LABORATORY STUDIES ON THE ALLELOPATHIC ACTIVITY OF THE GREEN ALGA, *SCENEDESMUS ACUMINATUS* CRUDE EXTRACTS AGAINST THE GREEN ALGA *CHLORELLA VULGARIS*.

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Abstract

The allelopathic activity of the green alga named *Scenedesmus acuminatus* against the green alga *Chlorella vulgaris* has been reported. Two algae were isolated, purified and identified from water of earthen fish ponds of Central Laboratory for Aquaculture Research, Abbassa, Sharkia, Egypt. *S. acuminatus* was cultured in mass culture. Algal mass was harvested at exponential growth phase and dried then extracted by the organic solvents (ethanol or methanol) using a soxhlet apparatus. Allelopathic activity of the crude extracts was investigated with agar-well diffusion and paper-disc assay to demonstrate efficiency of antialgal principles against *C. vulgaris*. Findings revealed that two crude extracts had an inhibitory effect against *C. vulgaris* where, large and small inhibition zone caused by Agar-well diffusion assay. Ethanolic crude extract was 23.6 and 51.6 mm at concentrations of 100 and 400 µl respectively. The largest and smallest clear zone caused by methanolic crude extract was 36.6 and 20 mm in favor of the above mention concentrations. On the other hand, the largest and smallest clear zone caused by paper- disc assay for ethanolic crude extract was 26.3 and 17.6 mm and for methanolic crude extract was 15.3 and 24 mm at concentrations of 10 and 40 µl respectively.
***Keywords*:** Allelopathy, *C. vulgaris*, Crude extract. *S. acuminatus*.

Introduction

Algae are important component of aquatic ecosystems. They form the oxygen necessary to consumer organisms. Microalgae are commonly used in the rearing of marine fish larvae they are either added directly to water in the rearing tanks (**Reitan *et al*., 1997**) or as food for rotifers.

Releasing chemicals and/or toxins by plants or microorganisms that affect their potential competitors for resources is known as allelopathy (**Lampert and Sommer, 1997**). Allelopathy is considered as an important process that occurs among all groups of marine and freshwater primary producers (**Gross, 2003 and Legrand *et al*. 2003**). Allelochemicals (natural plant toxins) are considered promising sources of herbicides (including algaecides). Due to their natural origin, many researchers have suggested that most allelopathic compounds will not only be biodegradable but also less polluting than traditional herbicides

(**Macias *et al*., 1998**) which means that most allelochemicals have short half lives compared to synthetic pesticides. It is difficult to isolate the bioactive compounds from phytoplankton due to its production in very small amounts. Under stress condition e.g., nutrient limitation; production of a highly active compound at low concentrations is a cost-effective strategy (**Leflaive and Ten-Hage, 2007**).

Allelopathic activity of green alga *S. acuminatus* crude extracts on growth of *C. vulgaris* was the specific objectives of this study.

Materials and Methods

Green algae *S. acuminatus* and *C. vulgaris* were isolated from Abbassa fish water ponds. Both species ware purified, identified and cultured in Bold's-Basal Medium BB.Medium (**Bischoff and Bold, 1963**) at pH 6.6.

Mass culture of green algae:-

Algal culture was obtained by cultivating of the green algae in 250 ml Erlenmeyer flasks, each contain 150 ml of the medium. Cultured flasks were stoppered with cotton plugs and sterilized in autoclave at 121.5 ºC and 1.5 atm. for 20 minutes, after cooling they were inoculated with 30 ml of the pre-culture of the green algae. The algal cultured was mentioned at 22 ± 2ºC and illuminated by florescent tubes with light intensity between 3000-5000 lux and a photoperiods of 19/5 h, light /dark cycles. Cultured flasks were shaken three times daily to prevent wall growth.

One more Erlenmeyer flask containing 500 ml of the BBM medium was inoculated by 100 ml of pre-culture green algae. This process was repeated having 1500 ml from pre-culture enough to inoculate the carboy.

Harvesting, extraction and processing the algal biomass:-

After 6 days algae reaching maximum growth, the circulation provided by the pumping system was stopped and the primary separation of the algal cells from the liquid phase was achieved by gravity separation forming thick sediment at the bottom of the carboy within 24 hrs. Supernatant was centrifuged at 2500 rpm for 20 minutes. The algal slurry obtained as thick sediment and by centrifugation was dried in an oven at 50 ºC for 24 hr.

