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A POTENTIAL ALGAECIDE FROM THE PRUNING WASTES OF GRAPE (*VITIS VINIFERA*) - STEMS AND LEAVES

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Abstract

A series of water quality and ecological problems are caused by algal blooms, and natural allelochemicals are considered as the source of algaecides due to their friendly advantages to environment. It has been reported that abundance of secondary metabolites are found in grape plants, and the extracts from the plant exhibit antioxidant, antibacterial and antifungal capacities. In order to reveal the anti-algal activity of grape pruning wastes and promote their usage, we analyzed the composition of the extracts from grape leaves and stems, and determined their effects on *Chlamydomonas reinhardtii*. The compounds identified from leaf and stem extracts included alcohols, aldehydes, ketones, acids, esters, phenolics, furans and terpenoids. Among these compounds, phenolics, furans and terpenoids were the main components. All of leaf and stem extracts can inhibit *C. reinhardtii* cell growth and reduce chlorophyll content and maximal efficiency of photosystem II photochemistry (Fv/Fm), and the inhibitory effects were enhanced with the increase of the extract concentration. The methanol extracts from grape leaves (MEL) contained the most compounds and had the strongest inhibitory effects. The response index (RI) of cell multiplication, chlorophyll a content, chlorophyll b content and Fv/Fm was -0.84, -0.83, -0.85 and -0.96, respectively, after the cells were treated with 12 mg·mL^{-1} MEL for 24 h. It has been reported that phenolics and terpenoids exhibit a wide range of antibacterial, antifungal and anti-algal properties, and they might be the main active ingredients to inhibit *C. reinhardtii* growth. Therefore, grape pruning wastes have a potential use value as an algaecide.

Key words: algaecide, extract, grape, leaf, stem

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1. Introduction

Eutrophication is a natural process concerning all habitats, and becomes more serious with the increasing input of nutrients, mainly nitrogen (N) and phosphorus (P) (Bhakta et al., 2016; Cloern, 2001). Eutrophication promotes the excessive growth of green algae and cyanobacteria, and even blooms, which causes a series of water quality and ecological problems (Dodds et al., 2009; Qin, 2009). Abundant volatile organic compounds (VOCs) released from these algae frequently lead to an unpleasant, earthy–musty odor in the water, and the odor mainly results from geosmin and 2-methyl borneol (Fujise et al., 2010). These algae also produce lots of toxins such as microcystin, hepatotoxins, neurotoxins, neosaxitoxins and anatoxin-a (Codd, 2000; Frangópulos et al., 2004), which can inhibit the growth of other algae (Li and Li, 2012; Sanna et al., 2004), aquatic plants (Pflugmacher, 2002) and zooplankton (Abrantes et al., 2006), and even poison fishes (Guzmán-Guillén et al., 2013). Moreover, algal toxins are also a hazard to human health through the usage of water for drinking and recreation (Hoeger et al., 2007).

Because of the severe effects of algal blooms on ecosystems and human health, extensive researches have begun to concentrate on the control of undesired algal growth. Some promising techniques have been

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developed, including the usage of yellow loess (Choi et al., 1998), copper sulfate (Costas and Lopez-Rodas, 2006; Song and Wang, 2015), biquaternary ammonium salt (Liu et al., 2004), TiO_2 (Kim and Lee, 2005), phosphorus inactivation (Lürling and van Oosterhout, 2013), sediment capping (Huang et al., 2011), and also biomanipulation, e.g. viruses (Garry et al., 1998) and bacteria (Cai et al., 2011; Park et al., 1998). Those methods seem to be efficient in control of the algae in experiments and fields, but they may have high financial costs (Huang et al., 2011; Kim and Lee, 2005; Lürling and van Oosterhout, 2013) and the potential of disastrous environmental consequences (Jeong et al., 2000; Song and Wang, 2015).

