Functional characterization of pressurized liquid extracts of *Spirulina platensis*

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Abstract

Three different parameters (temperature, solvent and extraction time) were studied regarding to pressure liquid extraction of antioxidant and antimicrobial compounds of *Spirulina platensis.* Two different antioxidant methods, β-carotene bleaching method and DPPH$^{\bullet}$ free radical- scavenging assay, were used to determine the optimal PLE conditions for antioxidant's extraction from *Spirulina platensis* microalgae. The selected conditions were as follows: extraction temperature equal to 115ºC, extraction time equal to 15 min and ethanol as extracting solvent. The main antioxidant compounds found in this extract were identified as zeaxanthin, a myxoxanthophyll-like compound and very polar phenolic compounds. Moreover, antimicrobial activity of different PLE fractions was tested against *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 11775, *Candida albicans* ATCC 60193 and *Aspergillus niger* ATCC 16404. Data obtained showed that the hexane and petroleum ether extracts were slightly more active than ethanolic extracts. As for water extracts, none of them were active against the microorganisms tested. Data indicated that both, 115ºC and 170ºC were the best extraction temperatures in order to optimize the extraction of antimicrobial compounds, whereas 9 min was the optimal extraction time. Besides, *Candida albicans* was the most sensitive microorganism to all *Spirulina* PLE extracts.

Keywords: *Spirulina platensis*; pressurized liquid extraction, antioxidant activity, antimicrobial activity

1 Introduction

Nowadays, interest in food products has increased since many studies have demonstrated its relationship with human health. Hence, a food can be regarded as functional if it is satisfactorily proved to beneficially affect to one or more target functions in the body, beyond adequate nutritional effects, either improving health or well-being and /or decreasing the risk of disease. Moreover, a functional food should be a natural food, a food to which a component has been added, or a food from which a component has been removed by technological or biotechnological means [1]. Therefore, nowadays food industries demand new food ingredients obtained by extraction of natural products, in order to developed novel functional foods.

Spirulina is one of the several alga genera that have attracted special attention due to their importance as human foodstuff and their in vitro and/or in vivo functional properties. Among these genera, *Spirulina platensis* has been extensively grown to obtain a protein-rich material of nutritional or industrial use (blue pigments) [2]. Several studies showed that *Spirulina platensis* or its extracts could posses physiological benefits as antioxidant, antimicrobial, anti-inflammatory, antiviral or antitumoral [3-7]. These functional properties have been attributed to different compounds such as, phycocyanins, carotenoids, phenolic acids and ω-3 and ω-6 polyunsaturated fatty acids [3,8-10].

Opposite to traditional solvent extraction techniques that use large quantities of toxic organic solvents, are labour-intense, need long extraction times, posses low selectivity and/or low extraction yields and can expose the extracts to excessive heat, light and oxygen, pressurized liquid extraction (PLE) uses less solvent in a shorter period of time, is automated, and involves retaining the sample in an oxygen and light-free environment [11,12]. Whereas other environmentally-friendly techniques, such as supercritical fluid

extraction (SFE), are frequently used to obtain functional compounds from natural sources, PLE has not been widely applied as a routine tool in natural product extraction. However, recent studies have demonstrated the advantage of PLE for the extraction of natural compounds from different matrices [12-16].

The aim of the present work was to study the antioxidant and/or antimicrobial properties of pressurized liquid extracts of *Spirulina platensis* obtained considering different extraction conditions (such as extraction time and temperature and extraction solvent). Thus, this study was performed as a screening to determine the optimal extraction conditions of functional compounds.

2 Materials and methods

2.1 Samples

Microalgae samples (*Spirulina platensis*) consisted of air-dried microalgae with 6% moisture mass, from Algamar (Pontevedra, Spain), stored under dry and dark conditions.

2.2 Chemicals

2,2-Diphenyl-1-picrylhydrazyl hydrate ($DPPH^{\bullet}$), BHT and ascorbic acid were obtained from Sigma-Aldrich (Madrid, Spain). Linoleic acid, Tween 20 and β-carotene was purchased from Fluka (Madrid, Spain). Methanol, ethanol, petroleum ether (bp 40-60 ºC) and chloroform were purchased from Panreac Quimica (Barcelona, Spain) and hexane, HPLC grade, from Lab Scan (Dublin, Ireland).