Dried algal biomass appeared in the form of small pellets were blended in an electric coffee mill. Resulting powder was submitted to lipid-soluble extraction with ethanol or methanol (95%) 1:15 (W:V) using a soxhlet extractor at 55-60ºC, all samples were refluxed until saturation (24 hrs) and the respective extracts were dried in an oven at 50ºC.

Screening of crude extract of *S. acuminatus* against *C. vulgaris*:-

(A)-Agar-well diffusion assay:-

Agar plate well-diffusion method was used as described by (**Desta, 2005**). Sterilized Bold's-Basal agar Medium poured in sterilized Petri Dishes. After

solidification, wells were cut from the plate using a sterile test tube (a sterile cork borer). A known volume of algal extract was introduced into each well and dried in room temperature. After drying, the plates were inoculated with 2 ml of the fresh (target) log phase growing organism and evenly distributed using a sterile glass spreader. Plates were incubated at 22 ± 2 ºC for a week in phytoplankton lab. Inhibition zones were measured with a ruler and compared with control well (well containing only the respective solvent). Experiment was carried out in triplicate.

(B)-Paper disk assay:

Sterilized Bold's-Basal agar Medium poured in sterilized Petri Dishes. After solidification; the plates were inoculated with 2 ml of the fresh (target) log phase growing organism evenly distributed using a sterile glass spreader. The empty sterile paper discs were dipped in the respective extracts and air-dried in the room temperature and placed on the agar medium using sterilized forceps (**Bauer *et al*., 1966**). The plates were incubated at 22 ± 2 ºC for a week in phytoplankton lab. Allelopathic activity was measured as mentioned above. Control discs soaked with respective solvents were run simultaneously. Disk diffusion assay was carried out in triplicate.

Allelopathic activity on growth inhibition was estimated by percentage of inhibition (PI), which is calculated by the following equation:-

Percentage of inhibition PI (%) = diameter of inhibition zone/diameter of growth ×100.

Considering that:-

Control inhibitor concentration = zero.

Diameter of growth = 90 mm.

Statistical analysis

Statistical analysis was performed using the analysis of variance (ANOVA) and Duncan's Multiple Test to determine differences among treatment means at significance level of 0.05. Standard errors were estimated. All statistics were run on the computer using the SAS program (**SAS, 2010**).

Results

Green algae which isolated from Abbassa fish ponds were identified as *Scenedesmus acuminatus* and *Chlorella vulgaris*. *S. acuminatus* is a small, non motile colonial green alga consisting of cells aligned in a flat plate. The cells are usually cylindrical but may be more lunate, ovoid or fusiform. Each cell contains a single parietal, plate-like chloroplast with a single pyrenoid.

C. vulgaris is a species of single-celled green algae; it is spherical shape, about 2 to 10µm in diameter, without flagella. Each cell has a bell-shape or cup shape parietal chloroplast with or without apyrenoid.

a- Agar- well diffusion assay:-

Diameters of inhibition zones due to ethanolic extract of *S. acuminatus* at concentrations of 100, 150, 200, 250, 300 and 400 µl were 23.3, 25.3, 30, 32.6, 42.6 and 51.6 mm, respectively. On the other hand, diameters of inhibition zones due to methanolic extract of *S. acuminatus* at concentrations of 100, 150, 200, 250, 300 and 400 µl were 20, 23, 23, 23.3, 33.3 and 36.6 mm, respectively. No clearing zones were noticed for control (respective organic solvents only).

The highest PI for ethanolic and methanolic extract was 57.4 and 40.66 %, respectively at concentration of 400 µl for each extract, while the lowest PI for ethanolic and methanolic extract was 26.22 and 22.2 %, respectively at concentration of 100µl for each extract. Results were shown in Table 1 and photo, 1, 2, 3 and 4.

Table (1): Allelopathic activity of ethanolic or methanolic crude extract of *Scenedesmus acuminatus* against *Chlorella vulgaris* growth by (Agar-well diffusion assay)

Quantity of algal extract (µl).	Diameter of inhibition zone (mm)	
	Methanolic extract	Ethanolic extract
100	23.6 ± 1.3 a	20±0.57a
150	25.3± 0.88 a	23±0.57a
200	30±0.57a	23±0.57b
250	32.6 ± 0.3a	23.3±1.8b
300	42.6 ±0.3a	33.3±3.3a
400	51.6±2.9a	36.6±3.3b

* letters a and b show that there's significant difference between ethanolic and methanolic extract at each treatment in the same raw, while data shown with the same letters aren't significantly different at $P < 0.05$.

b- Paper- disc assay:-

Diameters of inhibition zones due to ethanolic extract at concentrations 10, 15, 20, 25, 30, 35 and 40 µl were 17.6, 18, 22, 22.3, 23.3, 25.3 and 26.3 mm, respectively. On the other hand, diameters of inhibition zones due to methanolic extract at concentrations 10, 15, 20, 25, 30, 35 and 40 µl were 15.3, 16, 19, 20, 21.3, 22.6 and 24 mm respectively. No clearing zones were noticed for control.

