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Allelopathic Interaction between a Medicinal Plant; *Achillea santolina* L. and two Associated Soil Algae Species

Salama M. El-Darier[*] and Abdelfattah K. Metwally
Botany and Microbiology Department, Faculty of Science, Alexandria University, Alexandria, Egypt

ABSTRACT

Allelopathic effects of the aqueous extract of the medicinal plant; *Achillea santolina* on growth and distribution of two associated soil algae; *Chlorella vulgaris* (Chlorophyta) and *Lyngbya contorta* (Cyanophyta) were investigated under controlled laboratory conditions. The phytochemical analysis of *A. santolina* showed that it contains essential oils, flavonoids, glycosides, phenolic compounds, sterols, triterpenes and tannins. The algal flora inhabiting the soil away (control site) and underneath of *A. santolina* rooting zone were also identified. The results cleared a remarkable negative allelopathic effect of *A. santolina* aqueous extract on growth of *L. contorta*, and a detrimental positive effect on growth of *C. vulgaris*. Similarly, growth of *C. vulgaris* was significantly ($P \leq 0.01$) inhibited especially at the higher extract concentrations. Growth promotion ($p \leq 0.05$) of *L. contorta* was attained at high concentrations. The stress effect on protein profile and antioxidant activity was more prominent in *C. vulgaris* than *L. contorta.* The allelopathic effect of *A. santolina* extract may be due to the high concentrations of both flavonoids and total phenolics (7.98 and 29.72 mg/g, respectively).

Keywords: *Allelopathy, Achillea santolina, Chlorella vulgaris, Lyngbya contorta*, Growth, Active constituents.

INTRODUCTION

Allelopathy is a prevalent natural phenomenon in terrestrial and aquatic ecosystems. It is now widely accepted that plants, macrophytes and various microorganisms can produce and release chemicals into the surrounding environment (Gross, 2003). The modes of release for allelochemicals are volatilization, residue decay, leaching or root exudation (Bais *et al*., 2003). The phenomenon has been extensively studied in terrestrial habitats and harmful effects of plants on other plants or crops are quite well known. The involved allelopathic compounds (allelochemicals) have been explored as natural substitutes of pesticides for pest control (El-Darier *et al*., 2018).

Allelochemicals interaction is possible in aquatic as well as terrestrial environments. The benefit of producing the allelochemicals is a reduction of comp- etetion in the immediate environment. However, there are some categorical differences between allelepathy in an aqueous medium and allelopathy on a terrestrial substrate (Mulderij *et al*., 2007). Importantly, the continuous movement of water disperses any water soluble allelochemicals from its point of release, thus diluting it rapidly (lewis, 1986). The overall effects of the donor plant on growth of the target species were species - specific and inhibitory, stimulatory or none depend on the target species. Most of the allelopathic effects were conducted in aquatic environments, but few works have been done on the allelopathic effects between the terrestrial plants and the accompanied soil algae (Yang *et al.,* 2012). Allelochemicals are considered promising source of algaecides to control algal blooms (Hong *et al*., 2009). However, the role played by allelochemicals is mostly interpreted now in an ecological way, usually in some means of plant defense against other plants, pests or diseases.

The presence of oxygen in the cellular environment is an oxidative threat to cellular structures and processes of the organisms. Reactive oxygen species (ROS) are formed by the partial reduction of oxygen and can react with certain biomolecules, altering or inactiveating the biochemical activities. Algae minimize free radical damage by enhanced antioxidant defense (Chokshi *et al.,* 2017; Kazir *et al*., 2019).

Vegetative storage proteins (VSPs) are proteins that accumulate in vegetative tissues such as leaves, stems and tubers, depending on the plant species (El-Khawas and Shehata, 2005). The vegetative storage protein analysis function apparently to sequester temporarily excess amino acids that can later be released for recycle (Staswick, 1997), shoot regrowth and spring growth for perennials and trees (Bouchart *et al*., 1998). Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is most economical simple and extensively used biochemical technique for determination of molecular mass of protein subunits (Vishwanath *et al*., 2011).

The vital objective of the present work was focused on the allelopathic effects of water extract of *Achillea santolina* (donor species) on growth of two selected members of soil algae (recipient species); *Chlorella vulgaris* (Chlorophyta) and *Lyngbya contorta* (Cyanophyta). The two species were chosen on the basis of their flourishing in all the year round in two different ecological sites.