2.3. Extraction method

Spirulina platensis extractions were performed using a PLE (Sunnyvale, CA, USA), equipped with a solvent controller. Four different solvents (i.e. hexane, petroleum ether (bp 40-60ºC), ethanol and water) were used to achieve extracts with different

composition from a natural matrix. Moreover, extractions were performed at three different extraction temperatures (60ºC, 115ºC and 170ºC) and extraction times (3, 9 and 15 minutes). Previously, an extraction cell heat-up was carried out for a given time, which changed according to extraction temperature (5, 6 and 8 min heat-up were used for 60, 115 and 170ºC of extraction temperature, respectively). All extractions were performed in 11 ml extraction cells, containing 2.5 g of sample.

Extraction method was performed according to the procedure described by Herrero *et al*. [17]. The extracts were covered with aluminum foil and stored at –18ºC until dried. Organic solvents were removed under a stream of nitrogen gas, whereas a freeze dryer (Unitop 400 SL, Virtis, Gardiner, NY, USA) was used for water extracts. Afterwards, dry extracts were dissolved using the same solvent than during extraction to known concentration for antioxidant assays. In the same way, these solutions were stored at –18ºC and protected from light with aluminum foil.

2.4. β-carotene bleaching method

The procedure is based on a previously reported method [18] with slight modifications. 0.2 ml of *Spirulina* extracts (25 μg/ml, 50 μg/ml and 75 μg/ml of the extract in the liposome solution) or 0.2 ml of pure solvent (as control) were added to a reagent mixture, containing 0.2 ml of β-carotene solution (1 mg/ml in chloroform), 20 mg of linoleic acid, and 200 mg of Tween 20 and the final mixture was evaporated to dryness under a nitrogen stream. Fifty millilitres of distilled water were added and the mixture was vigorously shaken to form a liposome solution. The samples were then subjected to thermal autooxidation at 50ºC for 120 minutes. The absorbance of these solutions was measured at 470 nm using a Shimadzu UV-120-01 spectrophotometer (Shimadzu, Kyoto, Japan). Due to the coloration of the extracts, blank extracts were prepared, where 0.2 ml of chloroform were added instead of 0.2 ml of β-carotene solution. All

samples were assayed in duplicate. Butyl-hydroxy-toluene (BHT) (Sigma) (1 μg/ml) was used as standard. The antioxidant activity (AA) was calculated in terms of inhibition percent relative to the control using the following equation

$AA\ (\%) = [(R_{control} - R_{sample})/R_{control}] \times 100$

where

$R = \ln [Abs(t_0)/Abs\ (t_{120})]/120$

2.5. DPPH$^{\bullet}$ free radical- scavenging assay

The employed method was based on a procedure described by Brand-Williams et al. (1995) [19]. 100 μg/ml in the reaction mixture was tested in hexane, petroleum ether and ethanol extracts, whereas 250 μg/ml was tested in water extracts. 3.9 ml of DPPH$^{\bullet}$ solution (23.5 mg/l in methanol) were placed in test tubes and 0.1 ml of the extract was added. Absorbance was measured at 516 nm in a Shimadzu UV-120-01 spectrophotometer until the reaction reached a steady state. Methanol was used to adjust zero, DPPH$^{\bullet}$-methanol solution as a reference sample and ascorbic acid (2 μg/ml) as standard. Due to the coloration of the extracts, it was necessary to prepare a blank that consisted of 0.1 ml of *Spirulina* solution plus 3.9 ml of methanol. The radical scavenging activity of PLE extracts in the reaction medium was calculated from a calibration curve at 516 nm. Each determination was repeated twice.

2.6. TLC

Analytical-TLC was carried out in TLC plates (10 cm × 20 cm) cut from the commercially available sheets according to Jaime et al [20] procedure. 12 μl of 30 mg/ml extract solution were applied to 1 cm of the base of the silica gel layer and allowed to dry for a few minutes. Afterwards, the plate was eluted in a closed chamber with mobile phase. Two different mobile phases were used: petroleum ether/acetone (75/25) (eluent 1) and toluene/ethyl acetate/formic acid (50/40/10) (eluent 2).

