral, antimutagenic, and cytotoxic activities [6,7]. As a part of a screening program to search for new natural anticancer products, we report here the in vitro evaluation of the potency of methanol extract and its semi-purified fractions (F2-F3) of the red alga *Halurus equisetifolius* (*H. equisetifolius*) for growth and clonogenic inhibiting of three human cancer cell lines A549, HCT15 and MCF7.

MATERIALS AND METHODS

Sample collection and preparation of the methanol extract

The red alga *H. equisetifolius* was collected from the Mediterranean Sea, in various areas of the coastal region of Monastir (Tunisia), in June 2008, at a depth between 1 and 2 meters. The collected samples were cleaned by rinsing with sea water and distilled water, to remove associated debris and epiphytes. Identification of specimen was carried out in the National Institute of Marine Sciences and Technologies, Salamboo, Tunisia.

The seaweeds were then air dried in the shady at 30°C and finely powdered. 600 g of finely powdered seaweed material were packed in small bags (5x10 cm) of Whatman filter paper No. 1 and all bags were sealed and soaked in a methanol bath three times, steeping for 48h. The methanol extracts were combined and evaporated under vacuum at low temperature (<40°C) and then stored at -20°C until use.

Purification of the methanol extract

In order to localize the active fraction, methanol extract of *H. equisetifolius* was purified, using C₁₈ cartridges (Sep-pack, Supelco),

by gradient elution with methanol-water mixture (0%, 50% and 80% methanol) to give 3 fractions (F1, F2 and F3). Methanol solvent was removed from fractions recuperated using rotating evaporator at 35°C and distilled water was then added to the residues and the aqueous phases were lyophilized. The powdered fractions were stored at -20°C until use.

Preliminary phytochemical analysis

The methanol extract and its semi-purified fractions (F2, F3) were screened for the presence of the main active principles of red algae (sterols, glycosides, tannins, terpenoids and alkaloids) [8]. The identification of major chemical groups was carried out by thin-layer chromatography (TLC) using specific reagent [9,10].

Total Phenolic Content (TPC)

The TPC of methanol extract and its semi-purified fractions (F2, F3) from *H. equisetifolius* was estimated by the method of Taga [11]. Briefly, 100 µl aliquot of sample were mixed with 2.0 ml of 2 % Na₂CO₃ and allowed to stand for 2 min at room temperature. After incubation, 100 µl of 50% Folin-Ciocalteu's phenol reagents were added, and the reaction mixture was mixed thoroughly and allowed to stand for 30 min at room temperature in the dark. Absorbance of all sample solutions was measured at 720 nm using spectrophotometer (Jenway 6505 UV/Vis). A calibration curve of gallic acid (GA) (0.05-1 mg/ml) was prepared, and TPC was standardized against GA and expressed as mg GA equivalent per g of sample on a dry weight basis. All determinations were performed in triplicate.

Cell culture

The human tumor 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, all obtained from Biochrom AG (Berlin, Germany). They were grown on Flasks (Nunc, Denmark) at 37°C in a humidified atmosphere containing 5% CO₂. 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 [12-14].

Known number of A549, HCT15 or MCF7 cells (10^3) were transferred into 96-well plates (Nunc, Denmark) in a volume of 200 μ l 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 methanol extract or fractions to be tested. After drug exposure, the cells were washed with phosphate-buffered saline and then reincubated in fresh culture medium for a further 48h, then the culture medium was removed and 200 μ l of MTT reagent (diluted in culture medium, 0.5 mg/ml) was added. Following incubation for 4h, the MTT/medium was removed and DMSO (200 μ l) was added to dissolve the formazan crystals. Absorbance of the colored solution was measured on a microplate photometre (Bio-Tek Instruments) using a test wavelength of 570 nm and a reference wavelength of 630 nm. 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. All experiments were performed at least twice in triplicate. 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 Nicolaas with some modifications [15]. Known number of A549, HCT15 or MCF7 cells ($2 \cdot 10^4$) were transferred into six-well plates (Becton Dickinson Labware, 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 drug which is capable of bringing about 50% inhibition of colony formation.

