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VOLATILE METABOLITES COMPOSITION OF MICROALGAE *Chlorella vulgaris* AND ITS PLANT GROWTH INHIBITION EFFECT

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ABSTRACT

The volatile metabolites of green microalgae, *Chlorella vulgaris,* grown in outdoor condition were isolated by steam distillation and fractionated on a silica gel column (12×500mm) using different organic solvent mixtures. The volatile metabolites and obtained fractions were analyzed to identify their compositions using Gas liquid chromatography (GLC) and Gas Chromatography-Mass Spectrometry (GC/MS). Also, the influence of fractions at different concentration levels on ∝-amylase activity and coleoptile growth as well as germination of barley grains was evaluated. The obtained results showed that the volatile metabolites were a mixture of about 105 compounds of which 30 compounds were identified representing 86.38% of the total volatile components. These components were consisting of hydrocarbons, acids, alcohols, esters, aldhydes and ketones, which represented 32.86%, 23.93%, 15.62%, 8.02%, 3.24% and 2.71% of the total volatile components respectively. Hexadecene, octadec 9,12-dienoic, hexadecanol, methyl octadecanote, hexadecanal and hexadecanone were the major components detected in their fractions, respectively. The total volatile metabolites had a strong inhibitory action on the α-amylase activity and growth of coleoptile as well as germination of barley grains. On the other hand, the acid fraction had the highest inhibition effect compared with other fractions. In contrast, hydrocarbon fraction showed no effect in this respect.

INTRODUCTION

Interest in the possible use of bacteria, algae, yeast or fungi as a source of nutrients has increased rapidly during the past twenty years. This increase has been due to the shortage in protein sources parallel with increasing population. The microalgae have advantage over others because they are photoautotrophs and produce a simple and cheaper amount of biomass (Borowitzka 1986 and Ma *et al.* 1997). In addition, microalgae have proved to be a rich source of compounds with adverse structural features and interesting biological activities (Schwartz *et al*. 1988, Brown and Miller 1992 and Anggadiredja *et al.* 1997). These biological-active compounds have been identified as carbonyls, terpenes, fatty acids, isoprenylated and brominated hydroquinones, phycotene, fluorotannins and carotenoids which have served as potential compounds in the pharmaceuticals area including: antibacterial, antifungal, antiviral, anticancer agents and other properties (Kaker 1984, Schwartz *et al*. 1988, Kobayashi 1989, Mathew *et al.* 1995 and Morinoto *et al.* 1995).

The industrialization and urbanization reasulted in increasing of microalgae grown in fresh water which impart characteristic odours to the water (Juttner 1983, 1992, Rzama *et al.* 1995 and Borowitzka 1997). However, the microalgae can be used as biological system for removal of some toxic substances in aquatic environment which , microalgae degraded these substances and use it as carbon source (Travieso *et al*. 1999 and Fytianos *et al.* 1999). Thus, the interest in the use of microalage, eg., *Chlorella sp*. and *Scenedemus sp*., as a source of useful fine chemical products including acids, glycerol, lipids, enzymes, amins, antioxidant vitamins and pigments have been the subject of intense scientific investigation (Kay 1991, Mrechie *et al*.1995 and Ogbonna *et al*. 1998). In this regard, the volatile oils and their bioactivities of aquatic microalgae were not enough studied due to their lower amounts than in the higher plants (Kajiwara *et al.* 1993). However, this subject is becoming of increasing interest in the recent years. Some volatile compounds of aquatic microalgae were isolated and identified as alkenes, saturated and unsaturated aliphatic alcohols, aldehydes, ketones, esters, thioesters and sulfides (Juttner 1983, Kajiwara *et al.* 1993, Juttner 1992 and Rzama *et al.* 1995). These studies concerned with the chemical composition of some microalgae volatile oils but not with their bioactivities.

In the present study, the steam-volatile metabolites of microalgae grown in fresh water culture, *Chlorella vulgaris*, were isolated and analyzed using GC and GC mass spectrometry to identify their components. The bioactivity of the volatile metabolites was evaluated on α-amylase activity, the growth of coleoptiles and germination rate of barley grains.