The highest PI for ethanolic and methanolic extract was 29.2 and 26.6 % respectively at concentration of 40 µl for each extract, while the lowest PI was 19.5 and 17 % for ethanolic and methanolic extract respectively at concentration of 10 µl. The results were shown in Table 2 and photos, 5, 6, 7 and 8. No clearing zone noticed in control prepared for each charge of bioassay.

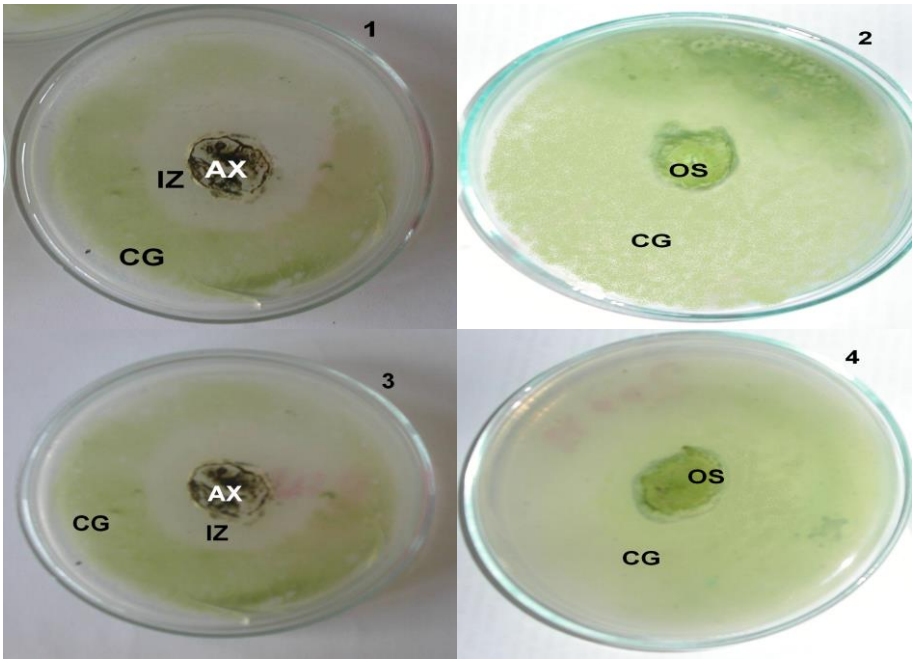

Photo (1): showing effect of *S. acuminatus* ethanolic crude extract on *C. vulgaris* growth. IZ=inhibition zone, AX=algal extract (ethanolic crude extract of *S. acuminatus*). CG= *C. vulgaris* growth.
Photo (2): Showing effect of organic solvent only on *C. vulgaris* growth using (Agar-well diffusion assay). OS= organic solvent (ethanol as control). CG= *C .vulgaris* growth.
Photo (3): Showing effect of *S. acuminatus* methanolic crude extract on *C. vulgaris* growth. IZ=inhibition zone, AX=algal extract (methanolic crude extract of *S. acuminatus*). CG= *C. vulgaris* growth.
Photo (4): Showing effect of organic solvent only on *C. vulgaris* growth using (Agar-well diffusion assay). OS= organic solvent (methanol as control). CG= *C.vulgaris* growth.

Table (2): Allelopathic activity of (ethanolic and methanolic) crude extract of *Scenedesmus acuminatus* against *Chlorella vulgaris* growth by (paper-disc assay)

Quantity of algal extract (µl).	Diameter of inhibition zone (mm)	
	Methanolic extract	Ethanolic extract
10	15.3±0.33	17.6±2.18
15	16±1.52	18±0.57
20	19±0.57	22±1.57
25	20±1.57	22.3±1.45
30	21.3±2.3	23.3±0.88
35	22.6±2.8	25.3±2.02
40	24±0.57	26.3±1.8

*There's no significant difference between ethanolic and methanolic extract at each treatment in the same raw.