Statistical Analysis

For all our experiments, a one-way ANOVA was used to analyze the differences between groups, followed by a Duncan's test with a threshold of significance of $P < 0.01$ and $P < 0.001$ to detect specific differences, using a statistical software package (STATISTICA edition 99 Maisons-Alfort- France). We used this post hoc test or multiple comparison tests, to determine the significant differences between a single control group mean and the remaining treatment group means in an analysis of variance setting.

RESULTS

Phytochemical analysis

The qualitative phytochemical screening of our seaweed showed the presence of sterols, alkaloids, tannins and terpenoids in both methanol extract and F3. Glycosides were found in F3. Fraction F2 contains only few terpenoids (Table 1).

The TPC of *H. equisetifolius* samples was measured according to Folin-Ciocalteu method. The Folin-Ciocalteu reagent determines total phenols, producing blue colour by reducing hetero polyphosphate molybdate tungstate anions. The level of phenolic in F3 fraction was higher than methanolic extract with $28,41 \pm 2.12$ and 4.55 ± 0.21 mg GAE/ g dried sample, respectively. Whereas no phenolic was found in F2.

Table 1: It shows qualitative phytochemical screening of the methanol extract and its semi-purified fractions (F2, F3) from the red alga, *Halurus equisetifolius*.

Samples	Terpenoids	Sterols	Alkaloids	Glycosides	Tannins
Methanol extract	++	++	+	-	+
Fraction F2	+	-	-	-	-
Fraction F3	+++	+++	++	+++	+

-: not detectable; +: low quantities; ++: high quantities; +++: very high quantities.

Evaluation of antiproliferative activity against tumor cell lines

In the first experiment, methanol extract was tested for its effect on inhibition of cell growth against three human tumor cell lines A549, HCT15 and MCF7 over a concentration range (12.5-1000 μ g/ml) to determine their potency (IC_{50} -50% inhibition of cell growth). Assay

was performed *in vitro* on exponentially growing cells. The activity was evaluated by measuring the levels of surviving cell after incubation for 24h with the test samples, using the MTT colorimetric assay [14,16]. This is the first step in our anticancer drug development program and is designed to identify those extracts with cytotoxic activity. The results of this primary screening are reported in Fig. 1.

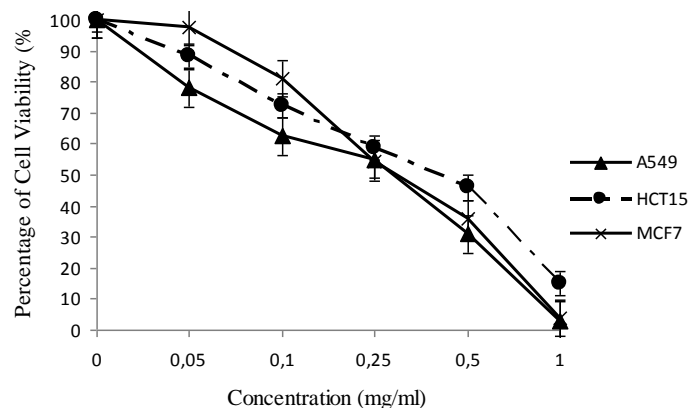


Fig. 1: It shows effect of the methanol extract from *Halurus equisetifolius* on cellular growth against three human tumor cell lines (A549, lung cell carcinoma, HCT15, colon cell carcinoma and MCF7, breast adenocarcinoma). Data are expressed as mean \pm standard deviation

Methanol extract exhibited a cytotoxic effect. 50% inhibition of cell growth was obtained at concentrations of 75, 60 and 175 $\mu\text{g/ml}$ respectively against human tumor cell lines A549, HCT15 and MCF7.

However within the series of fractions studied, only F3 revealed a significant activity against the three cell lines at concentration related manner, the results are shown in Figure 2.

