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Control of *Microcystis aeruginosa* toxic blooms by Moroccan medicinal plant-based algicides

Lamiaa Tebaa[a], Mountasser Douma[b], Zakaria Tazart[a], Khadija Mouhri[a], Mohammed Loudiki[a,*]

[a]*Water, Biodiversity and Climate Change Laboratory, Phycology, Biotechnology and Environmental Toxicology Research Unit, Faculty of Sciences Semlalia, University Cadi Ayyad, Av. Prince My Abdellah P.O. Box: 2390, Marrakech 40000, Morocco, emails: loudiki@uca.ac.ma (M. Loudiki), lamiaatebaa@gmail.com (L. Tebaa), zakaria.tazart@gmail.com (Z. Tazart), mouhri@uca.ac.ma (K. Mouhri)*

[b]*Environmental Microbiology and Toxicology Research Unit, Polydisciplinary Faculty of Khouribga (FPK), Sultan Moulay Slimane University, Morocco, email: douma_mountasser@yahoo.fr*

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ABSTRACT

Algaecide substances extracted from terrestrial plants are considered an ecofriendly and promising tool in the biocontrol of harmful cyanobacteria blooms (HCBs). We assessed the inhibitory effects of ethyl acetate (EA) extracts taken from the fresh leaves of three Moroccan medicinal plants (*Thymus maroccanus*, *Origanum compactum* and *Artemisia herba-alba*) on the toxic cyanobacteria *Microcystis aeruginosa*. The algicidal mechanism of the plant extracts was examined through physiological parameters (lipid peroxidation, protein content and antioxidant enzymes activity). High-performance liquid chromatography (HPLC) was used to characterize the chemical composition of each plant extracts and their specific algicidal compounds. Results revealed that *M. aeruginosa* growth was significantly inhibited by the three EA extracts in a concentration-dependent manner. After 8 d of application, the greatest inhibition rate reached 97.41%, 97.16% and 93.19% at 100 mg/L of *T. maroccanus*, *O. compactum* and *A. herba-alba* extracts, respectively. The chlorophyll-a and carotenoid concentration significantly decreased after 8 d of treatment. The malondialdehyde concentration, superoxide dismutase and catalase activities in *M. aeruginosa* cells increased significantly with increased extract concentration and exposure time as a response to oxidative stress induced by plant extracts. HPLC analyses of the three plant EA extracts indicated the presence of a total of 17 phenolic compounds. Rosmarinic acid, tannic acid and tyrosol were the most predominant compounds in all extracts, respectively. These potential main algicidal compounds could be resulting in the inhibition of *M. aeruginosa* growth and oxidative stress. Our findings highlight the potential of medicinal plant extracts as a new source of natural algaecides against HCBs.

Keywords: Anti-cyanobacterial activity; Ethyl-acetate plant extracts; Algicidal compounds; Antioxidant enzyme; Oxidative stress; *Microcystis* bloom control

1. Introduction

Harmful cyanobacterial blooms (HCBs) are a serious environmental concern in aquatic ecosystems worldwide [1,2]. *Microcystis* genera are the most common bloom-forming cyanobacteria [3–5]. HCBs cause severe water quality deterioration due to scum formation, toxin production, hypoxia, and production of taste and odors [6–8]. These bloom characteristics can lead to serious ecological, human and animal health impacts [9,10].

* Corresponding author.

To mitigate the potential harmful impact of cyanobacterial blooms, a number of chemical substances and physical treatments methods have been applied [11–14]. However, these methods are usually very expensive or potentially harmful to the environment. In recent years, biological methods using algicidal bacteria [15] and biopond-wetlands or top-down control methods using fish or large zooplankton have received more attention as ecofriendly alternatives [16–18]. Unfortunately, neither of these measures can adequately combat harmful algal blooms without disturbing other organisms in the trophic chain [9]. Other less polluting, more green and safe methods are still required to inhibit cyanobacterial growth.

Recently, seaweeds, macrophytes and terrestrial plants have acquired a special attention as a new source of bioactive compounds and an efficient alternative to the conventional techniques for cyanobacteria growth control [19–24]. Bioactive compounds isolated from plants are the potential agents of HCBs bio-control [25]. Extracts from parts of common terrestrial plants such as rice straw [26], rice hulls [27], oak bark [28], *Moringa oleifera* seeds [29] and barley straw [30] were also found to inhibit the growth of harmful algal bloom species, including cyanobacteria. Medicinal herbs as a group of terrestrial plants are known to be a potential reservoir of many bioactive substances [31]. Various papers have highlighted their powerful antimicrobial, insecticidal, antifungal, antiviral and antioxidant qualities owing to their rich content of phenolic compounds [32–34]. Apiaceae, Asteraceae, Ephedraceae, Papaveraceae, and Rutaceae are the most common families studied for their chemical potential [35–39]. The use of these plants in controlling *Microcystis* growth has been demonstrated as promising alternatives to synthetic chemical treatments. In recent years, several medicinal plants have shown algicidal potential for the control of harmful cyanobacteria such as *Conyza canadensis* and *Erigeron annuus* [38], *Radix Astragali* [39], Chinese gold thread (*Coptis chinensis*) [40], the root of rhizome of *Acorus tatarinowii*, cortex of *Phellodendron amurense* and fruits of *Crataegus pinnatifida* [41], *Artemisia annua* [42,43], *Salvia miltiorrhiza* [31], *Phellodendri chinensis* cortex, *Scutellaria baicalensis* and *Citrus reticulate* peel [44] as well as several traditional Chinese medicine herbs: *Ailanthus altissima* [45], *Galla chinensis* (nutgall) [46], shaddock and pomegranate peel, pomegranate seed pomegranate [47], peel tannin [48] and *Portulaca oleracea* [49].

The anti-cyanobacterial activity of terrestrial plants is still less exploited than that of aquatic macrophytes. Among terrestrial plants, Chinese medicinal plants are the most studied. However, as far as we know, few studies have been conducted to determine effects of Moroccan herb extracts on freshwater toxic microalgae [22,23]. Previous studies have investigated only the aqueous plant extracts, however using other types of solvent in extracts for cyanobacteria control may also be effective. Previous studies testing organic solvents such as methanol, ethanol, ethyl acetate, petroleum ether, and acetone have shown that ethyl acetate extracts present the highest inhibitory effect [38,41]. The Lamiaceae family includes several aromatic and medicinal plants including *Thymus*, *Origanum* and *Artemisia* species, which are often endemic and widely used in Morocco and the Mediterranean basin [50,51]. Several researchers have found that these medicinal plants are rich in polyphenols, flavonoids and tannins and constitute a good source of variety of bioactive compounds [52,53]. We aimed to assess the anti-cyanobacterial activity of ethyl acetate (EA) extracts from three Moroccan medicinal plants *T. maroccanus*, *O. compactum* and *A. herba-alba* on the growth of the toxic cyanobacterium *M. aeruginosa*, with a particular focus on characterizing the bioactive algicidal compounds within each extracts and understanding the mechanism of inhibition.

