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Allelopathic activity and chemical analysis of crude extracts from the macrophyte *Egeria densa* on selected phytoplankton species

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ABSTRACT

Egeria densa is an invasive macrophyte in Mexico and its allelopathic effects on phytoplankton species are poorly documented. Hence, we evaluated the inhibitory effects of different concentrations of crude extracts (hexane, ethyl acetate and methanol) from *E. densa* on *Scenedesmus acutus*, *Chlorella vulgaris, Monoraphidium* sp. and *Nitzschia palea*. The hexane extracts yielded less quantity of crude extract, while methanol resulted in higher amount. We detected 9-compounds, of which saponins, tannins, cardiac glycosides and phenols were most abundant. Agar diffusion in disc bioassays showed inhibition halos of different sizes depending on the extract, concentration, solvent type used and the tested phytoplankton species. In *C. vulgaris* and *S. acutus*, the inhibition halos of >20 mm diameter were observed in treatments containing ethyl acetate and methanol extracts. The results suggested that *E. densa* contained substances that could change the composition of phytoplankton communities through their inhibitory and/or allelopathic effects.

Keywords: Allelopathy, Chlorophytes, Diatoms, *Egeria densa*, Ethyl acetate, Hexane, Invasive species, Methanol, *Monoraphidium*, *Nitzschia palea*, *Scenedesmus acutus f. alternans*.

INTRODUCTION

Submerged macrophytes play a major role in structure and function of aquatic ecosystems (41). They (i). stabilize the clear water phase in mesotrophic and eutrophic water bodies in temperate regions (29,34), (ii). affect the food web in different ways as they are part of trophic chain serving as food resources for some macroinvertebrates and fish (7), (iii). provide zooplankton spatial refuge from predation and offer many surfaces available for plankton colonization (6), (iv). provide shelter to fish (23), (v). compete for nutrients and light with phytoplankton and periphyton (17) and (vi). influence other primary producers through the release of allelopathic substances (10,14,25). The competitive relationship between the primary producers (macrophytes vs phytoplankton) determines which stable state would persist in the medium, one without the macrophytes and high turbidity and other with macrophytes and clear water (33,35).

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Allelopathic compounds determines the dynamics of nutrients, water chemistry, microbial ecology, dominance, invasion and succession (5,19). The production of allelopathic substances by the macrophytes is an effective strategy against other photosynthetic organisms that compete for light and nutrients (13,42). The macrophytes inhibits the phytoplankton growth through the release of secondary metabolites (12,30). However, these chemical substances differentially affect phytoplankton species (26,43). *Egeria densa* is a native perennial submerged macrophyte in South America, which has been introduced in several water bodies around the world (32). This species has become a nuisance in temperate, subtropical and tropical systems due to its rapid growth (8). It interferes with various water uses [swimming, fishing, shipping and tourism (32,39)]. Despite its wide distribution and high abundance in many shallow lakes, there are few studies regarding the effects of *E. densa* to phytoplankton (27,28). Most allelopathic studies involving macrophytes do not characterize the chemical properties of the exudates. However, there is some evidence that heat-stable and moderately lipophilic compounds, phenolic acids, tellimagrandin II, fatty acids and sulphur compounds are present in the allelochemicals produced by macrophytes such as *Myriophyllum* spp., *Potamogeton* spp., *Chara* spp. and *Stratiotes aloides* (14). These substances are responsible for algicidal effects (3,15,42). However, further research is necessary to relate the chemical compounds present in the secondary metabolites with those extracted from the whole plant (40).

This study aimed to provide a basic characterization of crude extracts from *E. densa* and to evaluate their effects on the selected algal species, besides testing the allelopathic effects on two phytoplankton species (*Monoraphidium* sp. and *Nitzschia palea*) that coexisted with *Egeria densa* in the field and two others maintained in the laboratory (*Chlorella vulgaris* and *Scenedesmus acutus*). We hypothesized that the phytoplankton species coexisted with *E. densa* in nature would be less affected due to the allelochemicals from the macrophyte than those not from the same waterbody.

