Original Article

Antibacterial, Cytotoxic and Antioxidant Potentials of *Cladophora prolifera* (Roth) Kutzing Collected from the Mediterranean Coast of Morocco

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ABSTRACT

Background: Among marine organisms, seaweeds are a highly diverse group of organisms from which many new substances have been isolated and many of these compounds have been demonstrated to possess a large spectrum of bioactivities.
Objective: In this study, we aim to evaluate the antibacterial, cytotoxic and antioxidant activity of green algae, *Cladophora prolifera* (Roth) Kutzing, collected from the northern Mediterranean coast of Morocco.
Material and Method: The antibacterial activity was determined by disk diffusion method. After fractionation by column chromatography, the fractions from *C. prolifera* were tested against *Staphylococcus aureus* ATCC 25923 using the broth microdilution assay. The antitumor effect on human colon cancer cells was investigated via sulforhodamine-B (SRB) assay. Then the Crude extract has been tested in radical-scavenging assays to assess their antioxidant activity.
Results: In vitro screening of methanolic extract of *C. prolifera* showed specific activity to inhibit the growth of five virulent strains of pathogenic bacteria, *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus aureus* (ATCC 29213), *Enterococcus faecalis* (ATCC 29212) and *Klebsiella pneumoniae* (ATCC 700603). The obtained results indicated that the extracts of *C. prolifera* were cytotoxic against HT29 human colon cancer cells. In addition, based on the capacity of the algae to scavenge the ABTS radical cation, we revealed that *C. prolifera* extract presented a satisfactory antioxidant activity.
Conclusion: These results suggest that *C. prolifera* has a great biological potential, which could be considered for future uses in food, pharmaceutical and cosmetic industries.

Keywords: *Cladophora prolifera*, Crude extract, Cytotoxicity, Antibacterial activity, Antioxidant activity.

INTRODUCTION

In recent years, natural products have been playing a major role in the search for novel drugs or drug candidates against infectious diseases, inflammation, cancer and many other illnesses. They are an ongoing and inspiring source for researchers due to their enormous structural diversity and complexity.

The marine algae represents a largely unexplored source for the isolation of novel bioactive compounds and may become even more so as knowledge on marine natural products. Thus, macroalgae has been recognized as a promising source of bioactive secondary metabolites with antitumor[1,2], antibacterial[3,4], antioxidant[5,6], anti-inflammatory[5,7] and antifouling activities[8].

The chemical structures of these seaweeds derived bioactive substances are diverse, including brominated phenols, sterols, polysaccharides, peptides, proteins, acrylic acid, chlorophyllides, terpenes, phenols and heterocyclic carbons[9,10]. Some of these bioactive compounds are antimicrobial, anticancer and antioxidant agents. *Cladophora prolifera* (Roth.) Kutzing, is a green seaweed extensively distributed in the Mediterranean and Atlantic seas of Morocco. Chemical analyses of the species, revealed its richness in important mineral oligoelements[11]. The protein content is very high (24.62%)[12], especially when compared to other green algae utilized by the industry[13]. However, little information about its biological active substances is available. Therefore, numerous studies have demonstrated that the extracts of *C. prolifera* showed powerful antibacterial activities[14]. Many authors suggested that the antibacterial activities of macroalgae could be due to the fatty acids constituents[15]. Many fatty acids isolated then from *C. prolifera* showed anti-coagulant[16] anti-inflammatory[17], antiviral[18] and antihelmintic[19] activities. Hence, the main objective of this study was to assess the antitumoural, antibacterial and antioxidative potentialities of *C. prolifera* settled along the northern Mediterranean coast of Morocco. The anti-proliferative effect of *C. prolifera* on HT-29 human colon cancer cells was investigated. We also evaluated the antimicrobial activity of the macroalgae extract against gram-positive and gram-negative bacterial strains using the diffusion method. Finally, for assessment of antioxidant properties, the ABTS free radical decolorization assay was used.