2. Materials and methods

2.1. Preparation of plant extracts

Thymus maroccanus and *Artemisia herba-alba* were collected from two localities, Ourika (31°17′45″ N; 7°42′36″ W) and Tahanaout (31°21′05″ N; 7°57′03″ W) in Marrakech area (Morocco). *Origanum compactum* was collected from Beni-Mellal area (32°20′22″ N; 6°21′39″ W). All the plants were collected between March and April 2019. The plants were thoroughly washed with distilled water to remove all debris, impurities and surface-deposited materials. The leaves were dried away from sunlight at ambient temperature (20°C–25°C) then crashed into powder prior to extraction.

A 20 g sample of the leaf powder was extracted with 300 mL of distilled water at room temperature for 48 h. After filtration, the resulting liquid was collected for further fractionation according to Wang et al. [54] (Fig. 1). The final fraction of ethyl acetate (EA) was dried by rotary evaporation at 39°C, weighed and then stored at 4°C prior to the preparation of bioassay concentrations. Two milligrams of dried extract was resuspended in 2 mL of ethyl acetate for analysis by high-performance liquid chromatography (HPLC).

2.2. Cyanobacteria strain

The cyanobacteria strain *Microcystis aeruginosa* was sampled during the bloom period (October 2015) from the eutrophic reservoir Lalla Takerkoust (31°21′36″ N; 8°7′48″ W), Marrakech area. Then the strain was isolated and maintained in culture in Z8 medium under controlled conditions (temperature of 26°C ± 2°C; light intensity of 63 μmol m^{-2} s^{-1} and a light/dark cycle of 15/9 h [21,23].

2.3. Algicidal activity assays

The algicidal activity of plant EA extracts was assessed in a 0.5 L Erlenmeyer flasks filled with a 300 mL of Z8 growth medium (pH 7.4). Each flask was inoculated with *M. aeruginosa* culture at exponential growth phase with initial cell density of 2 × 10^6 cells/mL. The dried EA extracts were dissolved in dimethyl sulfoxide (DMSO) 0.1% (v/v) and added to *M. aeruginosa* cultures to obtain final concentrations of 25, 50, 75 and 100 mg/L. An untreated equal volume of cyanobacterial culture with 0.1% of DMSO was used as negative control. All cultures were incubated for 8 d in a culture room under the same conditions used to maintain initial *M. aeruginosa* cultures. All treatments were carried out in triplicates.

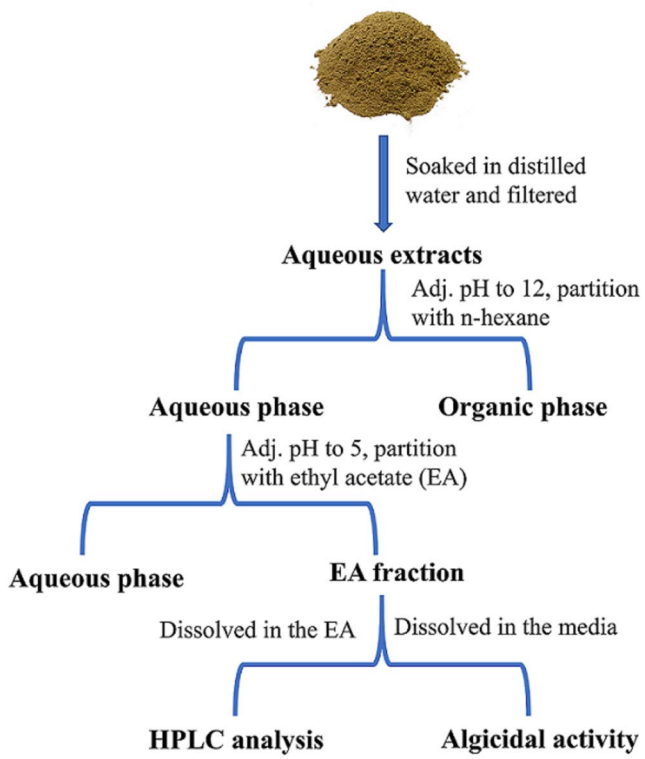

Fig. 1. Diagram for extraction of ethyl acetate extracts from *Thymus maroccanus*, *Artemisia herba-alba* and *Origanum compactum*.

2.4. Cyanobacterial growth and inhibition rates

Every day, a sample of 1 mL was collected from each culture, sonicated for 10 s at 50 kHz [55] and the *M. aeruginosa* cells were counted using a hemocytometer and a photonic microscope (Motic BA210, China). The inhibition rate (IR%) of *M. aeruginosa* growth was determined using the following equation:

$$\mathrm{IR}(\%) = \frac{(N_c - N_t)}{N_0} \times 100 \quad (1)$$

where N_c and N_t are the cell densities (cells/mL) in the control and treatment cultures, respectively.

2.5. Determination of chlorophyll-a and carotenoids

Chlorophyll-a and carotenoids concentrations were measured spectrophotometrically in triplicates and calculated following Lichtenthaler and Wellburn method [56]. Five milliliters of culture sample were centrifuged at 6,000xg for 15 min and the cyanobacterial cells were extracted in the dark with boiling ethanol (95%) at 4°C for 48 h. After extraction, the samples were centrifuged for 10 min at 5,000xg and the supernatant was collected for optical density (OD) measurements at the wavelengths of 665, 649 and 470 nm. Ethanol (95%) was used as a blank solution. Chlorophyll-a and carotenoids concentrations (μg/mL) were calculated according to Eqs. (2) and (3), respectively:

$$\left[\text{Chlorophyll-a}\right] = \left(13.95 \times \mathrm{OD}_{665}\right) - \left(6.88 \times \mathrm{OD}_{649}\right) \quad (2)$$

$$\left[\text{Carotenoids}\right] = \frac{\left[\left(1{,}000 \times \mathrm{OD}_{470}\right) - \left(2.05 \times \text{Chl-a}\right)\right]}{2} \quad (3)$$

2.6. Determination of malondialdehyde level

Lipid peroxidation was determined by measuring the malondialdehyde (MDA) level according to Li et al. [57]. Every day, a sample of 1 mL was collected from each culture and centrifuged at 4,000xg for 20 min. The cell pellets were then mixed with 2 mL of 10% (w/v) trichloroacetic acid (TCA). After centrifugation at 12,000xg for 15 min at 4°C, 2 mL of the supernatant was diluted with 2 mL of 0.6% thiobarbituric acid (in 10% TCA), then boiled for 15 min. The reaction terminated by putting the reaction tubes into an ice bath. After cooling, the samples were then centrifuged at 12,000xg for 10 min. The absorbance (OD) of the supernatant was measured at 532, 600 and 450 nm, using a reference mixture of 2 mL ultrapure water and 2 mL 0.6% TBA. The MDA concentration (μmol/L) was determined with the following equation:

$$\mathrm{MDA} = 6.45 \times \mathrm{OD}_{532} - \mathrm{OD}_{600} - 0.56 \times \mathrm{OD}_{450} \quad (4)$$

2.7. Determination of total protein content

The total protein content was determined according to the Bradford [58] method using the principle of protein-dye binding. In an ice water bath, 1 mL of *M. aeruginosa* culture sample was sonicated for 13 min, and then centrifuged at 4,000xg for 25 min. After sonication, 30 μL of the disrupted cell suspension was transferred to a test tube and incubated in 1.5 mL of Bradford reagent at room temperature in obscurity for 5 min. Absorbance of each sample was read at 595 nm.