MATERIALS AND METHODS

I. Macrophyte

Egeria densa was collected from Benito Juárez reservoir (State of Mexico, Mexico) (99º 25' 38” N and 19º 41' 22” W) located at an altitude of 2540 m above sea level. Fresh plants were rinsed by aged-tap water, later with distilled water and then dried for 48 h at 38 °C.

II. Crude extraction

Dry material (30 g) was soaked twice in 2.5 L flasks separately, each for 7 days, in hexane, ethyl acetate and methanol. Since the dried material was not powdered before it was soaked, this represented leachate; however, for the purpose of description, we treated this here as crude extract. The crude extracts were concentrated using rotary evaporator. The yield as percentage for each extract was calculated by dividing the dry extract obtained (g) with the initial plant dry weight and then multiplied by 100. The extracts were stored at 4 °C until for further analyses and bioassays. The extract weighing 400 mg was dissolved in one ml of one the employed solvents (see below). Negative controls using only solvents but without plant extract were prepared in the same way, while for positive controls we used juglone (5-hydroxi-1,4-naphthalenedione) at 50 µg DW per SensiDisk. The GC-MS analysis for methanol extract was conducted, however no peak was shown due to possible interference from polar substances, based on the type of analytical equipment used. Hence these data were omitted.

III. Thin layer chromatography

The hexanic crude extract was dissolved in hexane-dichloromethane (1:1) and elution conditions of dichloromethane-methanol (9.5:0.5). The ethyl acetate crude extract was dissolved in dichloromethane-methanol (1:1) and their elution conditions were dichloromethane-methanol (4:6). The methanolic crude extract was dissolved in ethyl acetate-ethanol-water (0.5:1:1) and their elution conditions were dichloromethane-methanol (3:7). The characteristics of the thin layer were in chromatoplates of slice gel 60 F_{254} (Merck).

IV. GC-MS

GC-MS observations were made using an Agilent technologies chromatographer model 6850 and MS model 5975C with a column RESTEK CORP RTX-50 with nominal length of 30 m, nominal diameter of 320 µm, nominal film thickness of 0.5 µm and initial flux of 0.9 ml $min.^{-1}$. Oven was set at the initial temperature of 70°C, initial time of 2 min. with a rate of 15°C $min.^{-1}$, final temperature of 290°C and run time of 16.67 min. Inlet split mode was set at an initial temperature of 250°C with helium as the type of gas used. The GC injector had an acquisition mode by scan with solvent delay of 2 min. and low mass of 35 and high mass of 600, with MS source of 230°C with a maximum of 250°C and MS quad of 150°C with a maximum of 200°C. The ionization mode was by electron impact with 70eV of ionization energy. The crude extracts were analyzed through phytochemical methods; their analysis was based on mass spectral fragmentation analysis compared with those reported in the library of the National Institute of Standards and Technology (16,36,38).

V. Phytochemical analyses

The phytochemical analysis of all extracts were done by standard methods (14).

Phytochemical screening: Chemical tests were carried out using extracts to identify various constitutes using standard methods as described by Sofowara (36), Trease and Evans (38) and Harborne (16).

Alkaloids was performed by Dragendorff's and Shonnenschein's tests, flavonoids with NaOH 10% and Shinoda's test, saponin by Foam, Liberman and Roshentaler's tests, tannins by FeCl3, Gelatin and $K_3(Fe(CN)_6)$, Coumarins volatiles by Erlich and KOH test, Cardiac glycosides by Legal´s and Baljet's tests, Cyanogenic glucosides by Grignard test, Free anthracene by Börntraguer test, Aldehydes and ketones by 2,4-dinitrophenol test and Phenols by Folin-Ciocalteu test.