MATERIALS AND METHODS

Plant Materials

(A) Collection and manipulation of Achillea santolina

The present study was conducted in the laboratory of Botany and Microbiology Department, Faculty of Science, Alexandria University, Alexandria, Egypt. Samples of the whole plant of *A. santolina* have been collected from different locations at the western Mediterranean coastal region of Egypt. The collected materials were washed with tap then with distilled water, dried in shade, then ground in a Wiley Mill to coarse uniform texture and stored in a desiccator until use.

[*] *Corresponding author e-mail: salama_eldarier@yahoo.com*

Twenty grams powder of the air dried donor plant were mixed with 1 liter autoclaved distilled water, shacked for 24h at 20°C and darkness and separated by centrifugation at 5000 r.p.m. The combined aqueous extract was evaporated to dryness. After evaporation the residue was redissolved in 100ml distilled water to form a stock solution (200 ppm). This was considered as the full strength concentration. Subsequently, it was kept in a refrigerator at 5°C until used. Series of dilutions (25, 50, 75, and 100% besides the control) were prepared from the stock solution and tested for their effects on growth and some metabolic activities of the two selected soil algae.

The presence of sterols or terpenes, carbohydrates or glycosides, tannins, flavonoids, saponins, alkaloids and coumarins was evaluated according to Harborne (1984). The essential oil was extracted from the fresh plant by hydrodistillation method using Clevenger apparatus (Odalo *et al*., 2005).

(B) Collection and manipulation of Chlorella vulgaris and Lyngbya contorta

Soil samples were collected from two different sites; the first was far away from the shadow of *A. santolina* (control site), while the second was established under the shadow of the species. The current study followed the method described by Cameron and Blank (1965) for soil algae identification. In this method soil samples were added into Petri dishes contained enrichment culture media and placed in culturing room with constant temperature and light (25°C and 4000 lux).

The enrichment culture medium that used for isolating the two soil algae was described by Rippka and Hard man (1993). *C. vulgaris* was cultured in Bold's basal medium (Bischoff and Bold, 1963) while *L. contorta* was cultured in MBL medium (Nichols, 1973). These two organisms were prepared as axenic cultures. Algal culture was grown in Erlenmeyer pyres -glass flasks capacity of 250 ml containing 100 ml culture medium to which different concentrations of *A. santolina* extract added to the culture media. The flasks were grown under controlled light and temperature culture chamber under a regime of 16h light / 8h dark and 25°C incubation temperature.

Growth Measurements

The growth of the investigated algae was determined couple of days by optical density using Perkin Elmer (Lambada 1) ultra violet spectrophotometer at wave length of 625 nm. The measurements of optical density (turbidity technique) were particularly suitable for determination of growth rate according to the following equation (Levasseur *et al*., 1993):

$$[K'] = Ln (N_2 / N_1) / (t_2 - t_1)$$

Where N_1 and N_2 = optical density at time 1 (t_1) and time 2 (t_2) respectively.

Inhibition percentage (IP) was calculated according to Zhen *et al*., (2008).

$$IP = [1 – (N/N_0)] \times 100$$

Where N_0 and N were the optical density in the control and treated cultures, respectively.

Protein electrophoresis

Proteins were separated by 15% SDS Polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). The percentage of polymorphism was determined according to the following equation:

Polymorphism (%) = [(Σ bands for sample - Σ com-mon bands for sample) / Σ bands of all samples] x100

Total antioxidant activity

The antioxidants capacity was performed by the reaction of antioxidants enzymes in the sample with a definite amount of exogenously provide hydrogen peroxide (H_2O_2) as described by Koracevic *et al*., (2001). The antioxidants capacity measured spectrophotometrically at 505 nm and calculated as follows:

$$Mm/L = A_B – A_{SA} \times 3.33$$

A_B = absorbance of blank

A_{SA}= absorbance of sample

All the data were subjected, where suitable, to standard one-way analysis of variance (ANOVA) ($p \leq 0.05$ was considered as significant) according to COSTAT 2.00 statistical analysis software supplied by CoHort Software Company (Zar, 1984); pairwise comparisons of means were performed using Least Significant Differences (LSD) at 0.05 probability level.