Afterwards, the developed silica layers were stained with a 0.5 mM $DPPH^{\bullet}$ radical as described on Jaime et al [20] procedure.

2.7. Microbial strains

The PLE extracts were individually tested against a panel of microorganisms including *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 11775, *Candida albicans* ATCC 60193 and *Aspergillus niger* ATCC 16404.

Bacterial strains stock cultures were kept on nutrient agar at 4ºC. *Candida albicans* was kept on sabouraud dextrose agar at 4ºC. *Aspergillus niger* spores were obtained in vitro from monoconidial cultures after incubation (7 days, 24ºC) on potato dextrose agar, harvested in sterile distilled water containing 0.1% tween 80 and stored at 4ºC until used as inoculums.

2.8. Determination of minimum inhibitory concentration (MIC) and minimal bactericidal and fungicidal concentration (MBC).

A broth microdilution method was used, as recommended by NCCLS, for determination of the minimum inhibitory concentration [21]. All tests were performed in Mueller-Hinton broth supplemented with 0.5% tween 20, with the exception of yeasts and fungi (Sabouraud dextrose broth + 0.5% tween 20). The inocula of bacterial strains were prepared from overnight Mueller-Hinton broth cultures at 37ºC. Yeasts and fungi were cultured overnight at 25ºC in sabouraud dextrose broth. Test strains were suspended in Mueller-Hinton (bacteria) or sabouraud dextrose (yeast and fungi) broth to give a final density 10^7 cfu/ml. The *Spirulina platensis* extracts dilutions in DMSO were ranging from 250 mg/ml to 10 mg/ml.

The 96-microwell plates were prepared by dispensing into each well 165 µl of culture broth, 5 µl of the inoculums and 30 µl of the different extracts dilutions. The final volume of each well was 200 µl. Plates were incubated at 37ºC for 24 h for bacteria and

at 24ºC for 48 h for yeast and fungus. Negative controls were prepared using 30 µl of DMSO, the solvent used to dissolve the microalgae extracts. Chloranphenicol and amphotericin B (Sigma, Madrid) were used as positive reference standards to determine the sensitivity of the microbial species used. After incubation, the MIC of each extract was determined by visual inspection of the wells bottom, since bacterial growth was indicated by the presence of a white "pellet" on the well bottom. The lowest concentration of the extract that inhibited growth of the microorganism, as detected as lack of the white "pellet", was designated the minimum inhibitory concentration. The minimum bactericidal and fungicidal concentration was determined by making subcultures from the clear wells which did not show any growth. Each test was performed in triplicate and repeated twice.

3. Results and discussion

In order to obtain a large variety of extracts from the microalga *Spirulina platensis*, accelerated solvent extraction (ASE) and four different solvents (hexane, petroleum ether, ethanol and water) were used.

A screening of several PLE conditions was carried out in order to evaluate the influence of solvent polarity, temperature and time, in the extraction of functional compounds from *Spirulina platensis*. Four different extracting solvents (hexane, petroleum ether, ethanol and water) were used. The choice of the solvent is based on their different polarity with dielectric constants equal to 1.9 for hexane, 4.3 for petroleum ether, 24.3 for ethanol and 78.5 for water. Different extraction temperatures (60, 115 and 170ºC) were selected along with different extraction times (3, 9 and 15 minutes).

3.1. Antioxidant activity of the PLE extracts

Two different methods were selected to determine the antioxidant activity of the PLE extracts. β-carotene bleaching test was used as reference of lipid peroxidation of unsaturated fatty acids, a mechanism that is widely known to cause many pathological effects. The most important reaction in lipid peroxidation is the autooxidation of unsaturated fatty acids that is known to proceed by a radical chain reaction [22]. In this method, antioxidant compounds intercept the free radical chain of linoleic acid autooxidation that avoid the extent of *β*-carotene bleaching, which can be monitored spectrophotometrically [23]. A clear advantage of this method consisted of the formation of a liposome solution, that allows knowing the antioxidant capacity of the extracts further their polarity. On the other hand, $DPPH^{\bullet}$ is a purple stable radical that turned out yellowish when reacts with antioxidant analytes, and the degree of discoloration indicates the scavenging potentials of the antioxidant extract. The activity of the extracts is attributed to their hydrogen donating ability [19].