2.8. Antioxidant enzymes activities measurements

2.8.1. Preparation of enzyme extract

Enzyme extracts were treated following the protocol of Li et al. [59] with minor modifications. Every day, *M. aeruginosa* cells were harvested by centrifugation of 5 mL of each culture at 4,000xg for 25 min at 4°C. The pellet was re-suspended in 0.1 M phosphate-buffer (pH 6.5) containing 1% (w/v) polyvinylpyrrolidone (PVP). Then the cells were degraded and homogenized by an ultrasonic cell pulverizer for 5 min in an ice bath. The homogenate was then centrifuged at 10,000xg for 10 min at 4°C. The supernatant was preserved as a cell-free enzyme extract and used for the following assays.

2.8.2. Superoxide dismutase activity

The superoxide dismutase (SOD) activity was assayed in triplicate according to Beauchamp and Fridovich [60] method. The reaction mixture containing 1 mL of the crude enzyme extract mixed with the SOD assay solution (0.80 mL potassium phosphate buffer (50 mM, pH 7.8), 0.3 mL nitro blue tetrazolium (NBT) (750 μM), 0.3 mL methionine solution (130 mM), 0.3 mL Na_2EDTA

solution (100 μM), 0.3 mL riboflavin solution (20 μM)). As SOD has the ability to inhibit the photochemical reduction of NBT, this assay utilized negative controls (silver paper wrapped around the test tube to mimic fully dark condition without any photochemical reduction of NBT), positive controls (deficiency of SOD activity in light with full photochemical reduction of NBT), and treatment groups (in light with SOD inhibition on photochemical reduction of NBT). One unit of enzyme activity was determined as the amount of enzyme needed for inhibition of 50% NBT reduction by monitoring absorbance at 560 nm. Absorbance was read using a Varian Cary® 50 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). SOD activity was expressed as U/mg protein.

2.8.3. Catalase activity

Catalase (CAT) activity was determined in triplicate by absorbance decrease being proportional to the consumption of H_2O_2 at 240 nm for 5 min according to the method of Rao et al. [61]. The reaction mixture contained 1 mL H_2O_2, 1.8 mL potassium phosphate buffer (100 mM, pH 7.0) and 0.2 mL enzyme extract for a total volume of 3 mL. Samples were incubated for 2 min at 37°C and the absorbance of the sample was monitored for 5 min at 240 nm using a Varian Cary® 50 UV-Vis Spectrophotometer.

2.9. HPLC to identify and quantify phenolic substances

Samples were first filtered through Whatman filter paper then through Millipore membrane filters (grade 42 and 0.22 mm, respectively). Two columns: reversed-phase (RP-18) column 250 mm × 4.6 mm, 5.0 μm and a 10 mm × 4.6 mm RP-18 pre-column (Agilent Technologies) were placed into a column furnace at 25°C. The HPLC system consisted of an automated injector (Shimadzu Corp., Kyoto, Japan SCL-10A series pumping system, SIL-10AD) coupled to an SPD 10A UV-visible detector (200–700 nm). Analysis and data collection were done using the Shimadzu LC Solution chromatography data station software. Two solvents (A and B explained below) were applied at 0.1 in a stable rate, with an injection volume of 1 mL/min 0 μL. All solvents employed were HPLC-grade. Solvent A consisted of acetonitrile (5%) and water (95%), solvent B was a phosphate buffer solution in water (pH = 2.6). The following proportions of solvent B were used for the elution program: 5% B (initial time), 13%–25% B (35 min), 25%–85% B (6 min), 85%–95% B (12 min), 100% B isocratic (7 min) at a constant flow rate of 1 mL per min at 20°C. Total run time was 60 min. Different standards (gallic acid, hydroxytyrosol, salicylic acid, hydroxybenzoic acid, syringic acid, rutin, rosmarinic acid, vanillin, 2-hydroxycinnamic, sinapic acid, p-coumaric, hydroxytyrosol, (−)-epigallocatechin, tannic acid, vanillic acid, ferric acid, fumaric acid, tyrosol) were run on the machine first, followed by EA plants extracts. The phenolic compounds of ethyl acetate extract are characterized according to their UV–Vis diode array detector at 280 nm spectrum, and they were identified by comparing their retention times (RT) to those of the standards.

2.10. Statistical analysis

All experiments were conducted in triplicate and data were reported as means ± standard deviation (SD). Statistical analyses were carried out between the treatment and control groups via a one-way ANOVA analysis. Post hoc differences between group means were checked with the Tukey test. *p*-values lower than 0.05 were considered significant. Statistical analyses were performed using the computer software Sigma Plot 12.5 for Windows.

3. Results

3.1. Effects of plant EA extracts on M. aeruginosa growth and pigments

The algicidal effects of the three medicinal plant extracts (*T. maroccanus*, *O. compactum* and *A. herba-alba*) at 25, 50, 75 and 100 mg/L on *M. aeruginosa* are shown in Fig. 2 as the growth kinetics and inhibition rates (IR). In opposition to the control group, the cell density of *M. aeruginosa* at the four concentrations tested was significantly ($p < 0.05$) lower after the 6th day of exposure. The reduction was greater for the three highest plant extracts concentrations, especially during the two last days (Figs. 2a, c, e). At 50 mg/L of *T. maroccanus* extract, *M. aeruginosa* growth was significantly inhibited (IR = 61.5%) compared with *A. herba-alba* and *O. compactum* extracts (IR = 44.17% and 32.11%, respectively). On the last day of treatment, *T. maroccanus* and *O. compactum* extracts showed strong growth inhibition at 100 mg/L and the maximum IR was 97.41% and 97.16%, respectively (Figs. 2b, d, f).