RESULTS

Phytochemical analysis of *Achillea santolina*

The phytochemical screening of *A. santolina* revealed that it contains essential oils, flavonoids, glycolsides, phenolic compounds, sterols/triterpenes, and tannins (Table 1). While, alkaloids, coumarins and saponins were absent. Data in Table 2 clearly demonstrated that the values of total flavonoids, total phenollics, and essential oils were 7.985 mg/g, 29.725 mg/g 0.65%, respectively.

Table (1): Qualitative evaluation of some active constituents in *Achillea santolina.*

Component	Currency
Alkaloids and /or nitrogenous bases	-
Coumarins	-
Essential oils	++
Flavonoids	+ + +
Glycosides	+ +
Phenolic compounds	+ +
Saponins	-
Sterols and / or triterpenes	+ +
Tannins	+

+, low concentration; ++, moderate concentration; +++, high concentration; -, Absent

Table (2): Quantitative content of total flavonoids, phenolics and essential oils in *Achillea santolina*.

Donor species	Total flavonoids (mg /g)	Total phenolic (mg /g)	Essential oil (%)
Achillea santolina	7.985	29.725	0.65

Description of soil algae

The algae grown in soils at the control site and underneath *A. santolina* were identified and represented in Table 3. The algal groups detected are related to Cyanophyta, Chlorophyta and Bacillariophyta. The total number of green algal species was higher than both the blue green algae and diatoms. Eleven species were isolated from the two collected soil samples. These species included four genera of blue green algae, five genera of green algae and two genera of diatoms. The eleven collected algal species revealed that the green algae were absent underneath *A. santolina*, while blue greens and diatoms were present in both sites.

Table (3): Algal identification at control site compared to those grown underneath *Achillea santolina* site.

Species of algae	Soil at control Site	Soil Underneath *A. santolina* plant
Cyanophyta		
Lyngbya contorta	+	+
Anabaena circinalis	+	+
Phormidium fragile	+	+
Oscillatoria sancata	+	+
Chlorophyta		
Chlamydomonas reinhardtii	+	-
Chlorococcum humicola	+	-
Chlorella vulgaris	+	-
Scenedesmus linearis	+	-
Oocystis borgei	+	-
Bacillariophyta		
Navicula viridula	+	+
Nitzschia palea	+	+

Consequently, the two algal species selected were *C. vulgaris* (absent in the soils from the shade of *A. santolina* plant) and *L. contorta* (isolated from the two types of soil).

Effect of *Achillea santolina* aqueous extract on optical density and growth rate of *Chlorella vulgaris* and *Lyngbya contorta*

(A) Chlorella vulgaris

Data concerning the effect of A. santolina extract on optical density and growth rate of C. vulgaris cultured for 14 days were recorded in Figure 1. These data revealed that the mean growth rate at control and at the treated cultures reached their maximum value at the 8th day of culturing. However, by increasing the concentration of the extracts values of growth increased gradually till the 12th day of culturing, then decreased gradually till the end of the experiment. In addition, the more the concentration of the extract, the less are the values of growth until the end of the experiment (14days of culturing).