The antioxidant activities of the Spirulina extracts are shown in Tables 1 and 2 for *β*-carotene bleaching and DPPH tests, respectively. Results indicated the presence of compounds with antioxidant capacity against $DPPH^{\bullet}$ radical and radical's chain reaction of linoleic peroxidation. Generally, similar behavior was obtained with both, β-carotene bleaching method and $DPPH^{\bullet}$ radical scavenging capacity. However, more important differences between extracts were observed with the β-carotene bleaching method.

When organic solvents were used, antioxidant activity increased as extraction temperature enhanced up to 115ºC. Moreover, the longer the extraction times, the higher the antioxidant activity as it is shown in the results obtained at 115ºC considering 3, 9 and 15 min. Higher extraction temperatures (170ºC) caused a decrease of antioxidant activity in these extracts. On the other hand, water extract at 170ºC showed the highest antioxidant capacity. This behavior could be due to the decrease of water

dielectric constant at subcritical conditions (high pressure and temperature). In these conditions, water was able to extract less polar compounds, explaining the increasing of antioxidant activity as temperature enhances in water extracts [24].

The extracts obtained with petroleum ether, hexane and ethanol showed similar antioxidant activity using the β-carotene bleaching method, whereas ethanol extracts scavenged slightly less $DPPH^{\bullet}$ radicals than petroleum ether or hexane extracts at the same extraction conditions. Furthermore, Herrero et al. (2004) [17] reported that extraction yield increased as extraction temperature and organic solvent polarity rises. Thus, ethanol turned out to be the most suitable solvent in extracting antioxidant components from *Spirulina platensis* since ethanol extracts showed a high antioxidant activity together with a high extraction yield. Besides, ethanol is considered as safe (GRAS solvent).

An analytical-TLC of PLE microalgae extracts was carried out to detect differences among *Spirulina* antioxidant compounds extracted considering the different solvents tested. Thus, TLCs of microalgae extracts with the four solvents at 115ºC and 15 min were compared (Figure 1). Two different mobile phases were used to achieve a good separation of polar (Figure 2) and less polar compounds (Figure 1).

Green, grey-greenish, orange-yellowish and orange bands were found in the TLC plates eluted with both eluent 1 and 2, in agreement with the colors of the main pigments described in *Spirulina platensis* [25,26]: chlorophyll a, α,β-carotene, zeaxanthin, β-cryptoxanthin and other minor carotenoids. Moreover, blue and violet bands were detected in ethanol extracts when eluent 2 was used as mobile phase. Higher amount of chlorophyll a together with a lower content of carotenoids was present in ethanol extract opposite to petroleum ether and hexane extracts. Moreover, pheophytin-like compounds

were found (R_f = 0.54, 0.43) mainly in petroleum ether and hexane extracts (Figure 1) as partial degradation of chlorophyll a [27].

Developed TLC plate was stained with 0.5 M $DPPH^{\bullet}$ solution to determine the compounds responsible of the antioxidant activity of the different extracts. Using this procedure, the compounds that showed antioxidant activity turned out brilliant yellow in the purple background. The intensity of the yellow color depends on the amount and nature of radical scavengers present in the extract [28]. Orange, orange-yellowish, blue and violet bands of Spirulina platensis extracts showed a high antioxidant capacity (Figure 1b and 2b). Moreover, pheophytin-like compounds also had a slight antioxidant activity.

As reported in a previous paper [20], bands at Rf = 0.94, 0.91, 0.84 and 0.72 correspond to β-carotene and β-carotene-like compounds, whereas band at Rf = 0.24 and 0.15 were identified as zeaxanthin and myxoxanthophyll-like compound, respectively. Besides, blue and violet bands found in TLC eluent 2, at Rf = 0.15 and 0.12, corresponded to very polar phenolic compounds. Thus, it can be concluded that carotenoids were the main antioxidant components of petroleum ether and hexane extracts, whereas phenolic compounds contributed in a higher extent to the antioxidant activity of ethanol extracts.