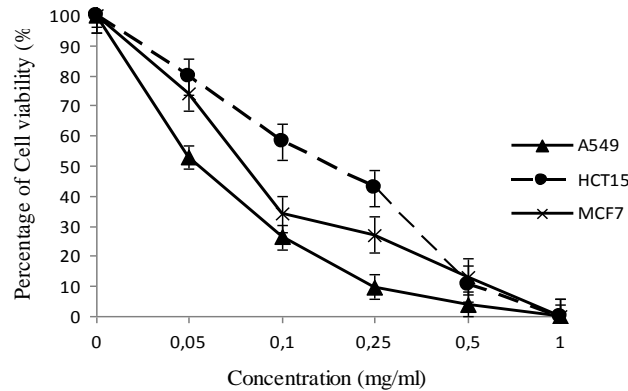


Fig. 2: It shows effect of the semi-purified fraction F3 from *Halurus equisetifolius*, on cellular growth against three human tumor cell lines (A549, lung cell carcinoma, HCT15, colon cell carcinoma and MCF7, breast adenocarcinoma). Data are expressed as mean \pm standard deviation

50% inhibition of cell growth was obtained at concentrations of 50, 31.25 and 62 $\mu\text{g/ml}$ respectively against human tumor cell lines tested A549, HCT15 and MCF7.

Since the inhibitory effects of the methanol extract and its semi-purified fraction F3 from the red alga *H. equisetifolius* on the inhibition of cell growth using the MTT colorimetric assay were established, we then examined their effects on cell viability by clonogenic inhibition assay against the same three human tumor cell

lines (A549, HCT15 and MCF7) with concentrations ranging from 50 to 1000 $\mu\text{g/ml}$. In our experiments, data for antiproliferative effects of methanol extract on A549, HCT15 and MCF7 cells showed a significant clonogenic inhibition at concentration-related manner, results are reported in Fig. 3.

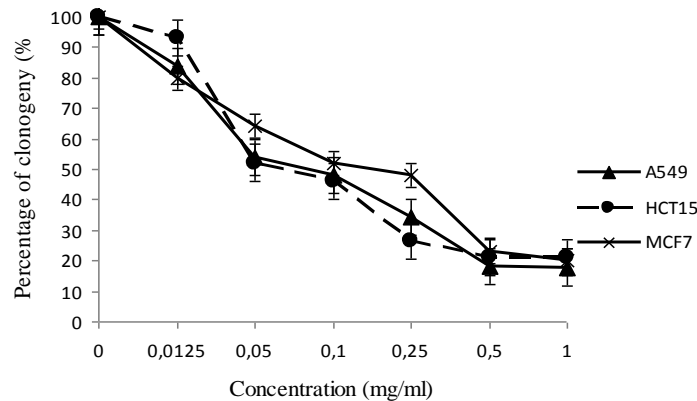


Fig. 3: It shows that the methanol extract induced clonogenic inhibition in human tumor cell lines (A549, lung cell carcinoma, HCT15, colon cell carcinoma and MCF7, breast adenocarcinoma). Data are expressed as mean \pm standard deviation

50% inhibition of cell growth was obtained at concentrations of 312, 343.5 and 313 $\mu\text{g/ml}$ respectively against human tumor cell lines tested A549, HCT15 and MCF7. In further experiments, F3 produced significant clonogenic inhibition too (Fig. 4).

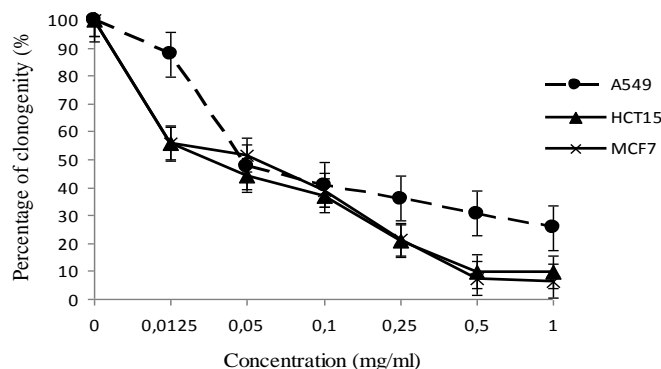


Fig. 4: It shows that the semi-purified fraction F3 induced clonogenic inhibition in human tumor cell lines (A549, lung cell carcinoma, HCT15, colon cell carcinoma and MCF7, breast adenocarcinoma). Data are expressed as mean \pm standard deviation

Concentrations resulting in 50% growth inhibition are 56.2, 157 and 81.25 $\mu\text{g/ml}$ respectively against A549, HCT15 and MCF7.