(i). **Flavonoids Test:** (Shinoda Test): About 0.5 g extract was dissolved in 1.5 mL of 50% methanol and warmed on steam bath. Metallic magnesium and 5 drops of concentrated hydrochloric acid were added. A red or orange colour indicates the presence of flavonoids aglycone.

(ii). **Alkaloids Test**: About 0.5 g extract was diluted to 10 ml with 1% aqueous hydrochloric, boiled and filtered. To 5 ml of the filtrate was added 2 ml of dilute ammonia. Chloroform 5 ml was added and shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 10 ml of acetic acid. This was divided into two portions. Shonnenschein's reagent was added to one portion and Draggendorff's reagent to the other. The formation of orange (with Shonnenschein's reagent) or reddish brown precipitate (with Draggendorff's reagent) was observed indicating the presence of alkaloids.

(iii). **Saponins Test**: To 0.5 g extract, 5 ml distilled water was added in a test tube. The solution was shaken vigorously and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously after which it was observed for the formation of an emulsion.

(iv). **Tannins Test**: About 0.5 g extract was boiled in 10 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride were added and observed for brownish-green or a blue-black coloration.

(v). **Steroids Test** [15]: One ml extract was dissolved in 10 ml of chloroform and equal volume of concentrated sulphuric acid was added by sides of the test tube. The upper layer turns red and sulphuric acid layer showed yellow with green fluorescence. This indicated the presence of steroids. Depending on the reagents used the inference varies. For example, if Shinoda reaction is positive then it indicated the aurones or chalcones and with 10% of NaOH, the presence of xanthones, flavones, anthocyanin and betacyanin was indicated.

V. Algal bioassays

The bioassays were done using the green algae *Chlorella vulgaris* (CL V-3 registered in the CICESE, Ensenada, Mexico), *Scenedesmus acutus* f. *alternans* (strain Hortobagy no. 72 University of Texas, USA). *Monoraphidium* sp. and the diatom *Nitzschia palea* isolated from Benito Juárez reservoir which coexisted with *E. densa*. Green algal species were separately cultured in Bold´s basal medium (4) in 2 L transparent bottles and under continuous illumination and aeration during 7 days. For the diatom, we added silica and H_2SO_4 0.1 N to the Bold´s basal medium to obtain pH 7. Log phase algae were separately harvested, centrifuged at 3000 rpm for 5 min., rinsed and re-suspended in distilled water for later use in bioassays.

Algal growth inhibition tests were performed using the single disk-diffusion method (2). We tested the effect of the ethyl acetate and methanol extracts on the phytoplankton growth using inoculation densities of 0.2 OD_{530nm} (optical density of culture at 530 nm) in the algal medium containing 1% bacteriological agar. Two concentrations of crude extracts impregnated in disk were placed onto agar plates (4 and 8 mg DW for disk) and dried using sterile air stream. After gelling the over layer, it was incubated for 9 days at 23 ± 2°C and constant illumination at 4300 lux with the lightmeter model 840020 Sper scientific.

VI. Statistical analyses

All data were analyzed using parametric tests (ANOVA) when data variances were homogenously (F_{max} test, $\alpha = 0.05$) distributed. Tukey post-hoc tests were applied when ANOVA tests revealed significant effects.

RESULTS AND DISCUSSION

With the evaporated extracts, the phytochemical profile was the first analysis, afterwards, the biological tests were done using two algal species (*Chlorella vulgaris* and *Scenedesmus acutus* f. *alternans*) maintained in laboratory and with two other phytoplankton species that coexisted with *Egeria densa* in the field (*Monoraphidium* sp. and *Nitzschia palea*). In hexanic crude extract, flavonoids were found in lowest intensity, saponins, volatile coumarins, cardiac glycosides, aldehydes and ketones were in moderate intensity. In the ethyl acetate crude extract, the chemical groups present were: cardiac glycosides, aldehydes and ketones, while saponins were in higher intensity. In methanol crude extract, aldehydes and ketones were found in less intensity, while cardiac glycosides and phenols were present in high intensity. However the alkaloids, tannins, cyanogenic glycosides and free anthracenes were not found in any crude extract (Table 1).