Two photosynthetic pigments were measured (chlorophyll-a and carotenoids) that can be used as indicators for both growth and physiological state of *M. aeruginosa*. Chlorophyll-a and carotenoid concentrations in treatments were significantly decreased during the 8 d of the experiment compared with the control group. The content of these two pigments was strongly inhibited at 100 mg/L concentration of all plant EA extracts from the 5th day of exposure (Fig. 3).

3.2. Effects of plant EA extracts on antioxidant enzymes activities in M. aeruginosa cells

To investigate the effect of plant extracts on the cellular oxidative defense system of *M. aeruginosa* cells, the CAT and SOD activities were evaluated at 100 mg/L of EA extracts (Figs. 4a and b). For both enzyme activities, the results showed a highly significant increase from the 3rd day of treatment ($p < 0.001$) compared with the control. CAT and SOD showed a maximum activity on the 4th day for both *T. maroccanus* and *O. compactum* with 452.38 U/mg protein, 453.12 U/mg protein and 449.48 U/mg protein, 705.20 U/mg protein, respectively. Maximum SOD activities for *A. herba-alba* were 373.28 U/mg protein and 399.38 U/ mg protein (Figs. 4a and b).

3.3. Effects of plant EA extracts on total protein and MDA contents

The effects of plant EA extracts (100 mg/L) on total protein content in *M. aeruginosa* cells, during 8 d of exposure

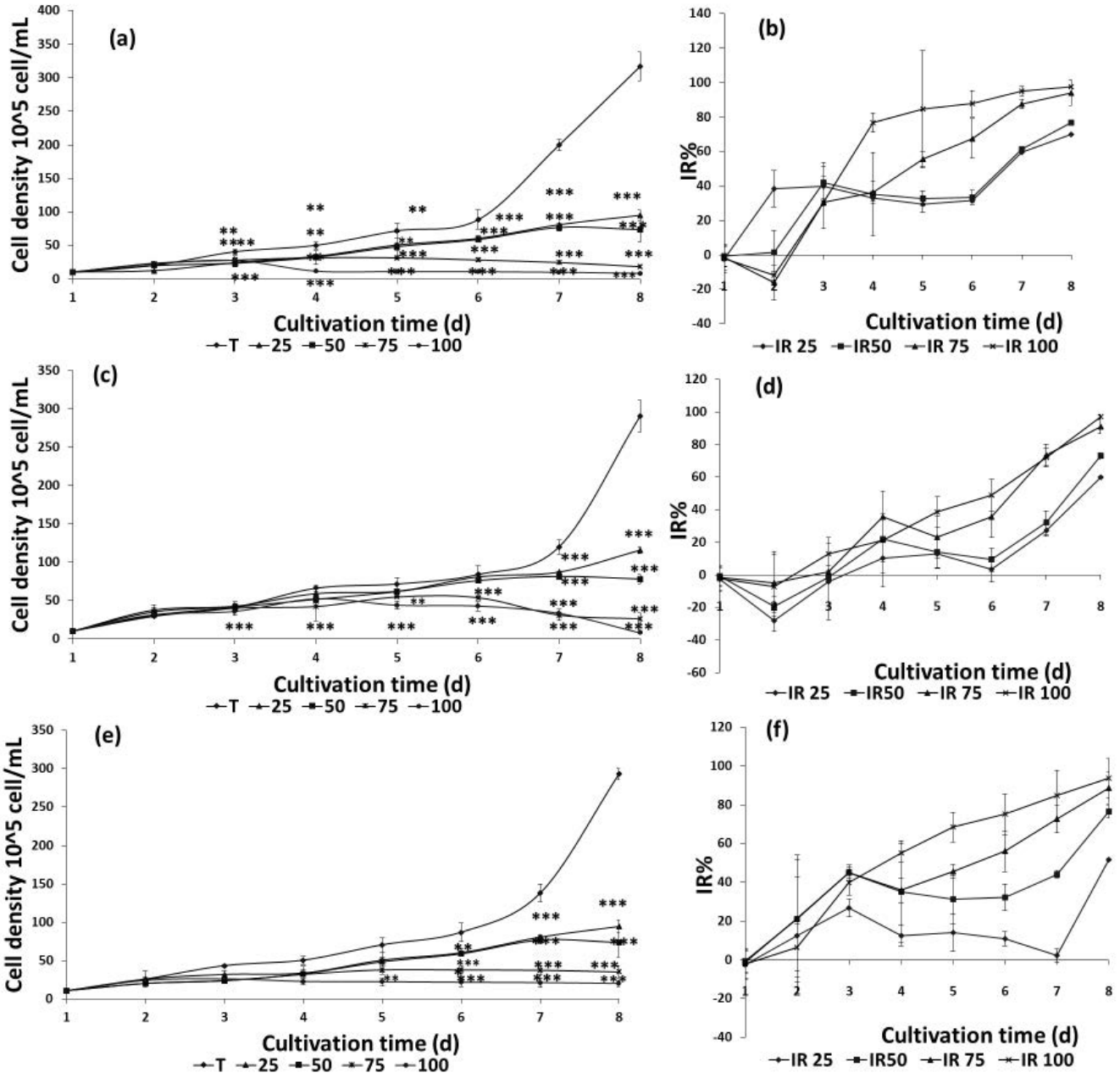

Fig. 2. Effects of plant ethyl acetate extracts on *Microcystis aeruginosa*. Growth and inhibition rate of *Thymus maroccanus* (a, b), *Origanum compactum* (c, d) and *Artemisia herba-alba* (e, f) ethyl acetate extracts. Data are means ± SD (n = 3). *p < 0.05 and ***p < 0.001 indicate significant differences compared with the controls.

are shown in Fig. 4c. From the 4th day of the experiment, the protein content of *M. aeruginosa* cells significantly decreased for all tested extracts compared with the negative control.

Malondialdehyde (MDA) is an indicator of lipid peroxidation as it is the final result of lipid peroxidation in cells [62]. As shown in Fig. 4d, from the 4th day of treatment MDA level in *M. aeruginosa* cells exhibited a significant increase in comparison with the control for the three plant EA extracts. The maximum MDA values were observed at day 8 for *T. maroccanus*, *O. compactum* and *A. herba-alba* with 0.60, 0.59 and 0.31 μmol /L, respectively.