MATERIALS AND METHODS

The algae

A pure culture of C*hlorella vulgaris* var. which has been isolated and identified by Shaheen *et al*. (1974) was mass-cultured in fresh water in 200 liter outdoor glass tanks. The culture was supplied with macro and micro elements according to Venkatraman *et al.* (1969). Carbon dioxide was used as a carbon source and the culture was aerated by mechanical motor at a rate of 15-20 turns/min. The pH of the medium was maintained between 6 and 7 during the growth period by adjusting the flow rate of CO_2 in the culture. The other details of conditions, cultivation and processing were achieved by El-Fouly *et al*. (1985). At the end of log phase of growth, the culture was harvested by centrifugation at 5000 rpm. for 10 min. and kept at 20°C until was used.

Isolation of volatile metabolites.

Suspension of *Chlorella vulgaris* cells (100 gm.) was placed in a flask (4 L) with double distilled water (2L). The volatile components were isolated by steam distillation and extracted as described by Liknes and Nickerson (1966).

Fractionation of volatile metabolites.

A portion of the volatile components was placed on a column (12×500 mm) of silica gel (Merk). The hydrocarbon fraction was eluted with pentane (150 ml). The polar fraction was then eluted with distilled diethyl ether (polar fraction I) and with an organic solvent mixture composed of diethyl ether and methanol (9:1) (polar fraction II). Finally, the acidic fraction was eluted with an organic solvent mixture composed of diethyl ether, methanol and acetic acid (45:4:1) as reported by Tava *et al.* (1991).

Identification and determination of volatile metabolites.

The volatile components and their fractions were analyzed using a Pye Unicam PU 4550 gas chromatography system equipped with a flame ionization detector (220°C). The coiled glass column (1.5m×4mm) packed with Diatomite C (100-120 mesh) and coated with 10% PEGA was used with nitrogen as the carrier gas. The oven temperature was programmed at 4°C/min from 60°C to 180°C and was hold at 180°C for 15 min. The injector temperature was 300°C. Gas flow rates for N_2, H_2 and air were 30, 33 and 330 ml/min. respectively. Peak identification was performed by comparing the relative retention times of each peak with those of known compounds. (co-chromatogram) Also, the essential oils were mixed with their major components and injected into GLC to verify of the peaks identity. Peak areas were calculated using a PU 4810 Philibs computing integrator.

Gas chromatography-Mass spectrometry (GC/MS) of volatile metabolites.

The volatile components and their fractions were analyzed by GC/MS using Hewlet Packard capillary GC-quadrupole MS system (Model 5970) flitted with a 50m×32mm, i.d., fused silica column coated with carbowax 20m (0.32mm thickness) programmed as follows: 60-180°C (4C°min^{-1}) GS-MS analysis were made in splitles mode with helium as the carrier gas at a flow rate of l ml min^{-1}. The mass spectrometer was operated at 70 eV. and the identification of the compound was based on a comparison of retention time and mass spectra with those of authentic samples and with literature data. Fragmentation patterns were used to determine molecular structure when reference spectra were unavailable.

Phytotoxicity bioassay:

The growth inhibitory activity of the volatile components of *Chlorella vulgaris* and their fractions were evaluated on grains of barley by using Edney and Rizvi (1996) method. Barley grains (5g) were soaked in 25 ml of tested volatile components sample at different concentration levels (100,250,500 and 1000ppm) or their fractions (250 ppm) for 4 hours at 28°C±1, then the grains were transferred to Petri dishes containing two disks of filter paper moistened with 20 ml of respective solution and incubated at 28°C±1 for 48hr. Grains with prominent radical growth were considered to have germinated. The germinated grains (%) were calculated. Then they were

incubated for 5 days in dark chamber at 28°C±1. At the end of experiment the coleopitle lengths were measured to calculate the percentage of inhibition compared to those of the control. Also, the ∝-amylase activity of 4hr imbibed grains were determined following Marambe *et al.* (1992) method. The activity of crude extract of ∝-amylase was determined by spectrophotometric method using starch as substrate and I_2/KI solution as reagent. The developed color was measured at 620nm. Standards of starch were analyzed simultaneously and ∝-amylase activity of crude extract was expressed as ≒g of starch degraded (1ml of crude extract/30 min)at 30°C. In all cases, replicates were prepared and any sample showed 20% reduction or greater at (100-1000ppm) levels from those control were considered active (Steven and Merrill 1980).