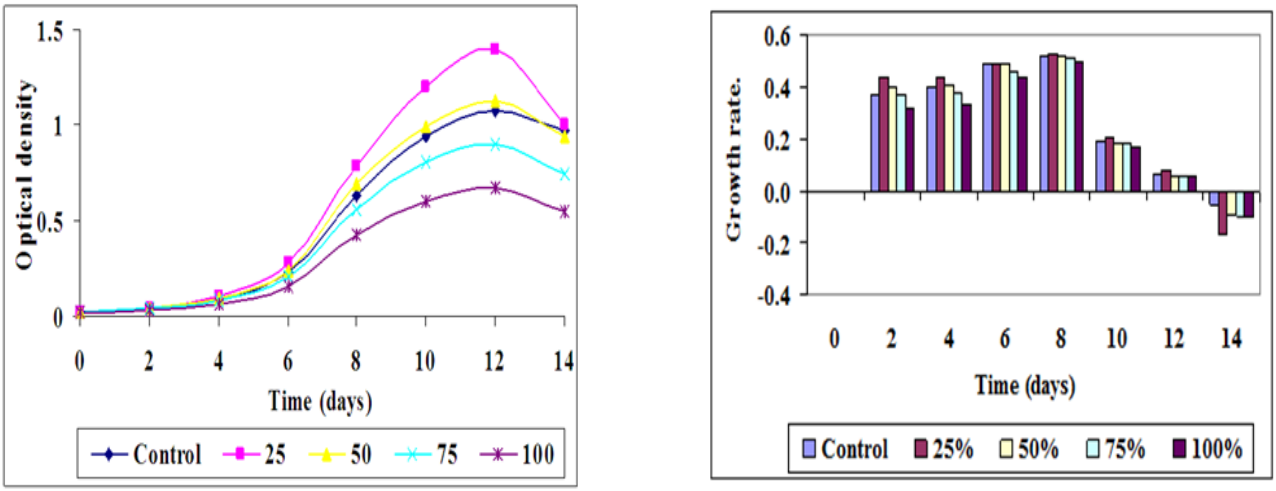

Figure (1): Optical density and the mean growth rate of *Chlorella vulgaris* cultured for 14 days under the effect of different concentrations of *Achilleasantolina* aqueous extract.

(B) Lyngbya contorta

Data concerning the effect of *A. santolina* extract on growth of *L. contorta* (optical density) were recorded in figure 2. It is clear from these data that growth rate and mean growth rate of the organism reached its maximum value at the 8th day of culturing. At 100% extract concentration, the inhibition percentage (IP) in growth rate of *C. vulgaris* at the 10th day reached 61.2% compared to the control**.** Notably, at the 14th day of culturing the IP was decreased to 44.2%. On the other hand, maximum IP (88.8%) in growth rate of *L. contorta* was attained at 2 nd day of culturing. A percent of about 35.7% increase at the 8th day of culturing was achieved relative to control. However, at the 14th day the percent of decrease reached 16 % at the same concentration (Figure 3).

Effect of *A. santolina* on protein pattern of selected soil alga

Different concentrations of *A. santolina*-aquaous extract showed a significant effect on protein pattern of two selected members of soil algae, *Chlorella vulgaris* and *Lyngbya contorta*, in which protein pattern were very difference between control and grown cells (Plate 1). Increasing the concentration of aqueous extract of *A. santolina* was dramatically affect the pattern of protein profile for *Chlorella vulgaris* and *Lyngbya contorta* (Plate 1, a and b, respectively).

DISCUSSION

Allelopathy is widely understood as the inhibitory or stimulatory effects of one plant; donor or test on another plant; target or recipient due to the release of biologically active biomolecules named allelechemicals (Chon and Nelson, 2010). The core objective of the present work was focused on the effect of different concentrations of water extract of *A. santolina* (donor species) on growth of two selected members of soil algae namely; *Chlorella vulgaris* and *Lyngbya contorta* (target species). However, few works have been carried out on the allelopathic effects between higher plants and soil microalgae (Yang et al., 2012).