3.4. Identification and quantification of phenolic compounds by HPLC

A HPLC technique was used to identify and quantify the major phenolic compounds in plant EA extracts. From the HPLC chromatogram (Fig. 5 and Table 1), 17 different chemicals were identified in the three organic extracts. Analyses of phenolic components from the EA extract of *T. maroccanus* revealed the dominance of rosmarinic acid (42.35% of the total identified compounds) followed by salicylic acid (21.98%), 2-hydroxycinnamic (8.09%), sinapic acid (7.37%), hydroxytyrosol (4.63%), syringic acid (3.22%),

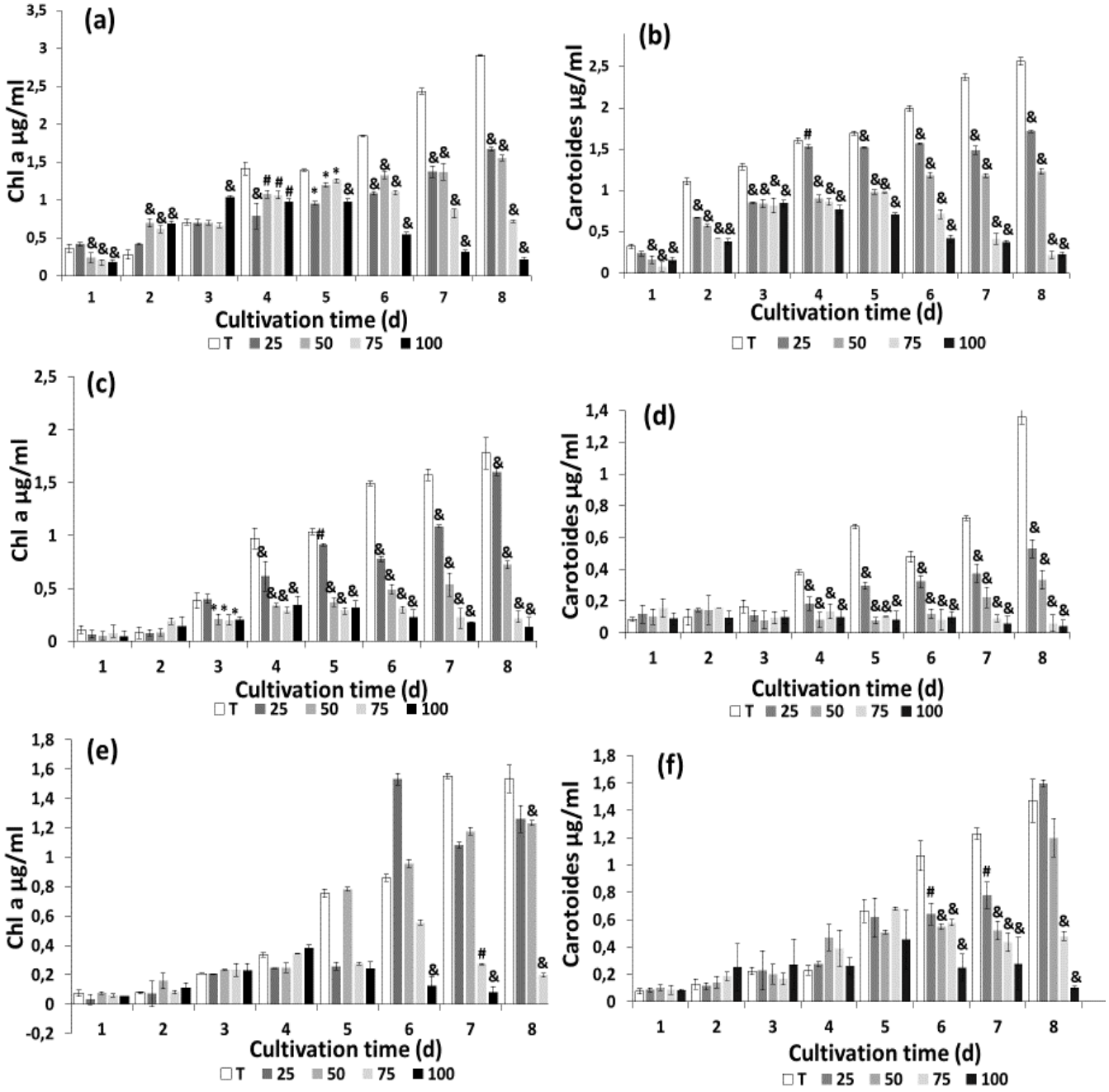

Fig. 3. Effects of *Thymus maroccanus*, *Origanum compactum* and *Artemisia herba-alba* ethyl acetate extracts on chlorophyll-a (a, c, e, respectively) and carotenoids (b, d, f, respectively) contents of *Microcystis aeruginosa*. Data are means ± SD (n = 3). *$p < 0.05$, #$p < 0.01$ and &$p < 0.001$ show significant differences compared with the controls.

vanillin (2.50%), rutin (2.23%), p-coumaric acid (1.84%), gallic acid (1.66%) and hydroxy benzoic acid (0.91%). For *O. compactum* extract, tannic acid (51.18%) is the major compound, followed by rutin (5.89%), vanillin (2.59%), 2-hydroxycinnamic (2.46%), ferric acid (2.07%), epigallocatechin (1.81%), hydroxytyrosol (1.70%) and vanillic acid (1.14%). The *A. herba-alba* extract was composed mostly of tyrosol (64.33%) followed by fumaric acid (9.78%), hydroxytyrosol (5.08%), p-coumaric acid (3.38%), rosmarinic acid (3.08%) and gallic acid (2.47%).

4. Discussion

Aquatic and terrestrial plants are a rich reservoir of substances that may inhibit or prevent the growth of microalgae including cyanobacteria and therefore may be