MATERIAL AND METHODS

Chemicals

Ethanol, acetone and methanol were obtained from Merck (Darmstadt, Germany). 2, 2-azino-bis (3-ethylbenzothia-zoline-6-sulfonic acid) diammonium salt (ABTS) and 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Potassium persulfate were procured from Fluka Chemical Co. (Buchs, Swizerland) and sulforhodamine-B (SRB) was purchased from Sigma-Aldrich (Taufkirchen, Germany).

Algal materials

Samples of *Cladophora prolifera* (Roth) Kutzing were collected from the northern Mediterranean coast of Morocco, during the summer of 2007 (Ksar-sghir

35º50’52.58’’N, 5º33’39.04’’O). The taxonomic identification of species was done by experts in these fields, using standard literature and taxonomic keys. Voucher specimens of all tested species were deposited in the herbarium of the Laboratory of Applied Algology-Mycology, Department of Biology, Faculty of Sciences, Abdelmalek Essaâdi University, 93002 Tetouan, Morocco.

In the field, epiphytes were removed from the algae. The associated debris and necrotic parts were also removed by rinsing the samples with sterile seawater. The samples were a shade dried, cut into small pieces and powdered in a mixer grinder (IKA R A11 basic, Sigma-Aldrich). The powder obtained was preserved cold at -12 °C.

Chemical extraction

Freeze-dried specimens of *C. prolifera* (5 g /200 mL) were extracted with methanol, toluene-ethanol (1:1) and ethanol solvents in a Soxhlet extractor for 8 h. After filtration, the solution was then evaporated under reduced pressure to obtain a residue, which was partitioned between H_2O and ethanol. The organic layer was evaporated to dryness to give an extract. The residue was weighed and stored in sealed vials in a freezer at -4°C until being tested. Before biological screening, organic extracts were dissolved in 2% dimethylsulphoxide (DMSO).

Bacterial strains

The strains used were, three gram-positive bacteria *Staphylococcus aureus 1* (ATCC 25923), *Staphylococcus aureus 2* (ATCC 29213) and *Enterococcus faecalis* (ATCC 29212) and two gram-negative bacteria Escherichia coli (ATCC 25922) and Klebsiella pneumoniae (ATCC 700603). The bacteria strains were obtained from the Department of Microbiology, Faculty of Pharmacy, University of Grenade, Spain. All cultures were kept on Brain Heart Infusion (BHI, Sigma) agar plates and stored at 4°C, except the initial stock cultures which were stored at -80°C in BHI broth containing 20% glycerol.

Antibacterial activity by disc diffusion assay

The screening of the antibacterial activity of the extracts was performed by the disc diffusion technique in agar-plated Petri dishes[20]. The cultivated agar plates were prepared by pipetting 25 µL of extract to each disc, placed on the agar Mueller-Hinton (pH 7.4 ± 0.2 at 25°C) and incubated at 37°C during 24 h overnight[21]. Inhibition results are expressed as the width of the clear halo surrounding each disc on cultivated agar plates. Methanol solvent without algal extract was also used as a negative control and antibiotic ampicillin (5 mg/disc) was used as a positive control for comparing efficiency. All experiments were performed at least in triplicate. Representative halos were those measuring a diameter superior to 10 mm[22].

The inhibition zone of bacteria around the disc was measured and the assay was scored positive (+) if it was <15 mm, doubly positive (++) if the zone was ≥ 15 mm, triple positive (+++) if the zone of inhibition was ≥ 19 mm and negative (-) if there was no inhibition of microbial growth.

HPLC and Thin layer chromatography analysis

Methanol extract was analyzed by HPLC and TLC; The HPLC separations were performed on a Waters 600E HPLC system coupled to an XTerra RP C18 column (4.7 x 250 nm) and to a Waters 486 UV visible tunable detector.