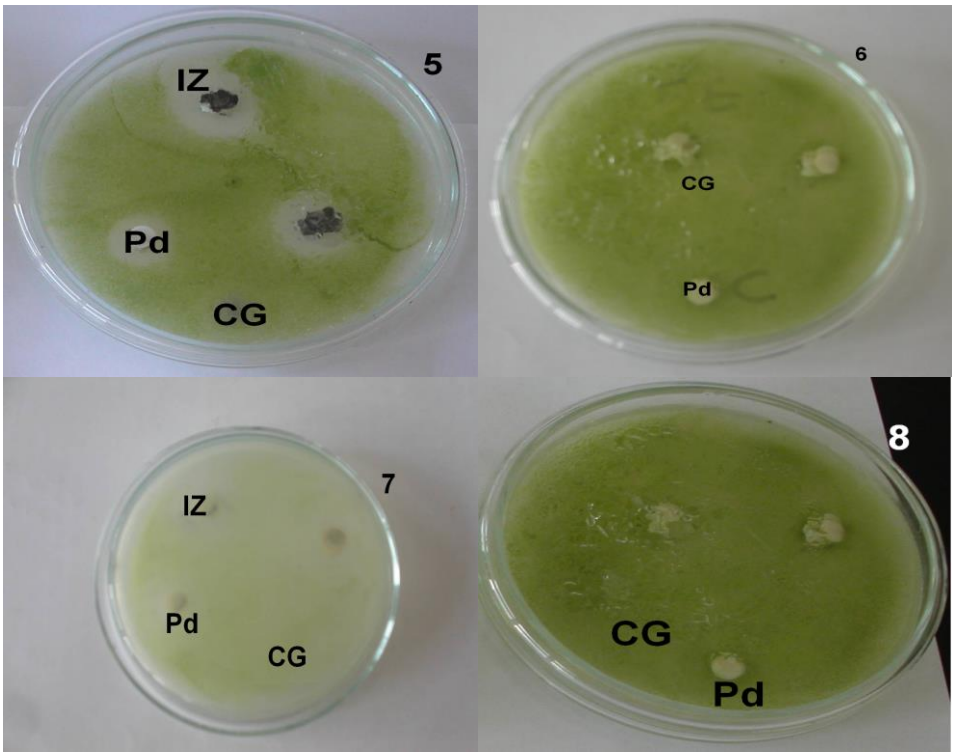

Photo (5): Showing effect of *S. acuminatus* ethanolic crude extract on *C. vulgaris* growth. IZ = inhibition zone, Pd = Paper disc impregnated with *Scenedesmus* ethanolic crude extract, CG = *C.vulgaris* growth.

Photo. (6): Showing effect of organic solvent only on *C. vulgaris* growth using (paper-disc assay). Pd = paper disc impregenated with organic solvent only (ethanol as control). CG= *C.vulgaris* growth.

Photo (7): Showing effect of *S. acuminatus* methanolic crude extract on *C. vulgaris* growth. IZ = inhibition zone. Pd = Paper disc impregnated with *Scenedesmus* methanolic crude extract. CG = *C. vulgaris* growth.

Photo. (8): Showing effect of organic solvent only on *C. vulgaris* growth using (paper-disc assay). Pd = paper disc impregenated with organic solvent only (methanol as control). CG = *C.vulgaris* growth.

Discussion

In particular, there has been a lot of interest in so-called allelopathic competition between two species, also due to their importance in many applications as for instance, in bio-remediation problems or laboratory biotechnological process. It is difficult for researchers to study allelopathic effects among aquatic organisms under natural conditions because factors such as nutrient and light competition, temperature and pH change can totally mask an allelopathic effect. So, it is necessary that attempts to identify allelopathic

interactions among aquatic organisms should be conducted in a controlled system (**Keating, 1977**). So our experiments were run under stable and controlled laboratory conditions. Temperature and light and illuminating periodicity play an important role in production of harmful substances and harvested the causative organism at the exponential growth phase as (**Egorov, 1985**).

Our results in agreement with **Jorgensen** (**1956**) who recorded that *Scenedesmus* formed substances that inhibited the growth of *Chlorella* and that *Chlorella* filtrates stimulated *Scenedesmus* growth rate. Also results agreed with those recorded by **Hulot *et al.*** (**2001**) who stated that *Chlorella* growth was impeded by the presence of *Scenedesmus*, *Chlorella* seemed to suffer during its invasion in a medium already occupied by *Scenedesmus*, these result explained the release of substance inhibiting *Chlorella* growth by *Scenedesmus*. However, **Grover** (**1991a, b**) ran phosphorus-limited cultures where phosphorus was supplied at a constant concentration or with varying periodic pulses leading to non-steady continuous cultures to study competition between *Chlorella* and *Scenedesmus.* In all cases, *Chlorella* outcompeted *Scenedesmus* whatever the order of introduction. The absence of allelopathic effects during **Grover's** (**1991a, b**) experiments might depend on temperature, which is known to play an important role in the production of harmful substances. Grover's experiments were run at 12°C while Jorgensen's at 20°C, Hulot's at 22°C and ours at 22±2°C.