Field observations as well as isolation and character-

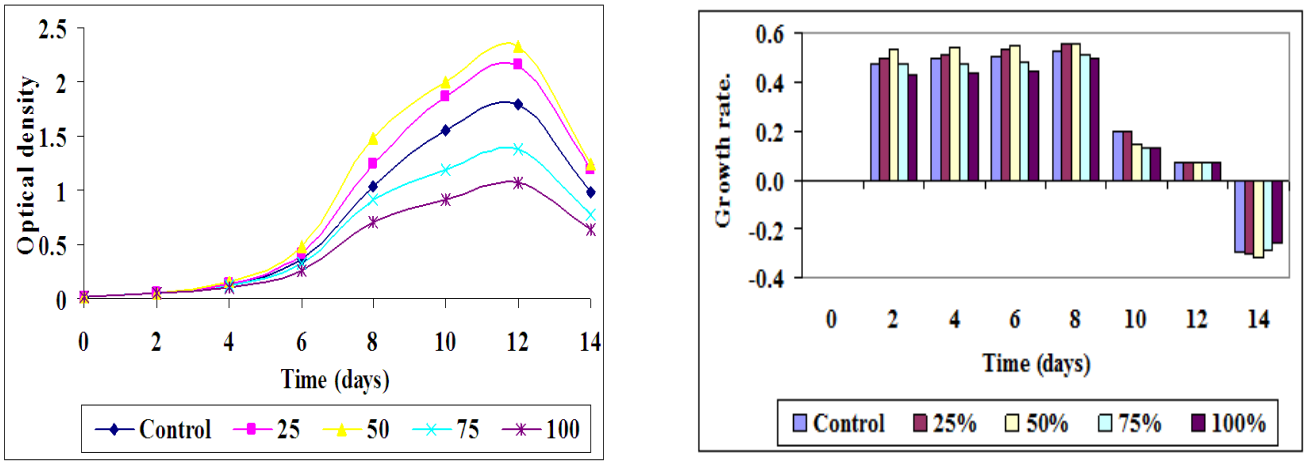

Figure (2): Optical density and mean growth rate of *Lyngbya contorta* cultured for 14 days under the effect of different concentrations of *Achillea santolina* extract.

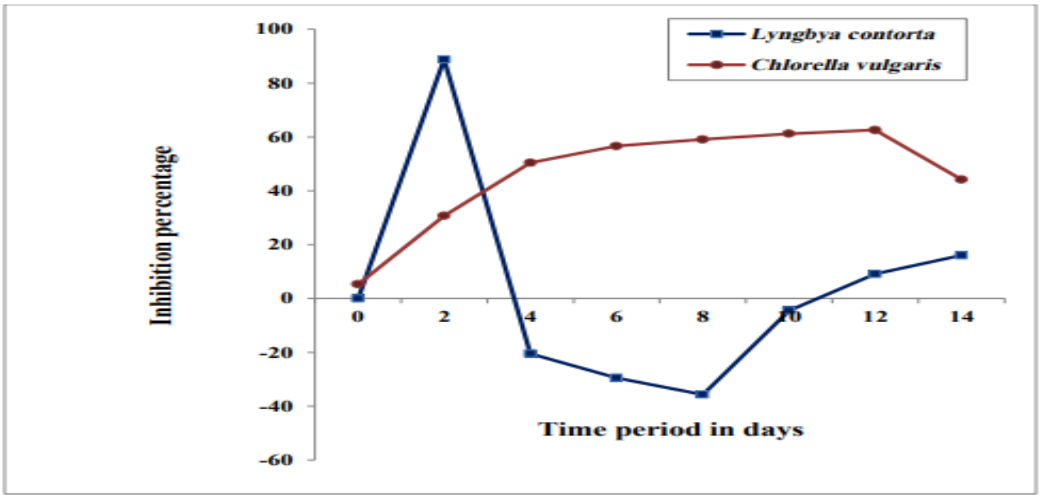

Figure (3): Inhibition percentage in growth of *Chlorella vulgaris* and *Lyngbya contorta* cultured for 14 days under the effect of 100% concentration of *Achillea santolina* extract.

ization of algal species indicated that members of Chlorophyta were dominant only in soils far away from *A. santolina* and nearly absent below shadow of *A. santolina*. This fact was supported by the idea of Morales-Flores *et al*., (2007). They reported that the photosynthetic machinery of chloroplasts is a preferred target for allelochemicals. Contrarily, the members of the blue green algae grew normally below or away from *A. santolina*. The phytochemical screening of *A. santolina* indicated that it contains essential oils, flavonoids, glycosides, phenolic, sterols/triterpenes, and tannins compounds. In addition, total flavonoids, total phenolic, and essential oil content attained values of about 7.985 mg/g, 29.725 mg/g and 0.65%, respectively. This may support the idea that the inhibitory effect of *Achillea santolina* extract probably due to the presence of high concentrations of these allelechemicals. This coincides with those obtained by different studies of Sasikumar *et al*., (2002) and El-Darier and El-Bakkosh (2011). Higher concentrations of allelochemicals influenced growth and different physiological processes during the period of plant growth (Olofsdotter, 1998).