Table 1. Phytochemical analyses of different extracts under different type of tests. +, ++, +++ symbols indicate the intensity of the presence of the different substances

Secondary metabolites	Reagent	Hexane	Ethyl acetate	Methanol
Alkaloids	Dragendorff	-	-	-
	Shonnenschein	-	-	-
Flavonoids	Shinoda	-	-	-
	NaOH 10%	+	-	-
Saponins	Foam	-	-	-
	Lieberman B.	++	++	-
	Rosenthaler	-	+++	-
				-
Tannins	Gelatin	-	-	-
	Fe3Cl	-	-	-
	K3(Fe(CN)6)	-	-	-
Coumarins volatiles	Erlich	++	-	-
	KOH y EtOH	-	-	-
Cardiac glycosides	Legal	-	-	++
	Baljet	++	+	+++
Cyanogenic glucosides	Grignard	-	-	-
Free anthracene	Börntraguer	-	-	-
Aldehydes and ketones	2,4-dinitrophenol.	++	+	+
Phenols	Folin-Ciocalteu	-	-	+++

Symbols. (-): Negative test (absence of turbidity, flocculation and precipitation). (+): Weak positive test (if the reagent has a slight opacity). (+ +): Positive test (if the product reactive and not a turbidity flocculation). (+ + +): Test strongly positive (if the reagent produces a precipitate or heavy flocculation).

The chemical composition of hexane and ethyl acetate crude extracts using GC-MS were presented in Tables 2 and 3. We detected 23 compounds from these crude extracts. The hexanic crude extract revealed more compounds (17 compounds) in lower percentage than ethyl acetate crude extract (6 compounds). The components showing match exceeding 80% of quality were selected. In the hexanic crude extract the main compounds were 2-pentadecanone, 6,10,14-trimethyl (19%), n-hexadecanoic acid (15.28%), n-propyl hexadecanoate (11%), tricosane (~11%) and heneicosane (~9%), while in the ethyl acetate crude extract nanocosane (32%), tetratriacontane (12%) and eicosane (10%) constituted more than 50%. The phytochemical analysis of hexane extract also yielded nonpolar substances in the plant, not recorded in previous studies.

Table 2. Chemical composition of crude hexane extracts of *E. densa.*

Retention time	(%)	Compounds	Quality
10.253	1.79	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl	94
11.748	1.66	Tetradecanoic acid	98
12.319	19.02	2-Pentadecanone, 6,10,14-trimethyl	90
12.563	2.27	1,2-Benzenedicarboxylic acid, buty l 2-ethylhexyl ester	90
12.634	2.59	Nonadecane	98
12.839	2.49	Hexadecanoic acid, methyl ester	93
13.012	3.44	Oxirane, 2-decyl-3-(5-methylhexyl)-, cis-(.+/-)-	68
13.121	15.28	n-Hexadecanoic acid	97
13.769	1.8	n-Propyl 9-hexadecenoate	81
13.878	10.96	n-Propyl hexadecanoate	95
13.91	8.86	Heneicosane	99
15.071	10.86	Tricosane	95
15.501	2.87	4,8,12,16-Tetramethylheptadecan-4-olide	93
16.143	2.6	Octadecane, 2-methyl-	94
16.322	1.22	Docosanoic acid, methyl ester	97
16.399	1.33	Cis-Z-.alpha.-bisabolene epoxide	90
16.508	1.68	1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester	86

% : Ratio of total extract amount.

Table 3. Chemical composition of crude ethyl acetate extracts of *E. densa.*

Retention time	(%)	Compounds	Quality
13.16	12.11	Tetratriacontane	91
13.93	5.99	Tetracosanoic acid, methyl ester	97
14.956	2.10	Tritetracontane	81
15.873	3.33	Z-14-Nonacosane	98
16.085	31.51	Nanocosane	97
16.143	9.90	Eicosane	96

% : Ratio of total extract amount.