RESULTS AND DISCUSSION

The volatile components of cultivated C*hlorella vulgaris* under outdoor conditions were obtained by steam distillation in a yield of 0.08% (v/w) with a fishy odor which were successively separated into their fractions by column chromatography. The volatile components and their fractions were analyzed by GC and GC-MS. The chemical compositions were identified by comparing their retention time and mass spectra with authentic reference compounds. Those without standard were tentatively identified by computer matching of their mass spectra with library mass spectra and the literature. The volatile metabolites of C*hlorella* were a complex mixture of about 105 compounds of which 30 compounds were identified (Table 1) and 75 compounds were not identified, representing 86.38% and 13.62% of the total volatile components respectively. The identified compounds were grouped as chemical class and their percentages are given in Table (1). The unidentified compounds were very low abundance and not listed. The C*hlorella vulgaris* volatile metabolites were composed of hydrocarbons, acids, alcohols, esters, and aldehydes as well as ketones, which presented 32.86%, 23.93%, 15.62%, 8.02%, 3.24% and 2.71% of the total volatile substances, respectively. Similar trend were observed in other microalgae, e.g., C*hlorella species* and S*cendesmus species*. where hydrocarbons and acidic compounds presented the major chemical classes as found by Juttner (1985) and Rzama *et al.* (1995).

In this study, the hydrocarbons make up the large part of volatile components collected from microalage *Chlorella vulgaris.* These compounds included saturated and unsaturated hydrocarbons, some of which contain one or two double bonds and a cyclic ring. The hexadecene was the major abundant (15.30%), as previously reported by Juttner (1992) and Rzama *et al*. (1995) in *Chlorella vulgaris* grown in Waste water, while B-pinene (7.63%) was the second most important compound. Other hydrocarbons such as dodecane (1.76%), heptadecene (2.11%) and octadecane (3.21%) were detected as minor compounds while heptadecane (0.74%) was determined as a trace constituent. These compounds in many microalage have been

reported as originating from decarboxylation of fatty acids, e.g., palmitic and stearic (Gelpi *et al.* 1970).

Table (1): Composition (%) of the green microalgae *Chlorella vulgaris* volatile metabolites.

Class	Compounds	%	Mode of I.D*
Hydrocarbons	α-pinene	2.11	a,b,c
	β-pinene	7.63	a,b,c
	Dodecane	1.76	c
	Hexadecene	15.30	a,b,c
	Heptadecane	0.74	c
	Heptadecene	2.11	c
	Octadecane	3.21	c
Acids	Dodecanoic	0.2	c
	Tetradecanoic	0.9	c
	Hexadecanoic	8.73	a,b,c
	Octadecanoic	2.2	a,b,c
	Octadec-9-enoic	0.73	c
	Octadec-9,12-dienoic	10.20	a,b,c
	Octadec-9,12,15-trienoic	0.97	c
Alcohols	Hexadecanol	10.80	a,b,c
	Octadecanol	2.30	a,b,c
	Nonadecanol	2.01	c
	Phytol	0.51	c
Esters	Methyl-hexadecanoate	2.20	a,b,c
	Methyl-octadecanoate	3.52	a,b,c
	Methyl-octadec-9-enoate	0.98	a,b,c
	Methyl-octadec-9,12-dienoate	1.32	a,b,c
Aldehydes	Hexanal	0.14	c
	Nonadecanal	0.90	c
	Hexadecanal	2.20	c
Ketones	Decanone	0.7	c
	Hexadecanone	1.70	c
	β-ionone	0.13	c
	α-ionone	0.18	c
Unidentified	----------	13.62	----------
Total		100	

***(a) Identified by co-chromatography with authentic sample, *(b) Identified by comparison of retention time and GC/MS data with those of authentic sample and *(c) Identified by comparison of GC/MS data with literature data.**

Seven fatty acids were identified in *Chlorella volatile* components, which were saturated and unsaturated monocarboxylic acid with chain lengths ranging from C_{12}-C_{18}. The saturated acids, $C_{12.0}$ (0.2%), $C_{14.0}$ (0.9%), $C_{16.0}$ (8.73%), $C_{18.0}$ (2.2%) and unsaturated acids $C_{18.1}$ (0.73%), $C_{18.2}$ (10.2%) and $C_{18.3}$ (0.97%) were detected in acid fraction of volatile *Chlorella* compounds. The most of acids were occurred in very low abundance and $C_{16.0}$ and $C_{18.2}$ were the most important one. These data are in agreement with the results obtained by Rzama et al. (1995).