Our results conclude that the alcoholic (ethanolic or methanolic) crude extracts of *S. acuminatus* are able to inhibit growth of *Chlorella*. In agreement with our results, **Souhaili *et al.*** (**2004**) stated that ethanolic and methanolic extracts of marine algae had an inhibitory effect against Gram-positive and Gram-negative organisms and fungi. Results showed that well – diffusion assay is better than paper disc assay where inhibition zones were greater than that of paper-disc assay because the later has a limited saturation capacity.

More research needs to find out the chemical nature of inhibitor(s) substance (allelochemical, or algaecide).

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دراسة معملية للنشاط المضاد (الاليلوباثى) لمستخلص خام من طحلب السينيديسمس ضد نمو طحلب الكلوريلا

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فى هذا البحث تم عزل وتنقية نوعين من الطحالب الخضراء وهما سينيديسمس اكيومينيتس و كلوريلا فولجارس من مياه الاحواض السمكيه الترابية بالمعمل المركزى لبحوث الثروة السمكية بالعباسة. تم استزراع مزرعة ذات كتلة حيوية كبيرة من طحلب السينيديسمس وقمنا بحصاده فى طور النمو المطرد (اللوغرتمى) وتجفيفه ثم استخلاصه بأستخدام كل من المذيبين العضويين (الايثانول اوالميثانول) بتركيز 95% بنسبة (15:1) (طحلب : مذيب عضوى) باستخدام السوكسيليت .

تم اختبارتأثير المستخلصات الخام من سينيديسمس اكيومينيتس على طحلب الكلوريلا وذلك باضافتها الى وسط غذائى صلب بطريقتين الأولى :عمل ثقب فى وسط الطبق ووضع المستخلص فيه والثانيه وضع اقراص من ورق الترشيح المشبعه بالمستخلص على الوسط الغذائى الصلب فى أطباق بترى وتم قياس منطقة التثبيط حول القرص المشبع بالمسطرة واظهرت النتائج الاتى:

اولا: طريقة عمل الثقب المضاف له المستخلص:

- مع مستخلص الايثانول كانت اعلى نسبه مئوية للتثبيط 57.4% مع قطرمنطقة التثبيط طولها 51.6مم بينما اقل نسبة مئويه للتثبيط كانت 26.22% مع منطقة تثبيط قطرها 23.6مم.
- مع مستخلص الميثانول كانت اعلى نسبه مئوية للتثبيط 40.66% مع قطرمنطقة التثبيط طولها 36.6مم بينما اقل نسبة مئويه للتثبيط كانت 22.2% مع منطقة تثبيط قطرها 20مم.

ثانيا: طريقة الاقراص الورقيه المشبعة بالمستخلص:

- مع مستخلص الايثانول كانت اعلى نسبه مئوية للتثبيط 29.2% مع قطرمنطقة التثبيط طولها 26.3مم بينما اقل نسبة مئويه للتثبيط كانت 19.5% مع منطقة تثبيط قطرها 17.6مم.
- مع مستخلص الميثانول كانت اعلى نسبه مئوية للتثبيط 26.6% مع قطرمنطقة التثبيط طولها 24مم بينما اقل نسبة مئويه للتثبيط كانت 17% مع منطقة تثبيط قطرها 15.3مم.

- المستخلصين قادرين على استخلاص المادة المثبطة من طحلب السينيديسمس واحداث التثبيط لطحلب الكلوريلا - طريقة عمل الثقب لها القدرة على اعطاء نتائج اوضح وذلك لامكانية اضافة تركيزات عالية من المستخلص على عكس طريقة تشبع الاقراص بالمستخلص وذلك لأن درجة تشبع القرص محدودة .

مما سبق يتضح ان طريقة عمل (نشاط) المادة المثبطة المستخلصة فى المستخلص الكحولى الخام لطحلب السينيديسمس قد احدثت تثبيط للكائن المستهدف الكلوريلا.

واستكمالا لهذه الدراسه فاننا سوف نقوم ان شاء الله بعمل عزل واستخلاص للمواد الفعاله المسببه لتثبيط النمو وتعريفها كيميائيا.