The column was washed with acetonitrile 100 % before and after analysis. A mixture of acetonitrile/water (90/10) was chosen as the optimal mobile phase with a

flow rate of 0.5 mL/min. The purification was archived after silica gel flash chromatography mono and bidimensional silica gel TLC. Seaweed extracts were applied and the chromatogram developed using different proportions of acetone/hexane/methanol as solvent. TLC plates were run in duplicate and one set was used as the reference chromatogram. Spots and bands were visualized by UV irradiation (254 and 366 nm) and H_2SO_4 spray reagent[23]. Fractions of the similar TLC profile were combined to get the final fractions, which were free from solvents, redissolved in an appropriate solvent after weighing and screened for antibacterial activity by disc diffusion methods as described above (25 µL solvent/ 6 mm disc).

Broth microdilution assay

The active extract and fraction of *C. prolifera* were tested by using the broth microdilution checkerboard technique for performing antimicrobial susceptibility testing. The microtitertrays, containing various volumes (25, 6.25, 3.12, 1.56, 0.78, 0.39, 0.19, 0.09, 0.048 and 0.024 µL) of methanolic extract and active fraction of *C. prolifera*, was prepared. Then, the bacteria strain was inoculated into the wells of 96-well microtiter and incubated at 37ºC for 16 to 20 h. The determination of the minimum inhibitory concentration (MIC, µL/mL) was then made by checking whether or not *S. aureus* grew in the various concentrations of the crude extraction and fractions. Turbidity of the broth or a button of cells at the bottom of the well is considered as evidence of growth.

Cell cultures

HT-29 human colon carcinoma cells were obtained from the American Type Culture Collection (ATCC, USA). The cells were maintained in McCoy's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin and was put in a humidified incubator at 37°C under an atmosphere of 5% CO_2. The *C. prolifera* extract was dissolved in DMSO and added to the culture medium, so that the final concentration of DMSO was less than 1%.

Sulforhodamine B assay

Sulforhodamine B (SRB) (Sigma–Aldrich, Germany) was used to test the effect of *C. prolifera* extract on cell growth and viability based on the method described by Vichai and Kirtikara[24]. The extract was dissolved in dimethylsulfoxide (DMSO) before diluting with the growth medium to a final DMSO concentration of <0.05%. The cancer cells were inoculated into 96 well plates in the growth medium at 5000 cells/well. After 24 h of incubation, the cells were exposed to various concentrations of *C. prolifera* extract (6.25, 12.5, 25, 50 and 100 µg/mL) and 5-fluorouracil (5-FU) (5, 10, 25, 50 and 100 µg/mL) used as positive control. The cells were then incubated for 48 h and 72h. The cells were fixed with TCA by gently adding 50 µL TCA (50%) to each well for 1 h at 4°C. The plates were then washed 5 times with deionized water and air-dried. The dried plates were stained with 100 µL of 0.4% (w/v) SRB prepared in 1% (v/v) acetic acid for 30 min at room temperature. The plates were rinsed quickly 5 times with 1% acetic acid to remove the unbound dye, followed by air-drying. The bound dye was solubilized in 2 mM Tris base (100 µL/well) for 5 min. Optical densities were read on a microplate reader (Spectrophotometer Labsystems Multiskan EX at $\lambda = 492$nm).

ABTS radical scavenging activity

Antioxidant activity was determined by ABTS free radical decolorization assay developed by Arnao *et al.*[25] with a slight

modification. The stock solutions included, 7.4 mM ABTS solution and 2.6 mM potassium persulfate solution. The mixture was maintained for 15 h in the dark at room temperature. The solution was diluted with ethanol to obtain the absorbance of 0.7 ± 0.2 units at 750 nm. To determine the scavenging activity, 10 µL ABTS reagent was added to 90 µL of EtOH and 10 µl of different concentrations of seaweed extract. The absorbance, monitored for 5 min, was measured spectrophotometrically at 750 nm using a microtitre plate reader. Trolox was used as standard. Percentage inhibition of the sample was calculated by the following equation:

$$\% \text{ Inhibition} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 expresses the absorbance of control; A_1 expresses the absorbance of the seaweed extract tested.