The alcohols group makes up the third major part of the volatile components collected from *Chlorella* (15.62%). The most abundant constituent of alcohols was hexadecanol, (10.8%) of the total volatile components. Other minor compounds were nondecanol and octadecanol. The saturated aliphatic alcohols are the predominant compounds in alcoholic fraction, while alcoholic monoterpenes occurred in trace amounts. The monoterpene alcohols are probably formed as intermediates compounds in microalge cells during the formation of higher terpene compounds, e.g., carotenoids (Rzama *et al.* 1995).

Ester group were characterized by esterified of saturated, mono and di-unsaturated fatty acids. The methyl ester of $C_{16:0}$, $C_{18:0}$, $C_{18.1}$ and $C_{18.2}$ were present as minor compounds. $C_{18:0}$ was the predominant compound in this fraction..

Ketone and aldehyde groups were relatively small and among these compounds, hexadecanone and hexadecanal were found as prevalent compounds. These compounds may be originated from the degradation of unsaturated fatty acids, while ionone could be formed during degradation of nor-carotenoid compounds (Kajiwara *et al.* 1993). Also, Juttner and Holfacher (1985) suggested that the aldehyde and ketone compounds were drived from carotenoids by oxidative cleavage of double bonds in various positions.

Several investigators have examined the volatile metabolites of *Chlorella* species grown in natural environmental conditions, e.g., Gelpi *et al*. (1970), Juttner (1983) and Rzama *et al.* (1995). There were qualitative and quantitative difference between the obtained results in the present work and that of aforementioned authors and also between them. The differences may be due to environmental conditions (e.g. the light intensity, temperature, etc.), degree of freshness of algal material and the age of algae cells (Juttner 1983).

The influence of steam volatile compounds of *Chlorella vulgaris* on $\propto$-amylase activity, coleoptile growth ,as well as germination (%) of barley grains are shown in Table (2). The germination (%) of barley was significantly reduced with the increase of volatile components concentration. The total volatile compounds of *Chlorella* at low concentrations of 100 and 250 ppm reduced the germination (%) of barely grains to 90 ± 10 and $82\pm15\%$

respectively while at higher concentration (1000 ppm) the germination (%) was reduced to 55±12%.

In coleoptile growth trials, the volatile components of *Chlorella* and their fractions possessed phytotoxic activity against the development of coleoptile lengths of barley grains. The coleoptile and roots lengths of barley were reduced by 70% when exposure to total volatile compounds of C*hlorella* at 500 ppm. In addition, the coleoptile of barley grains were more thick when they were exposed to volatile components of *Chlorella* and quite distinct from the thin control.

The ∝-amylase activity of barley grains exposed to total volatile compounds of *Chlorella* was reached 52±8% and 92±7% relative to control. Therefore, α-amylase activity was in similar to the other two phytotoxic activities trials. Thus, the volatile constituents of *Chlorella* may inhibited the enzyme of germinated grains that are involved in production of free sugar (e.g. amylase) and/or amino acids (proteases) as reported by many researchers (Marambe *et al*. 1992, Edney and Rizvi 1996 and El-Baroty 1997). The acids and polar fractions (P I and P II) obtained from *Chlorella* volatile components using column chromatography showed phytotoxic activities on germination (%), coleoptile lengths of barley grains but these activities were less than of whole volatile metabolites. However, among the fractions which were examined, acid fraction was found to be most toxic. The phytotoxicity of *Chlorella* volatile compounds fractions could be arranged in the following order of phytotoxicity:

acid fraction > polar fraction (PI) > polar fraction (PII) > hydrocarbon fraction

Table (2): Effect of steam volatile metabolites and their fractions of green microalgae *Chlorella* vulgaris on germination (%), coleoptile elongation (%) and ∝-amylase activity (%) of barley grains.