3.2. Antimicrobial activity of the PLE extracts

Four different microbial species, including a gram negative bacteria (*Escherichia coli*), a gram positive bacteria (*Staphylococcus aureus),* a yeast (*Candida albicans*) and a fungus (*Aspergillus niger*), were used to screen the potential antimicrobial activity of PLE *Spirulina platensis* extracts. The antimicrobial activity was quantitatively assessed by the determination of the minimum inhibitory concentration (MIC) and minimal bactericidal and fungicidal concentration (MBC).

Results obtained in all the extraction conditions tested in this study are shown in Table 3. As can be seen, extracts obtained using hexane, petroleum ether and ethanol showed antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*, meanwhile none of these extracts were active against the fungus *Aspergillus niger*. In general, the extracts obtained using hexane and petroleum ether were slightly more active than those obtained with ethanol. As for water extracts, none of them was active against the microorganisms selected. These data seemed to indicate that the extraction of the compounds responsible for the antimicrobial activity was related with the polarity of the solvent employed. In fact, the organic solvents with a low polarity were the most effective in extracting the antimicrobial compounds from the microalga, although ethanol, with a medium-high polarity was also able to extract antimicrobial compounds. Several authors [29, 30, 9] have attributed the cyanobacteria antimicrobial activity to different compounds; specifically the antimicrobial activity of a methanolic extract of *Spirulina platensis* was explained by the presence of γ-linolenic acid [31], an antibiotically-active fatty acid present in a high concentration in this alga [32]. The organic solvents used in this work, in particular hexane and petroleum ether, are able to extract the γ-linolenic acid, thus explaining the higher antimicrobial activity found in those extracts in comparison to ethanol extracts. When ethanol was used as extracting agent, some γ-linolenic acid could be extracted but the antimicrobial activity of such extracts should be attributed to more polar compounds.

The effect of the extraction temperature and time in the antimicrobial activity of the different extracts was also studied. Thus, for the same extraction time (9 min), an increase in the extraction temperature from 60ºC to 115ºC produced extracts with a higher antimicrobial activity. Meanwhile, when the temperature rose from 115ºC to 170ºC, the antimicrobial activity only increased in some cases. These data indicated that

115ºC and 170ºC were the best extraction temperatures in order to optimize the extraction of antimicrobial compounds, whereas 60ºC was too low for the total extraction of these compounds. In order to study the influence of the extraction time, the temperature was fixed at 115ºC and extractions were carried out at 3, 9 and 15 min. Data obtained showed that the antimicrobial activity of the extracts increased, only in some cases, when extraction time increased from 3 min to 9 min. In that way, the results seemed to indicate that 9 min was the optimal extraction time.

Results also showed that *Candida albicans* was the most sensitive microorganism to all *Spirulina* PLE extracts, with the lowest MBC values (15-10 mg/ml). The sensibility of the gram positive (*Staphylococcus aureus)* and gram negative (*Escherichia coli*) bacteria to microalga PLE extracts was quite similar, with the exception of petroleum ether extracts that showed a higher antimicrobial activity against *Staphylococcus aureus* than against *Escherichia coli*. The least susceptible was the fungus *Aspergillus niger*. In fact, none of the extracts tested were active against this fungus. These data were in agreement with those reported by Ozemir et al. (2004) [9], who indicated that a methanolic extract of *Spirulina platensis* was more active against *Candida albicans* than against *E. coli* and *S. aureus*. Besides, a previous work done in our laboratory with supercritical fluid extracts from this microalga, also indicated that *Spirulina* extracts showed a higher antimicrobial activity against *C. albicans* than against other microorganisms [33].