It is important to develop a new generation of algaecides that will be effective, economically favourable and environmentally friendly. For this reason, natural allelochemicals from plants have been considered as the source of potential agents (Lupoae et al., 2016; Ni et al., 2012; Pęczuła, 2013; Zhou et al., 2008). It has been reported that the extracts from *Rhizoma coptidis* and *Semen arecae* can inhibit the growth of *Alexandrium tamarense* (Zhou et al., 2007), and garlic solution can inhibit the growth of *A. tamarense*, *A. satoanum*, *A. catenella* and *Scrippsiella trochoidea* (Zhou et al., 2008). The extracts from *Artemisia annua* (Ni et al., 2012) and *Iris wilsonii* (Chen et al., 2012) have remarkable inhibitory effects on the growth of *Microcystis aeruginosa*, and the anti-algal activity compound is artemisinin in *A. annua*, and phenolics and tannin in *I. wilsonii*. Although lots of plants are found to have the inhibitory effects on algae, plant wastes from agricultural production will be more economically favorable and environmentally friendly in the usage, such as barley straws (Grover et al., 2007; Pęczuła, 2013) and rice hulls (Chung et al., 2007).

Vitis vinifera L. (grape) is widely cultivated in the world, and needs a lot of pruning in the growing season with producing massive wastes of stems and leaves. These wastes are discarded mainly in open fields, which may cause environmental problems due mainly to their high content of secondary metabolites such as organic acids, phenolics, flavonols, tannins, procyanidins, anthocyanins, lipids, carotenoids and terpenoids (Delíorman Orhan et al., 2009; Doshi et al., 2006; Felicio et al., 2001; Xia et al., 2010). It has been reported that the extracts from grape leaves and stems exhibit antioxidant, antibacterial and antifungal capacities (Anastasiadi et al., 2012; Batovska et al., 2008; Cárcel et al., 2010; Kosar et al., 2007). When lettuce is treated by grape leaf extracts, its polyphenol oxidase activity is inhibited markedly (Altunkaya, 2012). When breast, colon, renal and thyroid cancer cells are exposed to grape stem extracts, their growth is inhibited, indicating that the extracts have anticarcinogenic activity (Sahpazidou et al., 2014).

Since grape extracts contain abundant secondary metabolites and inhibit microorganism and cancer cell growth, they may have inhibitory effects on algae. However, to the best of our knowledge, there is no report about that. In order to reveal the anti-algal activity of grape pruning stem and leaf wastes and promote the usage of the wastes, we analyzed the composition of their extracts, and determined the effects of the extracts on cell growth, chlorophyll content and maximal efficiency of photosystem II (PSII) photochemistry (Fv/Fm) of *Chlamydomonas reinhardtii*, a model organism for algae (Funes et al., 2007).

2. Material and methods

2.1. Cell cultures

The *C. reinhardtii* strain CC-125 wild type mt+ [137c] from Dr E.H. Harris (Duke University, Durham, NC, USA) was cultured in liquid TAP (Tris-acetate-phosphate) medium (Gorman and Levine, 1965), and kept at a light/dark (16 h/8 h) regime at a temperature of 23 °C, and an illumination of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Cell cultures in mid-logarithmic phase were used for experiment. The density of the cell cultures was determined by using the blood cell counting plate, with each value being the means of 6 repeats.

2.2. Preparation of grape extracts

The pruning wastes from *Vitis vinifera* L. cv. Muscat Hamburg were collected in August from the vineyard in Wuqing District (117°03′ N, 39°38′ E), Tianjin, China. The separated stems and leaves were dried using drying oven at 80 °C, and crushed with a mortar and pestle. The crushed materials of 5 g were extracted with 50 mL 50% methanol and distilled water, respectively, at 20 °C for 48 h. The extracting solution was filtered by 0.2 μm filter membrane, and then its concentration was 100 $mg \cdot mL^{-1}$.