In thin layer chromatography shown are the data on the weight, yield and retention factor from the crude extracts (Table 4). In the hexanic crude extract, we got low yield (0.53%) with 5 spots; however this quantity of crude extract was not enough for

Table 4. Weight, performance and retention factor of the crude extracts of *E. densa* in Thin Layer Chromatography (TLC)

Extracts	Extract weight (mg)	Yield (%)	Retention factor
Hexane	162	0.53	0.33, 0.38, 0.44, 0.74, 0.85
Ethyl acetate	666	2.22	0.72, 0.89
Methanol	4385	11.19	0.73, 0.88

The yield (%) for each extract was calculated dividing the grams of dry extract obtained between grams of dry plant and its multiplication for 100.

biological analyses. Besides, it had low interaction with water due to its non-polar nature. From the ethyl acetate crude extract we obtained around 700 mg (2.22%) using 2 spots. The methanolic crude extract showed the highest quantity (4385 mg) which correspond to 11 % of the total dry biomass of *E. densa.* Their retention factors were 0.73 and 0.88.

For the bioassays we used juglone (5-hydroxi-1,4-naphthalenedione) as positive control, owing to its allelopathic properties to the tested phytoplankton species. Figure 1 illustrates the significantly different effects (Table 5, $F_{(3,52)}= 61.44$, p=0.001) to the tested phytoplankton species. The *post hoc* tests indicated four distinct groups. The growth of *Nitzschia palea* was most inhibited while *Scenedesmus acutus* was the least. With respect to the effects of *E. densa* effects, we observed that an increase in the extracts concentration increased the diameter of inhibition halo. The differences in the inhibition areas with extract types (ethyl acetate and methanolic) were significant ($F_{(3,43)} = 91.04$, $p < 0.001$). *Scenedesmus acutus* was most sensitive to ethyl acetate extract, while, *Nitzschia palea* was least sensitive to this (Figure 2).

Since the early 1980s, numerous studies have demonstrated the potential negative effects of macrophytes on different aquatic organisms including phytoplankton (10). Allelopathic effects among the different macrophyte species are long known. For example, *Hydrilla verticillata* severely reduced the biomass of other macrophytes (21). It is known that the exudates from macrophytes affect both algae and cyanobacteria. For example, using the crude extracts, Wang *et al.* (40) have reported that fatty acids, phenolic acids and hydroxy fatty acids of *Potamogeton* have allelopathic activity against *Microcystis aeruginosa*, they have also shown that the inhibitory effects were stronger with mixed chemical groups, than when compounds tested separately. On other hand, Mulderij *et al.* (26) found that moderately lipophilic extracts and exudates from *Stratiotes aloides* have caused differential effects to cyanobacteria ranging from stimulatory to inhibitory influence in their growth. In addition, it has been shown that methanolic extracts from *Elodea canadensis* and *E. nuttallii* containing hydrophilic and slightly lipophilic compounds inhibited the growth of phytoplankton and periphyton species (10). However, methanolic extracts from *E. densa* did not inhibit the *Selenastrum capricornutum* but was evident for *Microcystis aeruginosa* (27). This suggests that the allelochemicals in the methanolic extracts of *E. densa* have species-specific inhibitory effects. Further recently, it was found that the physical structure of the macrophyte had a role in the inhibitory effects of allelochemicals from *Egeria densa* to *Scenedesmus acutus* (11).

Table 5. Results of one-way analysis of variance conducted on the diameter of clearance zone of *S. acutus*, *C. vulgaris, Monoraphidium* sp. and *N. palea* exposure to 50 µg of juglone. DF = degrees of freedom, SS = sum of square, MS = mean square; F = F-ratio, *** = $p<0.001$.