RESULTS AND DISCUSSION

Antimicrobial activity

Methanol, ethanol and toluene-ethanol (1:1) extracts from the marine algae *C. prolifera* were assayed for their antibacterial activity by using agar diffusion and Broth microdilution methods.

The methanolic extract of *C. prolifera* showed some antimicrobial capacity against all tested organisms, with the major effect against *S. aureus* 1, moderate activity against *E. coli* (Table 1, Figure 1) and low activity against *K. pneumonia* and *Enterococcus faecalis*, but the extract was not active against *Staphylococcus aureus* 2 *(ATCC 29213)* (Table 1). The toluene-ethanol (1:1) extract of *C. prolifera* was active only against *Staphylococcus aureus* 1. However, the ethanol extract was inactive against all pathogenic organisms tested. The strain the most sensitive to the extract of *C. prolifera* was *S. aureus* 1. The negative control discs showed no zone of inhibition against all the pathogenic bacteria and the positive control Ampicillin showed an inhibition zone ranging from 13 mm to 16 mm against Gram positive and Gram-negative bacteria.

As for the tests with pathogenic bacteria, the extracts showed differences in their activity. This variation in their antibacterial activities may be due to the method of extraction, extraction solvent used and season at which samples were collected and may be attributed also to the effectiveness of the extraction methods[26]. Some studies showed that methanol extraction yielded higher antimicrobial activity than other organic solvents[27]. In this study, methanol was the best solvent for extracting the effective antimicrobial substances from the seaweed *C. prolifera*. Similar results have been already reported by other authors[28]. Taşkin *et al.*[29] reported that the methanolic extract of *C. prolifera* had broadest inhibitory activity against *Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis* and *Escherichia coli* O157:H7. The investigation of Ely *et al.*[30] indicated that the methanolic extract of *C. prolifera* had moderate antibacterial activity against *S. aureus* while the inhibitory activity of the extract of Turkish *C. prolifera* was better. Tuney *et al.*[31] investigated the antimicrobial activity of different organic extracts of the same genus of seaweeds collected from the coast of Izmir (Turkey). Different organic extracts of *Cladophora* sp. were assayed against test microorganisms. The authors reported that methanol and diethylether extracts did not show any inhibitor activity. Freile-Pelegrin and Morales[32] studied ethanolic extracts from different thallus regions (apical, basal and stolon) of *Caulerpa* spp. They reported that the stolon was the part having the highest antibacterial activity. The results of the current study revealed that Gram-positive organisms were more susceptible to the crude extracts of *C. prolifera*. Reichelt and Borowitzka[33] also reported that Gram-

positive bacteria were more effectively controlled by the majority of Australian algal extracted.

HPLC analysis and fractionation of the methanolic extract

Active methanolic extract of the *C. prolifera* (Rothpletz) Kutzing was analyzed by HPLC to identify the nature and the number of major constituents of the active extract. The obtained chromatogram is shown in the Figure 1. The chromatogram revealed the presence of a major compound that corresponds to the peak C (retention time, RT = 29 min). The other peaks correspond to minor compounds, A (RT = 27 min), B (RT = 28 min) and D (RT = 37 min) (Figure 1).

The methanolic extract was then separated using chromatography column. Twelve fractions were further obtained after silica gel flash chromatography; they were eluted using a gradient polarity (hexane, ethyl acetate and methanol). Each fraction displayed a major spot in TLC using different dyes and UV light.

The obtained fractions were then tested against *Staphylococcus aureus* 1 (Table 2). Tree fractions (CPF3, CPF4 and CPF7) of petroleum ether extract of *C. prolifera* showed high growth inhibitory activity against *Staphylococcus aureus* 1. However, only one fraction (CPF5) showed moderate activity and seven showed no activity against the tested bacteria (Table 2).