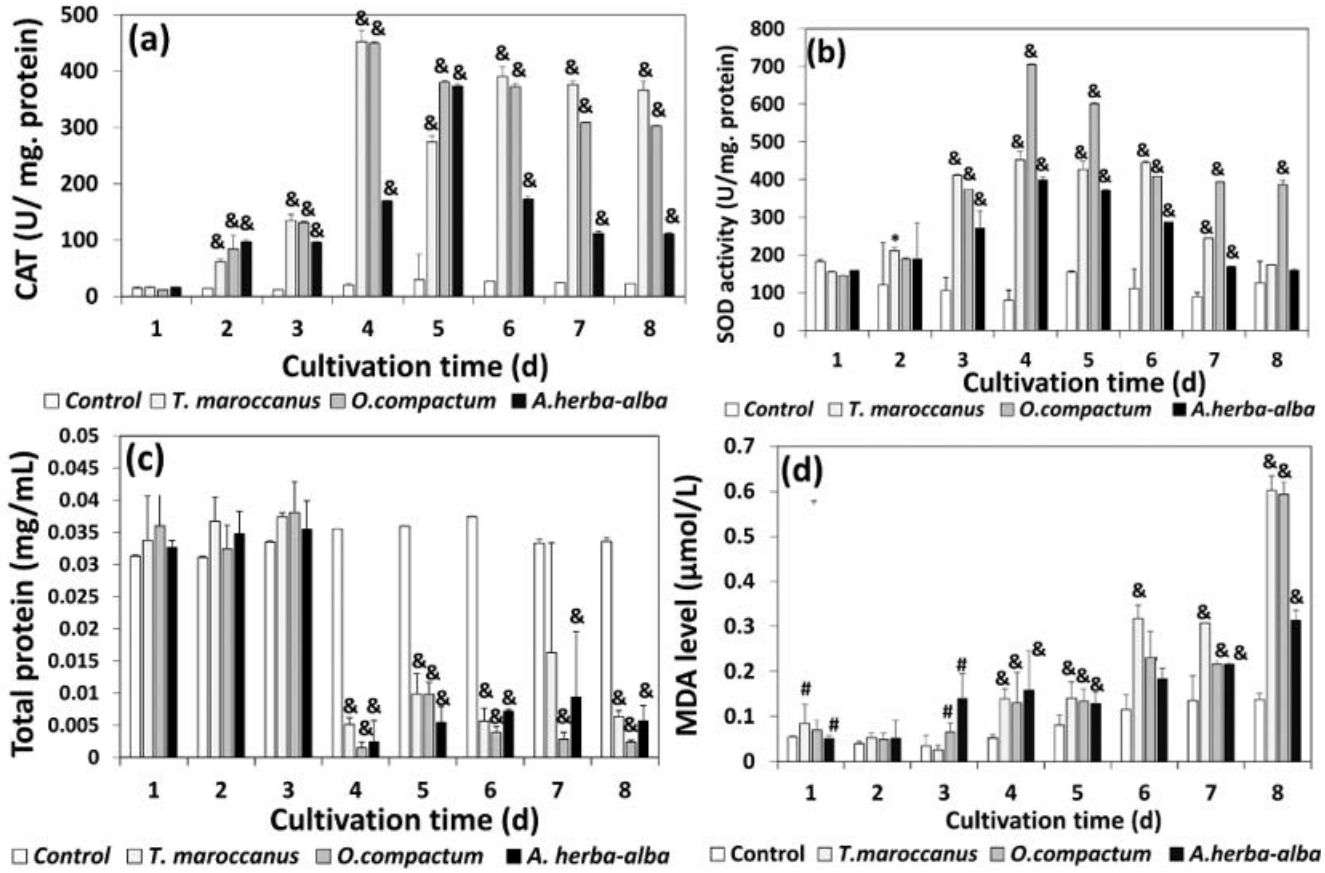

Fig. 4. Catalase (CAT) (a), superoxide dismutase (SOD) (b) activities, total protein (c) and malondialdehyde (MDA) concentration (d) in *Microcystis aeruginosa* cells after treatment with 100 mg/L plant ethyl acetate extracts. Data are means ± SD (n = 3). *p < 0.05, #p < 0.01 and &p < 0.001 show significant differences compared with the corresponding controls.

capable of controlling the proliferation of HCBs [24,63]. Although medicinal and aromatic plants are a reliable source of bioactive substances [32,62], few studies have reported their anti-cyanobacterial activity using plant aqueous extracts [22,23,45,46], organic extracts [38,41] or essential oils [64]. The present study investigates, for the first time, the anti-cyanobacterial activity of ethyl acetate extracts from three Moroccan medicinal plants (*T. maroccanus*, *O. compactum* and *A. herba-alba*) on the growth of the toxic cyanobacterium *M. aeruginosa*.

The results showed that EA extracts of the all three plants tested have a significant anti-cyanobacterial activity against the toxic cyanobacterium *M. aeruginosa* in a concentration-dependent way. With increased concentration and exposure time, the cell density and chlorophyll-a content of *M. aeruginosa* were significantly reduced. The EA extracts of *T. maroccanus* and *O. compactum* at 100 mg/L caused the strongest inhibitory effect on *M. aeruginosa* growth and the IR was greater than 97% in the last day of exposure. The *T. maroccanus* extract had the most effective algicidal activity even at the low concentration (25 mg/L) with an IR higher than 70% compared with *O. compactum* and *A. herba-alba* extracts. The latter are strongly effective only from concentrations higher over 50 mg/L. Our finding confirmed previous results showing that among the three herbs tested, aqueous extract of *Thymus* had a high algicidal effect (IR = 95%, 93%) on *M. aeruginosa* [22,23]. Furthermore, our experimental results are in agreement with previous studies showing that several medicinal plants extracts present a strong anti-cyanobacterial activity such as *Artemisia annua*, *Artemisia lavandulifolia*, *Conyza canadensis*, *Erigeron annuus*, *Coptis chinensis*, *Stephania tetrandra*, *Chelidonium majus* and *Phellodendri chinensis* [40,43,44,65].

Several recent studies have showed that aqueous and organic (e.g., methanolic, ethanolic, acetonic, ethyl acetate) extracts of medicinal plants had algicidal effects on *M. aeruginosa* [38,41,44,65]. However, the highest activity was frequently achieved with organic extracts. Indeed, numerous research demonstrates that petroleum ether (PE), ethyl acetate (EA) and ethanol extracts of three Asteraceae plants *Artemisia annua*, *Conyza canadensis* and *Erigeron annuus* could effectively inhibit the growth and photosynthetic activity of HABs [38]. Among these organic extracts, ethyl acetate solvent frequently presents the highest inhibitory effect [38,41,44,65]. Chen et al. [66] found that *Cinnamomum camphora* fresh leaves methanolic extract had a strong inhibitory effect against *M. aeruginosa*. Likewise Meng et al. [45] showed that *M. aeruginosa* growth was highly inhibited (IR = 90) by *Ailanthus altissima* ethanolic extract at 200 mg/L. These findings reveal the algicidal potential of the bioactive substances extracted from these medicinal plants.