Source of Variation	DF	SS	MS	F
Algal species	3	3636.57	1212.19	61.44***
Error	52	4603.32	19.73	

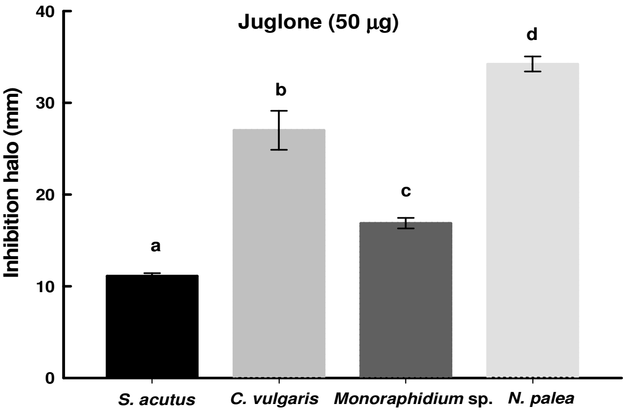

Figure 1. Inhibition halo produced by 50 µg of juglone (5-hydroxi-1,4-naphthalenedione) on the selected phytoplankton species. Lowercase letters indicates a statistically significant difference with one-way ANOVA and the post-hoc Tukey test ($p<0.05$). Data based on sixteen replicates.

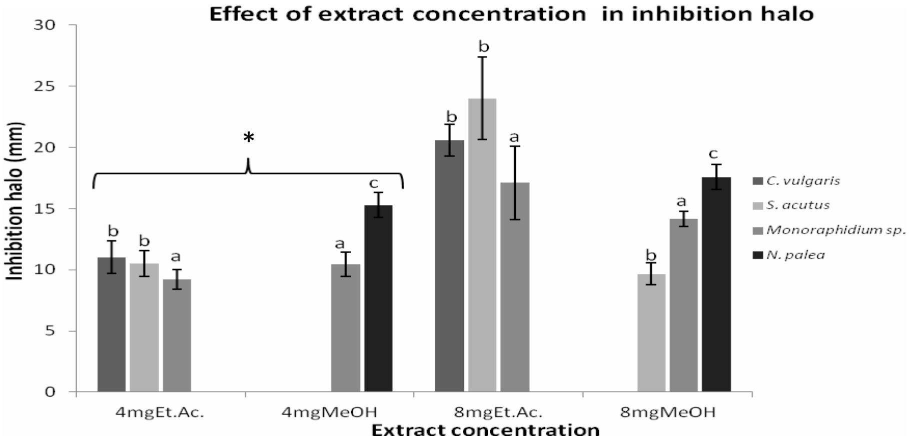

Figure 2. Inhibition halo produced by of 4 and 8 mg per sensidisk of ethyl acetate and methanolic crude extracts of *Egeria densa* on *S. acutus, C. vulgaris*, *Monoraphidium* sp. and *N. palea* (treatments without effect were discard). Lowercase letters indicates a statistically significant difference with two-way ANOVA and the post-hoc Tukey test ($p<0.05$). Data based on five replicates. The asterisk (*) indicates significant differences between the concentrations of crude extracts.