Comparative data of the minimal inhibitory concentrations (MIC) of methanolic extract and active fractions of *C. prolifera* are presented in Figure 2. The fractions of *C. prolifera* extract, CPF3, CPF4, CPF5 and CPF7 (eluted with ethyl acetate and hexane) showed MIC values of 3.12, 6.25, 1.56 and 3.12 µL/mL, respectively. In addition, the inhibitory effect of fractions of microdilution method showed higher efficiency compared to the crude extracts (Figure 2).

The identity of the peaks was confirmed by TLC with known standards. Organic acids and phenolic compounds, especially polyphenols or tannins have been shown to have antimicrobial activities[34,35]. Rosell and Srivastava[15] detected small amounts of free organic acids and polyphenols in the algal extracts and consequently pointed out that these substances play only a minor role in the antibacterial activities recorded in their study. We think that the antimicrobial activity shown by *C. prolifera* is attributed to the presence of fatty acids in the fractions obtained from this alga. The noteworthy capability of fatty acid to produce antimicrobial activities has been also reported by Kabara[36], McCracken *et al.* [37] and Rosell and Srivastava[15] as well as the antibiotic activity from ten Xantophyta was associated with the presence of organic acids, unsaturated fatty acids and phenolic compounds. While, Vaskovsky[38] and Zhukova *et al.*[39] reported the presence of fatty acids, in the algae *Cladophora rudolphiana* and also in some species of the genera Rhodomela, Gracilaria, Sargassum, Ulva, Enteromorpha and Zostera, Dunaliella and Chlorella, ranging from 14:0 to 20:5 with predominating unsaturated fatty acids.

Cell proliferation inhibition

To identify the anti-cancer phytochemicals potential, we examined the cytotoxicity of the seaweed *C. prolifera* extract on the HT-29 human colon cancer cells using the SRB method with 5-FU as a positive control. The result of the cytotoxic activity is shown in Figure 3. The percentages of growth inhibition of HT-29 cells by the *C. prolifera* extract at various concentrations were determined as the percentage of viable treated cells in comparison with viable cells of untreated

controls. The *C. prolifera* extract exhibited a dose- and time-dependent inhibitory effect on the HT-29 cancer cell growth. The algae extract was not efficient at low concentrations, but showed moderate toxicity at high concentration, furthermore the *C. prolifera* cytotoxic effects on HT-29 cell lines were less than the positive control 5-FU.

After treatment of the cells with the extract for 48 and 72 h, cell numbers decreased and cell death rates increased, the IC50 value of *C. prolifera* was 82.8 and 69.29 µg/mL for 48 and 72 h, respectively (Figure 3).

C. prolifera did not show significant cytotoxicity after treatment during 48 h at concentrations from 6.25 to 25 µg/mL, but it inhibited 10.46 ± 4.8% and 40.32 ± 0.9% cell proliferation of HT-29 at the concentrations of 50 and 100 µg/mL, respectively. After 72 h, the concentrations of 50 and 100 µg/mL of *C. prolifera* extract resulted in 25.84 ± 4.3% and 57.97±1.5 % inhibition in HT-29 cell growth, respectively.

Therefore, the current study demonstrated for the first time, to the best of our knowledge, that the extract of *C. prolifera* exhibits antiproliferative activity against HT-29 colon cancer cells. In fact, only a few investigations on the antitumor potential of Cladophora species have been reported. Feng *et al.*[40] reported the potential of a novel vanillic acid derivative (IC50 = 3.7 µM) and its sulfate adduct (IC50 = 1.7 µM) isolated from *C. socialis* to prevent cancer by inhibiting considerably the activity of protein tyrosine phosphatase 1B (PTP1B). Drugs targeting phosphatases are considered as promising novel cancer therapies[41]. Recently, Tang *et al.*[42] isolated some cyclic tetrapyrrolic photosensitizers from *C. patentiramea* and demonstrated that they exhibit an important phototoxicity against HL60 promyelocytic leukemia cells. To date, a number of photosensitizers derived from natural products have been clinically approved for the photodynamic therapy of specific cancers[43]. Furthermore, Laungsuwon and Chulalaksananukul[44] found that the ethyl acetate extract of *C. glomerata* possesses growth inhibitory activity (IC50=1420 µg/g) against the KB cell lines (epidermoid carcinoma of the oral cavity) *in vitro*.