Conclusions

In this work we have demonstrated the ability of pressurized liquid extraction to produce extracts with antioxidant and/or antimicrobial activities from a natural source such as *Spirulina platensis* microalgae. Results show that the optimal extraction

conditions in terms of antioxidant activity were those using ethanol as extracting agent and considering an extraction temperature equal to 115ºC and 15 minutes of extraction time. Moreover, zeaxanthin, myxoxanthophyll-like compound and very polar phenolic compounds seemed to be the main responsible of the antioxidant activity in ethanol extracts of *Spirulina platensis*. Thus, since ethanol is found to be the best extracting solvent for antioxidants of *Spirulina platensis*, PLE can be used as an environmentally-friendly and fast process for obtaining antioxidant extracts to be used as ingredients in the food industry. As for the antimicrobial activity, the extracts obtained using hexane and petroleum ether were slightly more active than those obtained with ethanol, being 115ºC and 170ºC and 9 min the best extraction temperatures and time in order to optimize the extraction of antimicrobial compounds from *Spirulina platensis*.

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Table 1. Antioxidant activity (%) of *Spirulina platensis* PLE extracts at different conditions using the β-carotene bleaching method. Different concentrations of extracts are considered; a concentration of BHT equal to 1 μg/ml in the reaction mixture is also included.

Sample			25 μg/ml	50 μg/ml	75 μg/ml
Solvent	T (ºC)	Time (min)			
Hexane	60	9	17	59	74
	115	3	62	75	83
	115	9	53	75	83
	115	15	74	82	86
	170	9	2	22	43
Petroleum ether	60	9	17	49	71
	115	3	33	63	76
	115	9	27	65	75
	115	15	44	77	85
	170	9	2	24	51
Ethanol	60	9	41	60	79
	115	3	8	42	69
	115	9	64	83	92
	115	15	72	83	90
	170	9	8	45	74
Water	60	9	4	20	24
	115	3	6	24	29
	115	9	7	17	35
	115	15	9	21	42
	170	9	15	36	48
BHT			81		

Table 2. DPPH$^{\bullet}$ radical scavenging activity (%) of *Spirulina platensis* PLE extracts at different conditions. Different concentrations of extracts are considered; a concentration of ascorbic acid equal to2 μg/ml in the reaction mixture is also included.