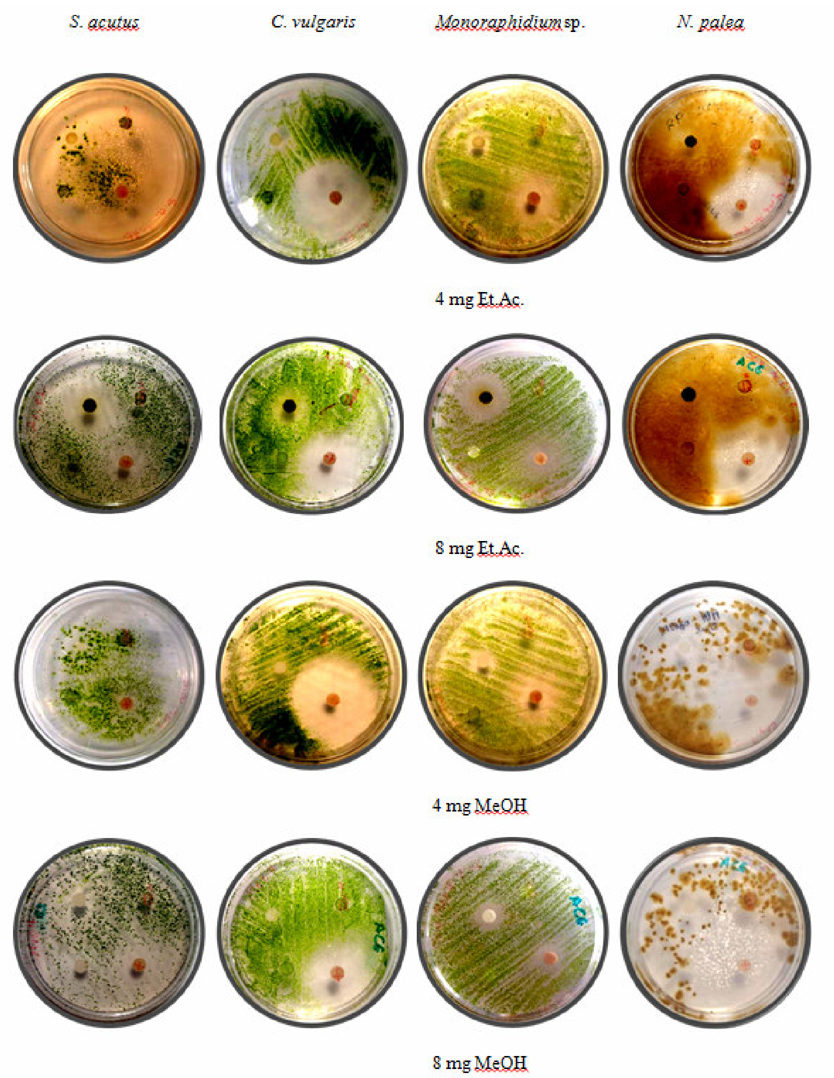

Figure 3. Images of inhibition of 4 and 8 mg ethyl acetate and methanolic crude extracts on *S. acutus, C. vulgaris*, *Monoraphidium* sp. and *N. palea.*

The allelopathic activity of macrophytes on different species of phytoplankton is generally determined using the species isolated from various waterbodies or laboratory-cultured taxa (20). However, only a few studies have used natural phytoplankton species (9,18,26) and much less information is available on the phytoplankton species that coexist with macrophytes in the field (15). In this work, we tested both these conditions: allelopathic effects were compared with phytoplankton species cultured in the laboratory and with those that co-existed with the macrophyte in nature. It has been hypothesized that allelopathic interactions among aquatic plants would be more intense if the species originate from different environments (1,9,14). For example, Erhard and Gross (10) tested allopatric and sympatric phytoplankton species and found that *Chlorella* cf. *vulgaris* (isolated from the waterbody containing *Elodea nuttallii*) was not inhibited by extracts from *E. canadensis* and *E. nuttallii*, while phytoplankton species from laboratory cultures were severely affected. However, our findings do not support this because algal species isolated from *Egeria densa* beds were more severely inhibited by the allelochemicals than those maintained in the laboratory cultures.

We observed that the sensitivity of tested phytoplankton species to increasing concentration of chemicals differed depending the crude extract type. It is generally known that an increase in the concentration of total phenolic compounds from the macrophytes lead to an increase in the inhibition area (3,42). However, substances released from the same macrophyte may affect the phytoplankton species differently. For example, chemicals released from *Chara* had affected differentially *Scenedesmus obliquus*, *Selenastrum capricornutum* and *Chlorella minutissima* (24).