Both methanol extract and F3 semi-purified fraction of *H. equisetifolius* showed, *in vitro*, significant antiproliferative activities. However F2 had no activity. The IC₅₀ values clearly indicated that the semi-purified fraction F3 had a much more potent effect, on the three human tumor cell lines tested, than methanol extract. In term of cell line sensitivity, similar responses were observed against the three human tumor cell lines.

DISCUSSION

According to the American Cancer Society, the global burden is expected to grow as 27 million new cancer cases and 17.5 million cancer deaths simply due to the growth and aging of the population by 2050. Natural derivatives play an important role to prevent the cancer incidences as synthetic drug formulations cause various harmful side effects to human beings. Of the anticancer compounds extracted so far, the marine algal contribution is 65.63%. Owing to a diverse chemical ecology, the marine organisms especially marine flora have a great promise for providing potent, cheaper, and safer anticancer drugs, which deserve an extensive investigation [17].

The MTT reduction and clonogenic assays are two functional tests to predict the response of tumors to cytotoxic drugs. The MTT colorimetric assay [14,16] is based on the ability of metabolically active cells to convert the pale yellow MTT to a blue formazan product, which is quantifiable spectrophotometrically and the clonogenic assay or colony formation assay [15] is an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony. The colony is defined to consist of at least 50 cells. This clonogenic assay has been used in the ensuing decades for a large variety of studies with many types of cells. The assay detects all cells that have retained the capacity for producing a large number of progeny after treatments that can cause cell reproductive death as a result of damage to chromosomes, apoptosis [18].

In the current study, both methanol extract and F3 fraction of the Mediterranean red algae, *H. equisetifolius* showed, *in vitro*, a significant antiproliferative activity ($P < 0.001$) against three human cancer cell lines A549, HCT15 and MCF7. The IC₅₀ values clearly indicated that the semi-purified fraction F3 had the most potent effect on the three human tumor cell lines and these results are in correlation with its total phenolic content.

The marine floras are rich in medicinally potent chemicals predominantly belonging to polyphenols and sulphated polysaccharides. And the present paper showed the presence of polyphenols especially in the semi-purified fraction F3 from *H. equisetifolius*. The chemicals have displayed an array of pharmacological properties especially antioxidant, immunostimulatory, antiprotozoal, antimycobacterial and antitumor activities [17]. The phytochemicals possibly activate macrophages, induce apoptosis, and prevent oxidative damage of DNA, thereby controlling carcinogenesis. Polyphenolic compounds inhibit cancer cells by xenobiotic metabolizing enzymes that alter metabolic activation of potential carcinogens, while some flavonoids can also alter hormone production and inhibit aromatase to prevent the development of cancer cells [19]. The mechanism of action of anticancer activity of phenolics is by disturbing the cellular division during mitosis at the telophase stage. Phenolics reduce the amount of cellular protein and mitotic index, and the colony formation during cell proliferation of cancer cells [20]. No previous studies were reported about the cytotoxic activity from the same genus *Halurus*. However other genres of red algae were evaluated for this activity. In fact *Dasya villosa* lectin purified from marine red alga *Dasya villosa* have antitumor activity against hepatic carcinoma H₂₂ tumor in mouse [21]. The crude extract of the red alga *Corallina officinalis* displayed a significant ($P < 0.001$) cytotoxic effect towards L6 cells at 90 µg/ml (IC₅₀ = 88.6 µg/ml) [22]. The alcoholic extract of the red alga *Acanthophora spicifera* exhibits tumoricidal activity on Ehrlich's ascites carcinoma cells developed in mice at a dose of 20 mg/kg, comparable to the standard drug, 5-fluorouracil [23].

CONCLUSION

The main substances biosynthesized by red algae with antiproliferative and antitumor potential include polyphenols, sulfated

polysaccharides and terpenes, and their presence in the semi-purified fraction F3 may be responsible for its antiproliferative activity.

All these findings support the need for further investigations to clarify the features underlying the antiproliferative potential of this fraction. Biochemical and molecular studies carried out using the fraction in different animal models to establish their therapeutic efficacy, and subjected to HPLC and LC&MS analyses to identify and characterize the efficacious bioactive compound(s) in *H. equisetifolius*.

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