More recently, Cha *et al.*[45] reported a skin cancer protective effect of 3, 4-dihydroxybenzoic acid isolated from *C. wrightiana* Harvey by protecting human keratinocytes against UVB-induced apoptosis and oxidative stress.

Thus, Future researches are needed to elucidate the antiproliferative mechanism of *C. prolifera*, also the identification of the bioactive compounds of this seaweed will lead to their evaluation for uses in medicine, food production and cosmetic industry.

ABTS radical scavenging activity

Antioxidant activity of the *C. prolifera* extract was determined by the ABTS assay, which evaluates the radical-scavenging activity of extract toward the $ABTS^{\bullet+}$ radical cation[46]. This method is one of the most commonly used methods for measuring antioxidant activity, which measures the capacity of a compound to scavenge $ABTS^{\bullet+}$ radical. It is recommended for use in plant extracts because the maximum absorption at at the wavelength of 734 nm avoids color interference in plant extracts[47].

The extracts of *C. prolifera* showed good antioxidant activity and effective scavenging of the ABTS radical (Figure 4) and this activity was lower to Trolox. The percentage of inhibition was 97.77% and 70.32% for the Trolox and *C. prolifera* extract, respectively, at the concentration of 200 µg/mL.

Previous studies have indicated high antioxidant activity in the genus Cladophora[48,49]. Zubia *et al.*[50] reported that the extracts of *C. prolifera* exhibited also relatively high DPPH radical scavenging activities (IC50=16.66 mg/mL). Phenols and polyphenols such as flavonoids which are a large group of compounds, widely found in macroalgae. These compounds exert potent antioxidant activity not only because of their capacity to donate electrons or hydrogen atoms, but also because of their stable radical intermediates[51]. In the study done by Soltani *et al.*[49] high phenolic content has been measured for *Cladophora* species showing a maximum of 3077 ± 105 mg gallic acid equivalent per gram of dry weight of extract. It is well known that the increase in the levels of flavonoids in the daily diet may reduce the incidence of certain human diseases. The health benefits of flavonoids may be due to their interaction with various biological systems and show antioxidant capacity, free-radical scavenging activity, anticancer activity, and cardiovascular disease prevention, while some flavonoids exhibit potential for anti-HIV functions[52,53]. Therefore, the phenolic content can be the responsible for the antioxidant activity of the algae *C. prolifera*.

CONCLUSION

The first chemical description of *Cladophora prolifera* (Rothpletz) Kutzing from Mediterranean coast of Morocco was presented. The investigation of its methanolic extract revealed remarkable activity against some pathogenic bacteria. We noticed that the methanolic extract of the green seaweed *C. prolifera* (Rothpletz) Kutzing exhibits an effective antibacterial activity against *S. aureus*, *E. coli* and *Ent. faecalis*. Then we attempted to fractionate and purify the active substances from this seaweed. Twelve fractions of *C. prolifera* extract was purified, but tree of this fraction showed satisfactory antibacterial activity. Further researches are needed to isolate the active fraction (CPF7) for a subsequent structural and biological characterization. Screening of antioxidant activity of *C. prolifera* extract exhibited strong radical-scavenging potential (ABTS test). Furthermore, in antiproliferation assays the extract of *C. prolifera* showed significant activity as an inhibitor of the growth of the HT-29 cells.