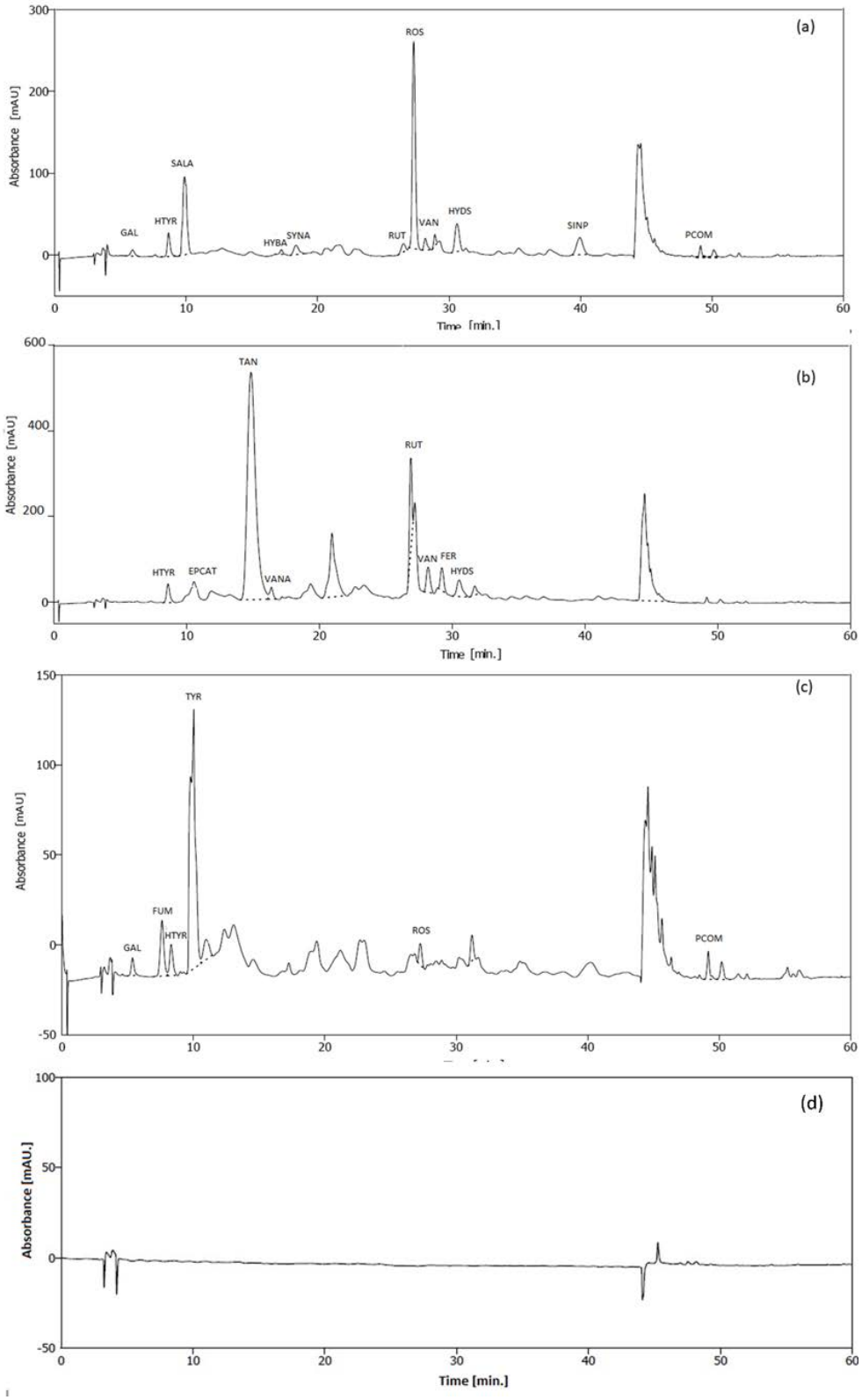

Fig. 5. HPLC chromatogram recorded at 280 nm for the main phenolic compounds identified in the plant ethyl acetate extracts: (a) *Thymus maroccanus*, (b) *Origanum compactum*, (c) *Artemisia herba-alba* and (d) blank. EPCAT: (−)-epigallocatechin, HYDS: 2-hydroxycinnamic, FER: Ferric acid, FUM: Fumaric acid, GAL: Gallic acid, HYBA: Hydroxybenzoic acid, HTYR: Hydroxytyrosol, PCOM: p-coumaric, RUT: Rutin, SALA: Salicylic acid, SINP: Sinapic acid, SYNA: Syringic acid, ROS: Rosmarinic acid, TAN: Tannic acid, TYR: Tyrosol, VANA: Vanillic acid, VAN: Vanillin.

Table 1
Concentrations (mg EGA/g DM) of the major phenolic substances detected in the plant ethyl acetate extracts

Plant materials	Reten. time (min)	Area (mAU s)	Compounds	Concentrations (mg EGA/g DM)	% of compounds
	5.4	158,413	Gallic acid	6.46	1.66
	8.7	446,596	Hydroxytyrosol	17.98	4.63
	9.9	2,131,926	Salicylic acid	85.40	21.98
	17.2	84,914	Hydroxybenzoic acid	3.52	0.91
	18.4	310,043	Syringic acid	12.52	3.22
	26.7	213,690	Rutin	8.67	2.23
Thymus maroccanus	**27.3**	**4,110,231**	**Rosmarinic acid**	**164.53**	**42.35**
	28.2	239,826	Vanillin	9.71	2.50
	28.9	152,191	Unknown	6.21	1.60
	30.6	783,097	2-Hydroxycinnamic	31.44	8.09
	40	712,721	Sinapic acid	28.63	7.37
	49.1	175,291	p-Coumaric	7.13	1.84
	50.2	155,579	Unknown	6.34	1.63
	8.7	770,810	Hydroxytyrosol	6.79	1.70
	10.5	824,183	(−)-Epigallocatechin	7.25	1.81
	14.8	**23,337,872**	**Tannic acid**	**204.70**	**51.18**
	16.4	517,417	Vanillic acid	4.56	1.14
	20.9	4,931,839	Unknown	43.28	10.82
Origanum compactum	26.7	2,682,705	Rutin	23.55	5.89
	28.2	1,176,554	Vanillin	10.34	2.59
	29.2	942,461	Ferric acid	8.29	2.07
	30.6	1,119,369	2-Hydroxycinnamic	9.84	2.46
	31.7	397,255	Unknown	3.51	0.88
	44.5	8,876,158	Unknown	77.87	19.47
	5.4	149,620	Gallic acid	6.11	2.47
	7.6	600,117	Fumaric acid	24.12	9.78
	8.7	310,531	Hydroxytyrosol	12.54	5.08
	10	**3,964,999**	**Tyrosol**	**158.72**	**64.33**
Artemisia herba-alba	11	339,743	Unknown	13.71	5.56
	27.3	186,695	Rosmarinic acid	7.59	3.08
	31.2	210,598	Unknown	8.54	3.46
	49.1	205,164	p-Coumaric	8.33	3.38
	50.2	173,379	Unknown	7.06	2.86

HPLC analysis of the three investigated plant extracts showed a rich variety of the chemical constituents. The chromatographic profile of EA extract from *T. maroccanus* showed a high content of rosmarinic acid (RA) (42.35%), followed by salicylic acid (21.98%), as a major phenolic component. This result is in agreement with other studies confirming that among the identified phenolic compounds, RA an ester of caffeic acid was found to be the most important [67]. Furthermore, studies have shown that RA has antibacterial properties [68,69]. For the *O. compactum* EA extract, a great variability of phenolic compounds was observed with a dominance of tannic acid (51.18%). El Babili et al. [70] have reported a similar chemical composition of the aerial part of *O. compactum*. The *A. herba-alba* extract was dominated mostly by tyrosol (64.33%). In contrast, among phenolic acids commonly occurring in several *Artemisia* species, caffeic and vanillic acids were commonly dominant [71].