According to the present study, the considerable inhibition of algal growth parameters may be due to the inhibitory effect of allelochemicals, such as, phenolic, terpenoids and flavonoids, which could affect growth directly or by altering the mobilization of storage compounds during growth. This suggestion has been confirmed by using different concentrations of *A. santolina* extract and its effect on the total antioxidant activity in *Chlorella vulgaris* and *Lyngbya contorta*. The data showed that *Chlorella vulgaris* displayed a significant decrease in total antioxidant activity, meanwhile, *Lyngbya contortawas* was not affected (Table 4).

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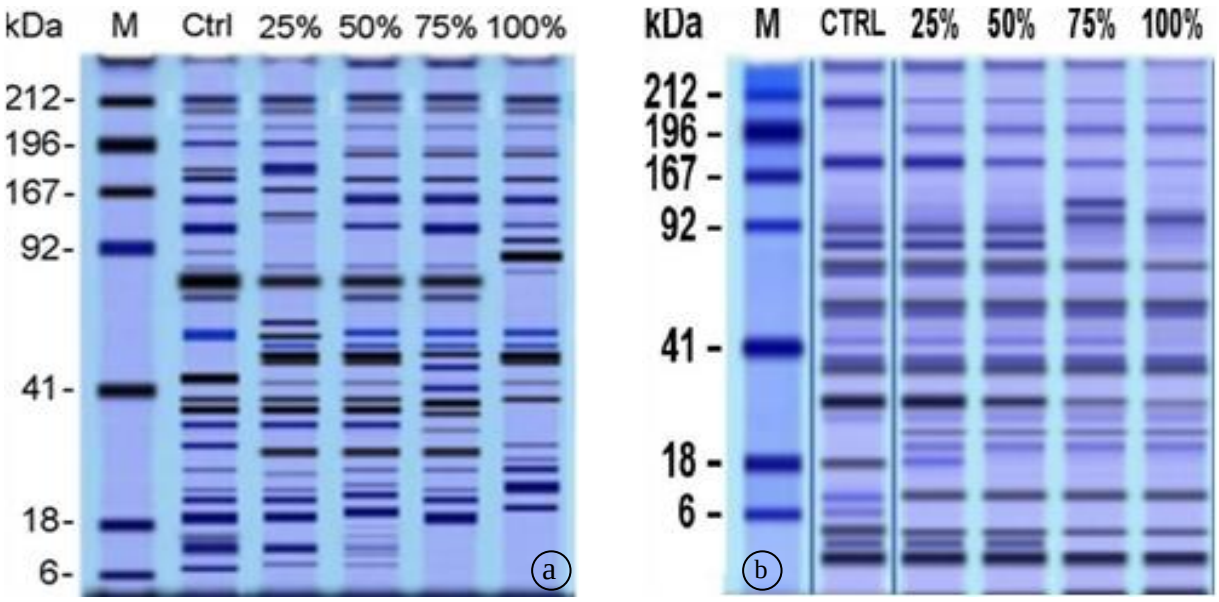

Plate 1: Patten of protein profile for *Chlorella vulgaris* (a) and *Lyngbya contorta* (b) cells cultured for 10 days under the effect of different concentrations of *Achillea santolina* aqueous extract.

Table (4): Effect of different concentrations of *Achillea santolina* extract on the total antioxidant activity in *Chlorella vulgaris* and *Lyngbya contorta*.

Algal species	Control	Extract concentration (%)			
		25	50	75	100
Chlorella vulgaris	9.4	6.5	4.5	2.5	2.4
Lyngbya contorta	7.2	7.3	7.6	7.7	7.8

Likewise, mobilization; a process which usually takes place rapidly during early stages of algal growth seems to be delayed or decreased under allelopathy stress conditions (Muscolo *et al*., 2001; Gniazdowska and Bogatek, 2005). Data proved that in case of *C. vulgaris* the more the concentration of the extract, the less is the values of growth. These results coincided with those obtained by Ahrabi *et al*., (2011). Similarly, Li and Hu (2004) observed that extracts from some macrophytes inhibited the growth of *Chlorella pyrenoidosa*. Conversely, in case of *L. contorta*, the growth increased by increasing the concentration of the extract.This may explain why *L. contorta* was found in soil at the shadow of *A. santolina* where it flourished well and the growth increased by increasing the concentration of the extract. Furthermore, Ferrier *et al*., (2005) found that

the aqueous extract of barley straw liquor significantly increased growth of *Oscillatoria lutea* and *Navicula* sp. While *Ulothrix fimbriata*, *Scenedesmus quadricauda* and *Chlorella vulgaris* showed no significant difference from controls. Moradi and Ismail (2007) and Ghezelbash *et al*., (2008) reported that reduction in growth rate is due to the decrease in the photosynthetic rate.