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Table 1. Antibacterial activity of methanol, toluene-ethanol (1:1) and ethanol extracts of *C. prolifera* (Rothpletz) Kutzing against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* 1 ATCC 25923, *Staphylococcus aureus* 2 ATCC 29213, *Enterococcus faecalis* ATCC 29212 and *Klebsiella pneumoniae* ATCC 700603

Treatment	*E. coli*	*S. aureus 1*	*S. aureus 2*	*E. fae*	*K. pneum*
Methanol	++	+++	-	+	+
Toluene-ethanol (1:1)	-	+	-	-	-
Ethanol	-	-	-	-	-
Ampicillin	+	+	nt	++	++

nt: Not tested

Table 2. The antibacterial screening of separated fractions against *Staphylococcus aureus* 1 ATCC 25923

Fraction	Antibacterial activity
CPF1	-
CPF2	-
CPF3	+++
CPF4	+++
CPF5	++
CPF6	-
CPF7	+++
CPF8	+
CPF9	-
CPF10	-
CPF11	-
CPF12	-

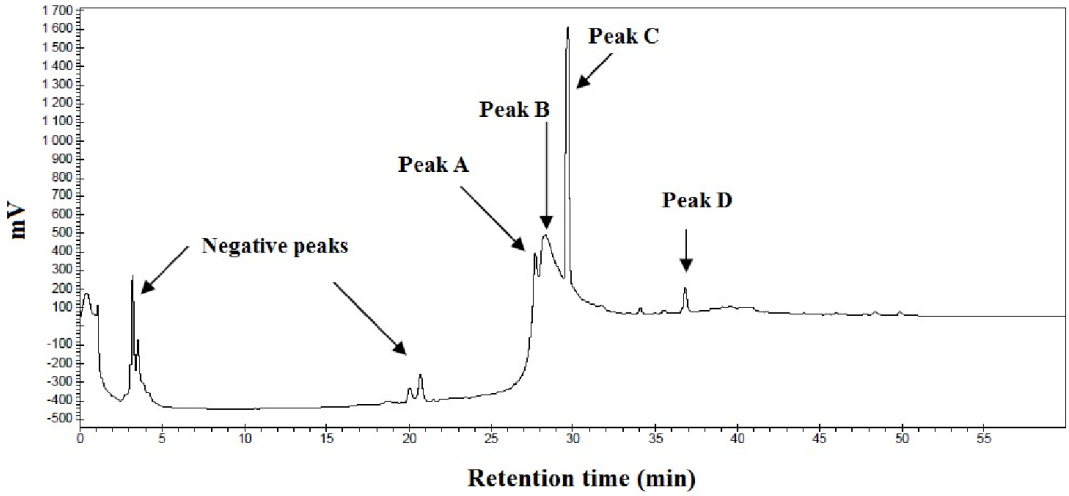

Figure 1. HPLC chromatogram of the analysis of the methanolic crude extract of *C. prolifera*

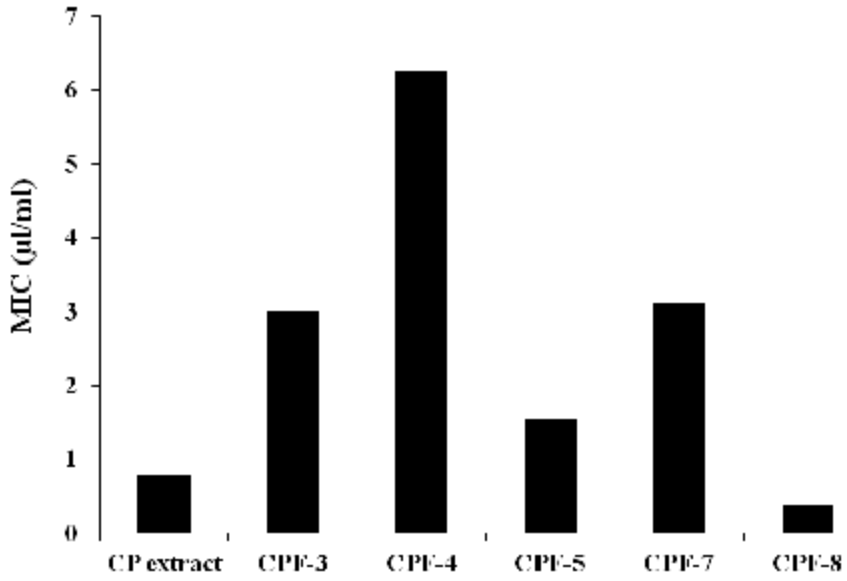

Figure 2. Minimal inhibitory concentration (µL/mL) of the crude extract and fractions of *C. prolifera* against *Staphylococcus aureus* 1 ATCC 25923