Natural compounds from plants used to control harmful algae are more environmental friendly when compared with conventional synthetic organic algaecides [72]. Plant extract components showing inhibitory activity against cyanobacterial growth include phenolic compounds from *Acacia decurrens* [73], a naphthoquinone isolated from *Juglans nigra* [74], tannins of barley straw [75,76] and quinone composite from *Salvia miltiorrhiza* [31]. An inhibitory effect has also been demonstrated in certain terpenoid constituents liberated from waterweed [77,78], artemisinin isolated from *Artemisia annua* [41,43], Juglone from Juglans [79] and Berberine from golden thread [41]. These plant compounds may be relatively effective agents for the inhibition

of cyanobacterial growth. The three major components (rosmarinic acid, tannic acid, tyrosol) from *T. maroccanus*, *O. compactum* and *A. herba-alba*, respectively, could have high potential as algicidal compounds against *M. aeruginosa* growth. The additive and synergistic effect of all the potential algaecides could be even greater [79].

The increase of antioxidant enzyme concentrations under an excess of reactive oxygen species (ROS) caused by plant extracts stress suggested that the free radicals content (O_2^-, H_2O_2, OH^-) increases under environmental stress [80,81], destroying the physicochemical properties of cell membranes [82] and disrupting the physiological activity of *M. aeruginosa*. Zhao et al. [83] used the eucalyptus leaves as an inhibitor to control the growth of *M. aeruginosa*. This inhibitory effect led to the reduction in photosynthesis, disruption of the cell membrane integrity and inhibition of esterase activities of the cyanobacterial cells. The present work supports that plant-based natural algaecides have multisite action, including impairment of cyanobacterial photosynthesis and antioxidant enzyme systems [84–86]. This mode of action is another strong advantage, which differs from synthetic and conventional algaecides [87]. The allelochemical substance artemisinin, isolated from *Artemisia annua* by Ni et al. [43,88,89], has previously been shown to have significant inhibitory effects on *M. aeruginosa*. Decreased photosynthesis in overexposure to elevated artemisinin may be the result of blocking the acceptor side of electron transport and deactivation of PSII reaction centers [42,88]. Allelochemicals in an aqueous *Spartina alterniflora* extract, including cyclohexane, heptane, 2-cyclohexen-1-one, hexadecenoic acid, 2,4-di-tert-butylphenol and hydrocinnamic acid, may play an important role on inhibiting *M. aeruginosa* by decreasing chlorophyll-a and weakening photosynthesis [90].

The activity of different antioxidant enzymes exhibited specific patterns in different stress conditions. MDA is the final product of lipid peroxidation, which reflects the degree of damage of *M. aeruginosa* cells [91]. Our result demonstrated that there was an increase in MDA level by the three tested plant extracts with highest values for treatments by *T. maroccanus* and *O. compactum*, which may indicate that the polyunsaturated acids in cell membranes may be attacked by oxygen radicals, and that the cyanobacterial cells were subjected to serious oxidative stress. The cell membranes of cyanobacteria are made of unsaturated phospholipids, which are vulnerable to ROS. MDA, as the breakdown material of this degradation process, has been commonly referred to as a biomarker for vital sign of cell membrane oxidative damage [86]. Significant differences ($p < 0.001$) between control and treatment groups were especially apparent from the third to the seventh day of the experiment. Similar results were reported by Meng et al. [45]. and Tazart et al. [20] who found that MDA content increased significantly with exposure concentration and exposure time extends. Many studies confirmed that most antioxidant enzyme activities are enhanced under stress conditions, such as SOD and CAT activities [92–95]. SOD acts as catalysts in the reaction of O_2^- (a precursor of ROS) converting it into the relatively low cytotoxic H_2O_2 [93,96], while CAT can convert H_2O_2 into H_2O and O_2, protecting the cell against the oxidative damage caused by H_2O_2 [95]. When *M. aeruginosa* cells were treated with plant extracts, SOD and CAT activities increased significantly. To mitigate oxidative damage, the antioxidant defense mechanism acts to remove excess ROS. Elevated SOD and CAT activities indicate a stronger capacity for elimination of excess ROS; SOD is generally considered the first line of the defense system against the potential toxicity of ROS. Excess ROS can lead to severe cell injury, and even death. [97]. Our results are consistent with other studies reporting the increase in the earlier stage of SOD activity in response to oxidative lesions of *M. aeruginosa* induced by the crude extract of tubers of *Sagittaria trifolia*. However when exposure time reached 48 h, SOD activity decreased notably, which may reflect the degradation of the detoxification process in cyanobacterial cells [59].

Changes in protein content here were similar to other studies on the growth of *M. aeruginosa* under different concentrations of several plant extracts. The significant decline in soluble protein concentrations in our research suggests that plant extracts block the protein biosynthesis ability of cyanobacteria. The decrease in total protein level can be associated with decreasing rates of cell photosynthesis, which restricts the quantity of sugars/monosaccharides and various other organic substances that provide carbon supply for protein biosynthesis [98,99]. Protein insufficiency could disturb or alter the normal workings of the physiological metabolism, even causing the death of the cells, which may be one of the inhibition mechanisms. Wang and Xu [100] also proved that the photosynthetic center of photosystem II is affected by artemether and dihydroartemisinin, which reduce the extracellular alkaline phosphatase activity with a greater artemether inhibition capacity compared with dihydroartemisinin and artemisinin at the same dose [100]. MDA level and the antioxidant defense system demonstrated in terms of physiology that *M. aeruginosa* cells were subjected to oxidative damage, which was probably caused by the bioactive compounds from the Moroccan herbs EA extracts.

To date, the methodological approaches used for the control of cyanobacterial blooms by terrestrial plants have been mostly laboratory-based bioassay using plant extracts, purified compounds or essential oils. However, the feasibility of large-scale application of these alternative approaches is still dependent on the optimal dose to be applied (based on the volume of water and cell density) and therefore the amount of extract and plant material required. Therefore, there is still much research to be done in order to address these issues before these treatments can be used in the field. Extraction of allelochemicals from terrestrial plants that produce them in large quantities and in a profitable way, testing their synergistic potential interactions in macrocosms and environmental conditions prior to their application can be an effective alternative to the techniques currently in use.

5. Conclusion

Our results highlighted that ethyl acetate extracts of three Moroccan herbs *T. maroccanus*, *O. compactum* and *A. herba-alba* has an inhibitory effect on *M. aeruginosa* growth, with strong algicidal activity. Characterization of these organic plant extracts revealed some major compounds that were presumed responsible for *M. aeruginosa* growth inhibition and cell oxidative damage.

Consequently, these plants could be considered as potential producers of anti-cyanobacteria compounds and might be as new sources of natural algaecides against HCBs. Moreover, further research using other plants is needed with a focus on the search for their major active compounds. Thus, more detailed potential mechanisms of the inhibitory effect, such as the effects on the process of photosynthesis and respiration of *M. aeruginosa* should be further studied.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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