The destructive effect of *A. santolina* extracts on protein profile was more prominent in *C. vulgaris* than in *L. contorta* which coincided with results obtained by Ahmed (2010) and El Taher (2012). Therefore, changes in protein composition of these organisms may also be involved in adaptation processes (Hagmann *et al*., 1990). Protein synthesis in the stress adaptation mechanism in the two studied algae revealed the occurrence of species stressed proteins for a particular environmental stress factors (*A. santolina* extract). Maha (2003) reported that, Nostoc linckia cultured under stress conditions showed the appearance of two new bands compared with control. In addition, Ahmed (2010) stated that, the tolerance of an organism to stress conditions could be achieved through synthesis or accumulation of new proteins. These results are nearly in harmony with the results of the present study. *A. santolina* extract stress may trigger the expression of the antioxidative defense systems. This is evident from the fact that, total antioxidants including non-enzymatic and enzymatic antioxidants, showed in general an increase with increasing stress indicating a cellular capacity to overcome the stress. This conclusion can be substantiated if the degree of stress is not so high to concentration limit that may cause damage and death of the organism (Hamid and Sibi, 2018; Kazir *et al*., 2019). The same conclusion was also observed for *C. vulgaris* where the total antioxidant activity began to decrease at the higher concentration of *A. santolina* extracts.

Finally, we concluded that Chlorophyta species were dominated only in soils of the control site of *A. santolina*, while Cyanophyta and Diatoms species were identified in both types of soil. *C. vulgaris* and *L. contorta* were chosen because both organisms flourish all the year in their sites. In conclusion, future studies on *A. santolina* interactions with soil algae and microorganisms must be focus on other algal groups in order to understand the effect of allelochemicals from higher plants on soil organisms. More experiments however, are needed to prove the general existence and importance of allelopathy in these ecosystems under different environmental and geographical conditions.

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التداخل الأليلوباثي بين النبات الطبي؛ البعيثران واثنين من أنواع الطحالب الموجودة بالتربة

سلامه الضرير وعبدالفتاح متولي

قسم النبات وعلم الاحياء الدقيقه, كليه العلوم, جامعه الاسكندريه، مصر

الملخص العربـــــي

لقد تم معمليا إختبار التأثير الاليلوباثي للمستخلص المائي للنبات الطبي؛ *Achillea santolin* على نمو وتوزيع اثنين من الطحالب الموجودة بالتربة وهما *Chlorella vulgaris* (الطحالب الخضراء) و *Lyngbya contorta* (الطحالب الزرقاء). وقد أظهر التحليل الكيميائي النباتي لـ *A. santolina* إحتوائه علي زيوت أساسية، فلافونويد، جليكوسيدات، مركبات فينولية، ستيرول، ترايتيربينات وعفصيات. كما تم التعرف على الطحالب التي تقطن التربة بعيدًا (موقع التحكم) وتحت منطقة الجذر لـ*A. Santolina* .

وقد أوضحت النتائج وجود تأثير أليلوباثي سلبي ملحوظ من المستخلص المائي *A. santolina* على نمو *L. contorta* , وتأثير إيجابي على نمو*C. vulgaris* . وبالمثل، فقد تم تثبيط النمو للأخير بشكل كبير ($p \leq 0.01$) خاصة في التركيزات العليا للمستخلص بينما تم تعزيز النمو ($P \leq 0.05$) من *L. contorta* عند التركيزات العالية. ومن ناحية أخري فقد كان تأثير الإجهاد الأليلوباثي على البروتين والنشاط المضاد للأكسدة أكثر وضوحًا في *C. Vulgaris* مقارنةً بـ *L. contorta* .

وقد لخصت النتائج إلي أن التأثير الأليلوباثي لمستخلص *A. santolina* قد يكون بسبب التركيزات العالية لكل من الفلافونويد والفينولات الكلية (7.98 و 29.72 ملغم / جم ، على التوالي).