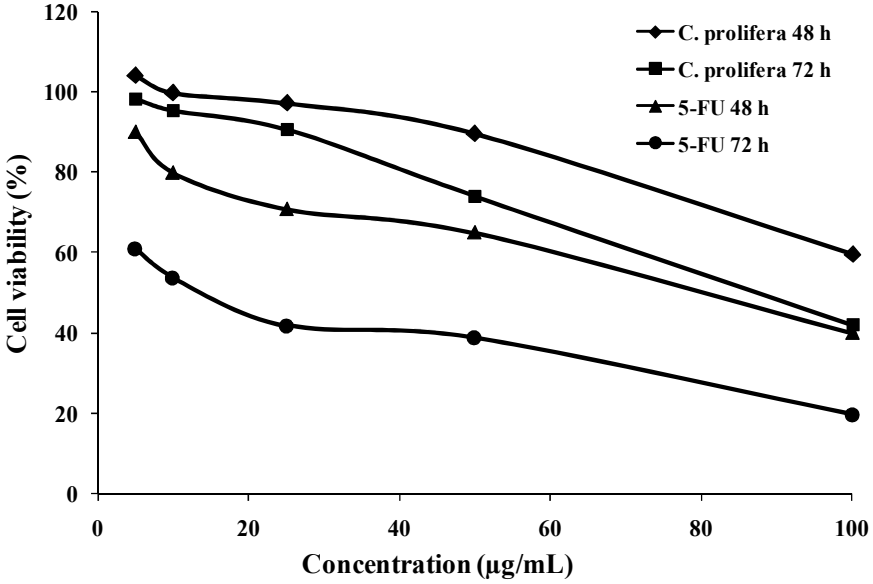

Figure 3. The percentages of HT29 cells growth inhibition by various concentrations of *C. prolifera* extract after treatment of the cells with the extract for 48 and 72 h

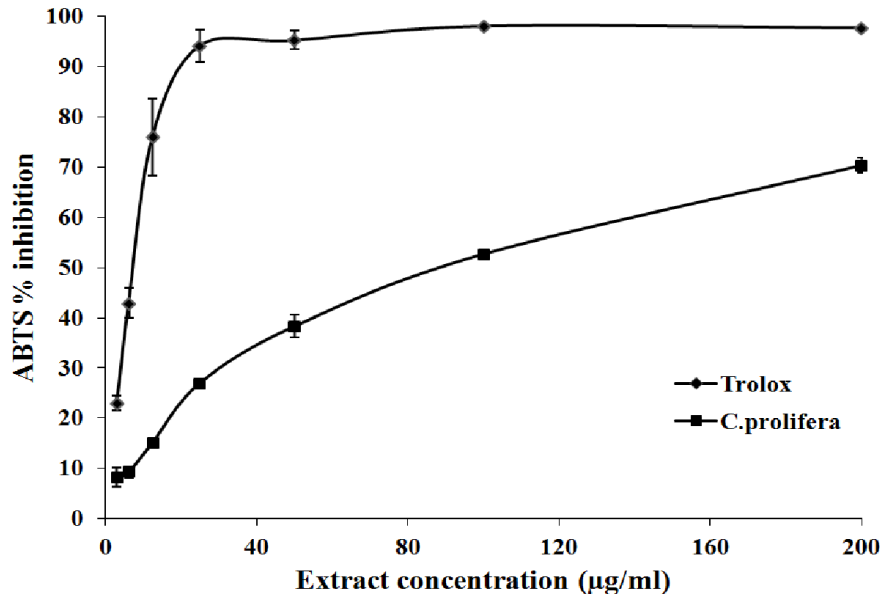

Figure 4. Radical-scavenging activity (ABTS test) of *C. prolifera* extract