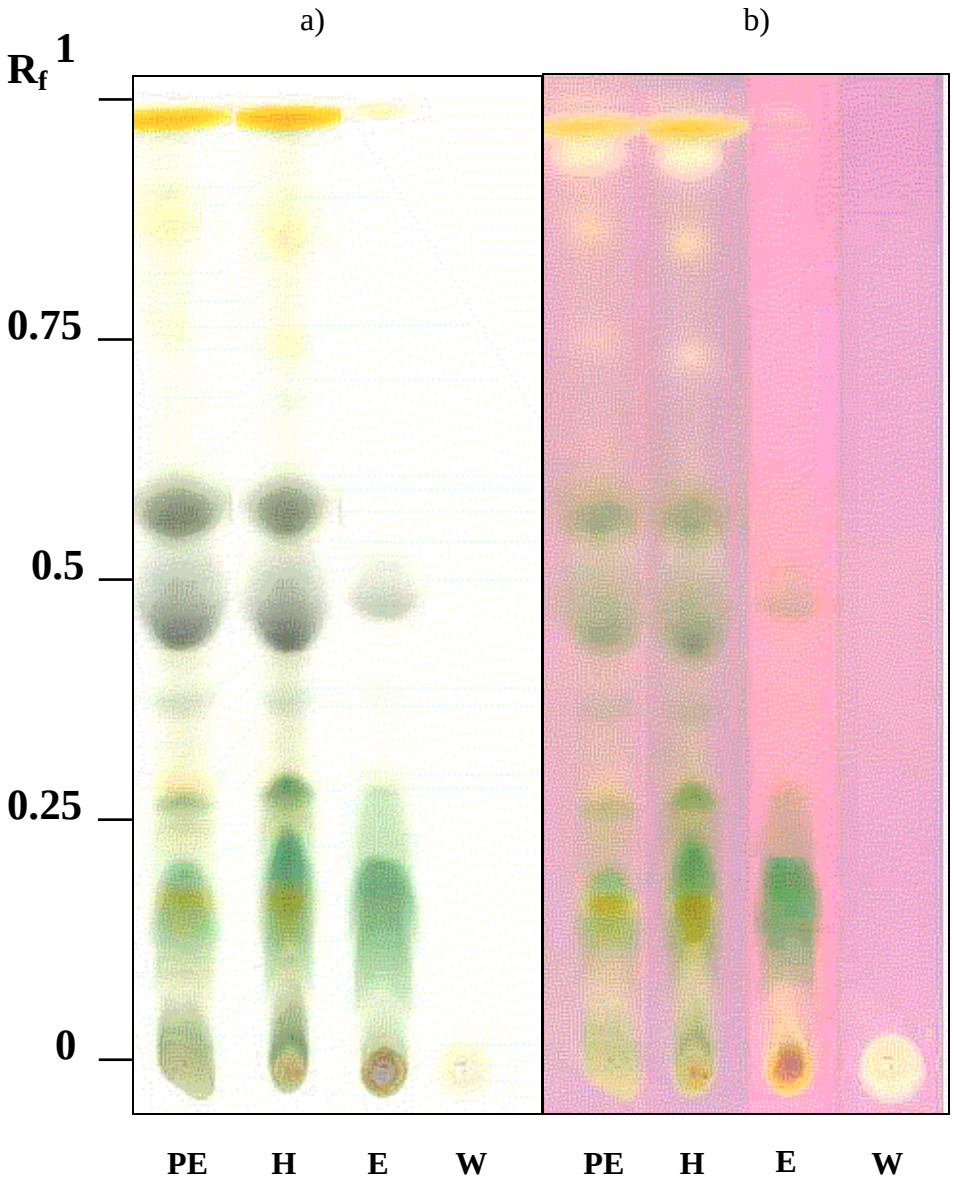
Sample			100 μg/ml	250 μg/ml
Solvent	T (ºC)	Time (min)		
Hexane	60	9	60	
	115	3	67	
	115	9	67	
	115	15	68	
	170	9	45	
Petroleum ether	60	9	60	
	115	3	65	
	115	9	67	
	115	15	66	
	170	9	46	
Ethanol	60	9	50	
	115	3	59	
	115	9	58	
	115	15	55	
	170	9	50	
Water	60	9		35
	115	3		35
	115	9		36
	115	15		39
	170	9		48
Ascorbic acid			44	

Table 3. Antimicrobial activities of different PLE extracts of *Spirulina Platensis*

Sample			*E. coli*	*St. aureus*	*C. albicans*	*A.niger*
Solvent	T (ºC)	Time (min)	MBC[1]	MBC	MBC	MBC
Hexane	60	9	30	30	15	>35
	115	3	25	30	15	>35
	115	9	25	30	15	>35
	115	15	25	30	15	>35
	170	9	25	15	10	>35
Petroleum ether	60	9	30	30	20	>35
	115	3	25	25	20	>35
	115	9	25	15	15	>35
	115	15	25	15	15	>35
	170	9	25	15	15	>35
Ethanol	60	9	30	30	20	>35
	115	3	30	30	20	>35
	115	9	25	25	20	>35
	115	15	25	25	20	>35
	170	9	25	25	15	>35
Water	60	9	>35	>35	>35	>35
	115	3	>35	>35	>35	>35
	115	9	>35	>35	>35	>35
	115	15	>35	>35	>35	>35
	170	9	>35	>35	>35	>35
Reference Antibiotic			10	10	100	150

(1) MBC, minimum bactericidal concentration. MBC values given as mg/ml for samples and μg/ml for antibiotic

Figure 1. Analytical-TLC of *Spirulina platensis* PLE extracts at 115ºC and 9 minutes; eluent 1 a) without $DPPH^{\bullet}$; b) stained with 0.5 mM $DPPH^{\bullet}$ solution.



PE: petroleum ether; H: hexane; E: ethanol; W: water

Figure 2. Analytical-TLC of *Spirulina platensis* PLE extracts at 115ºC and 9 minutes; eluent 2 a) without DPPH$^{\bullet}$; b) stained with 0.5 mM DPPH$^{\bullet}$ solution.