Based on the results of the analysis realized here, we observed that extracts of ethyl acetate and methanol contained certain secondary metabolites in common such as cardiac glycosides, aldehydes and ketones; however in the extract of ethyl acetate, we found saponins which were not detected in the methanolic extract; in the methanolic extract phenols were found which were not present in the ethyl acetate extract. In addition, the ethyl acetate extract inhibited the growth of *C. vulgaris*, which was not found in the methanolic extract and so saponins may be involved in this inhibition (9,14). Furthermore, *N. palea* was inhibited by methanol extract but not the other. Accordingly, based on the phytochemical profile it appears that phenols of methanolic extract may be involved in inhibiting the growth of *N. palea* (27,28). It has been observed that the steroidal saponins or triterpenes are often responsible for the observed changes in the permeability of the plasma membrane and some promote or inhibit sprouting and rooting (14), whereas phenols (a group of secondary metabolites) have strong inhibitory effects on phytoplankton (3, 9).

It is necessary to characterize the allelopathic substances present in the crude extracts, as they contain unknown concentrations of chemicals and each chemical may have different effects on phytoplankton species. Juglone, used in this study, is known for its allelopathic effects to both terrestrial and aquatic organisms (37). Randall and Bragg (31) have reported differential inhibitory effects of juglone on phytoplankton species: *Anabaena flos-aquae* was drastically inhibited, *Nitzschia palea* was most inhibited, *Scenedesmus acuminatus* was less inhibited which supports with our data and the *Scenedesmus acutus* was less inhibited.

In a classic work, Nakai *et al.* (28) evaluated the effects of nine species of macrophytes including *E. densa* on three species of blue-green algae. They observed that only *Cabomba caroliniana* and *Myriophyllum spicatum* inhibited the growth of the tested blue-green algae, the effect being stronger from *M. spicatum*. Using a single initial addition and semi-continuous addition of *M. spicatum*-conditioned medium, they further noticed that single additions did not significantly inhibit the phytoplankton growth. On the other hand, in the treatments with the semi-continuous addition a strong inhibition was observed which increased with increasing macrophyte density suggesting that allelochemicals from the macrophyte are continuously secreted. This supports our observations for the treatments with ethyl acetate extracts on the growth of green algae. Nakai *et al.* (28) stated that the inhibitory effect is species-specific, which also corresponded with our findings.

Macrophyte invasion modifies the composition and community structure of local plankton. Our study showed that *Egeria densa*, native to Brazil-Uruguay-Argentina, had affected the phytoplankton growth. In addition, in its natural habitat too, *E. densa* had caused considerable changes in the phytoplankton composition through its allelochemicals (39). Thus, as demonstrated by Bais *et al.* (1) allelopathy is a strategy for invading new habitats through the release of chemical substances that inhibit the growth of competing species. However, the intensity of allelopathic effects of *E. densa* in Mexican waterbodies is not yet available. Such studies need to be undertaken in future works so as to understand the impact of invasive macrophytes in the natural waterbodies. The effect of allelochemicals from macrophytes on the composition and abundance of phytoplankton possibly depends on the origin of algal and macrophyte species. Thus if a given phytoplankton species co-exists in nature with the macrophyte, it is possible that such phytoplankton species may have developed strategies to minimize the allelopathic effects from the macrophytes. Our study however has shown that the growth of laboratory-cultured algae as well as the phytoplankton species that coexisted in nature with *E. densa* was inhibited by the presence of macrophyte-allelochemicals. This suggests that the phytoplankton adaptation in nature to counter the adverse effects of allelochemicals from macrophytes is possibly much less. This has other consequences in natural waterbodies, especially for controlling the toxic cyanobacteria through the allelochemicals from macrophytes as show in Nakai *et al.* (28).

CONCLUSIONS

These inhibition bioassays did not show different effects between the phytoplankton species that coexist with *Egeria densa* in the field and those maintained in laboratory conditions. We found that *E. densa* produces more than one allelopathic compound. The growth inhibitory properties varied depending on the extraction method, the phytoplankton species used and the secondary metabolites involved. In these interactions mainly phenols, cardiac glycosides, aldehydes and ketones were found in methanolic and saponins cardiac glycosides, aldehydes and ketones were in ethyl acetate extract

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