Treatments	Concentrations	Relative germination (%)	Coleoptile	∝-amylase
Control	-------	100	100	100
Total volatile metabolites	100 ppm.	90 ±10	86 ± 6	92 ± 7
Total volatile metabolites	250 ppm.	82 ±15	79 ±11	84 ± 8
Total volatile metabolites	500 ppm.	63 ± 9	70 ± 8	60 ±10
Total volatile metabolites	1000 ppm.	55 ±12	63 ±10	52 ± 8
Acid fraction	250 ppm.	62 ±10	58 ± 7	55 ±12
Hydrocarbon fraction	250 ppm.	95 ± 4	98 ± 3	95 ± 9
Polar fraction (PI)	250 ppm.	70 ± 6	79 ± 7	62 ±10
Polar fraction (PII)	250 ppm.	76 ±10	80 ± 6	68 ± 9

(±) indicates standard deviation of the mean (S.D). The treatment showed 20% reduction or greater from those of control (100%) were considered active (Steven and Merrill 1980).

Similar results were obtained by El-Baroty and Abdel-Lattif (1997) who found that the whole components of the volatile oil of *Artemisia herba alba* had potent bioactivity than the individual compound and these might be due to the synergetic effect between the components. However, allelopathic action drived from algae plays an important role in marine ecology. For instance, Edney and Rizvi (1996) reported that the long chain fatty acids had phytotoxic activity against germination of sorghum at concentration levels of 10-100 ppm. Also, Ueda and Kato (1982) found that jasmonic and fasmonic methyl esters isolated from *Chlorella* and *Spirulina* cells have a regulatory activities on plant growth.

Finally, The potency of *Chlorella* volatile metabolites which are similar to an allelopthic action could make these volatile metabolites as a promising agent for emerging integrated pest management (IPM) strategies for wood control. In addition, the use of natural volatile components had become over welcoming in recent years than the synthetic herbicides from both environmental and health consideration (Bagchi et al. 1997). That the most of synthetic chemicals are more hazardous due to their long persistence, non-target toxicity, carcinogenic and mutagenic activities was reported by Duke et al. (1988). These volatile oil are characteristic by fat solubility, volatility, ephemeral nature, easily biodegradation and recognized as safe (Mishra and Dubey 1994).

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مكونات الجزء المتطاير للطحالب الخضراء الدقيقة *Chlorella vulgaris* وتأثيراتها المثبطة على نمو النبات

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****قسم النبات-المركز القومى للبحوث-الجيزة.**

تم دراسة مكونات الجزء المتطاير للطحالب الخضراء الدقيقة بعد فصله بالتقطير فى بخار الماء ثم تفريده من خلال عمود يحتوى على السيلكاجيل بإستعمال العديد من مخاليط المذيبات العضوية المختلفة. وتم إستعمال التحليل الكروماتوجرافى الغازى (GC) والتحليل الكروماتوجرافى الغازى المرتبط بمطياف الكتلة (GC/MS) للتعرف على مكونات الجزء المتطاير الكلى ومكونات الأجزاء الناتجة بعد التفريد. بالأضافة لدراسة تأثير المكونات المتطايرة الكلية وكذلك أجزائها بتركيزات مختلفة على إنبات ونمو السويقة ونشاط إنزيم الألفا أميليز لحبوب الشعير.

وقد أوضحت النتائج المتحصل عليها أن الزيت المتطاير هو مخلوط يحتوى على حوالى 105 مركب عضوى أمكن التعرف على 30 مركب عضوى منها تمثل 86.38 % من الزيت الكلى. حيث تنتمى

هذه المركبات إلى الهيدروكربونات والأحماض والكحولات والإسترات والألدهيدات والكيتونات والتى تمثل 32.86 % ، 23.93 % ، 15.62 % ، 8.02 % ، 3.24 % ، 2.71 % من الزيت المتطاير الكلى على التوالى. وقد إتضح أن الهيدروكربون (ك16 الغير مشبع) والحامض (ك9،12 ك18 الغير مشبع) والكحول (ك16) وإستر الميثايل (ك16) والألدهيد (ك16) والكيتون (ك16) هى المكونات الرئيسية فى المجموعات السابقة. كما إتضح أن الجزء الكلى المتطاير ذو تأثير مثبط قوى على إنبات ونمو السويقة ونشاط إنزيم الألفا أميليز لحبوب الشعير وأن المكونات الحامضية للزيت ذات أعلى تأثير تثبيطى إذا ماقورنت بالمكونات الأخرى. ولم يتضح تأثير مثبط واضح للهيدروكربونات.