50 mL methanol extracts was distilled to 5 mL at 64.5 °C (the boiling point of methanol) using rotary evaporator to remove the methanol. The distilled extracts were supplemented to 50 mL using distilled water to keep the extract concentration of 100 $mg \cdot mL^{-1}$. Then, the methanol extracts from grape leaves (MEL), water extracts from grape leaves (WEL), methanol extracts from grape stems (MES), and water extracts from grape stems (WES) were used to treat *C. reinhardtii* cells.

2.3. Treatments with grape extracts

Grape extracts of 0.25 mL, 1 mL, 2 mL and 3 mL were added into 22 mL TAP medium, and the medium was supplemented to 25 mL using distilled water to keep the extract concentration of 1 $mg \cdot mL^{-1}$, 4 $mg \cdot mL^{-1}$, 8 $mg \cdot mL^{-1}$ and 12 $mg \cdot mL^{-1}$, respectively. 22 mL TAP medium with addition of 3 mL distilled water was the control. Cell cultures in mid-logarithmic phase were centrifuged at 5 000 g for 5 min, and the cells were resuspended in these medium to give a cell density of 4×10^6 cells per mL. After 12 h and 24 h treatment, the cell density, chlorophyll content per 10^6 cells and Fv/Fm per 10^6 cells were determined.

2.4. Determination of chlorophyll content

C. reinhardtii cells were collected by centrifugation and the pellets were resuspended in 3 mL 80% acetone. After removal of insoluble materials in the pigment extracts by centrifugation, the content of chlorophyll was determined with Arnon's method (Arnon, 1949).

2.5. Measurement of photosynthetic efficiency

The Fv/Fm was determined according to our previous method (Zuo et al., 2012a) with portable fluorometer PAM-2100 (Walz, Germany). Cells from 1 mL cell suspensions were collected by centrifugation and resuspended in 10 μl of the same culture medium. The resuspended cells were pipetted on a piece of filter paper fixed in the clip of the Handy-Pea and incubated in darkness for 15 min.

2.6. Analysis of grape extracts

A volume of 10 mL grape extracts (methanol was removed from the methanol extracts by distillation for eliminating the influence of methanol) were extracted by 1 mL ethyl acetate, and analyzed by GC-MS. The GC (7890A, Agilent Technologies Company, Santa Clara, California, USA) was run with a 30 m×0.25 mm HP-5MS capillary column and a 0.25 μm film phase. The temperature of the column was programmed to increase from 50 °C to 180 °C at a rate of 20 °C·min^{-1} and kept for 4 min. Then it was increased to 220 °C at a rate of 10 °C·min^{-1} and kept for 15 min. The MS (5975C, Agilent Technologies Company, Santa Clara, California, USA) was run under the following conditions: EI mode of ionization energy at 70 eV and source temperature at 230 °C, mass range between m/z 28 and m/z 450, I/F at 250 °C and quadrupoles temperature at 150 °C. The qualitative and quantitative analyses of the GC/MS data were obtained from NIST/EPA/NIH Mass Spectral Library (NIST 08) (National Institute of Standards and Technology, Gaithersburg, USA). The peak area was used to indicate the content of the compounds.

2.7. Calculations and statistical analyses

Each experiment had three replications, each of which was repeated three times. Oneway ANOVA was used to compare the effects of treatments. Statistical analyses were performed using SPSS 12.0 (SPSS, Chicago, IL, USA), and the graphs were drawn with Origin 8.0 (Origin Lab, USA).

The response index (RI) was calculated using the Eq. (1) in Williamson and Richardson (1988):

$$RI = \begin{cases} 1 - C/T & \text{when } T \geq C \\ T/C - 1 & \text{when } T < C \end{cases} \quad (1)$$

where C is control response and T is treatment response. RI ranges from -1 to +1, with positive values indicating stimulation by the treatments and negative values indicating inhibition by them, relative to the controls.

3. Results

3.1. Composition of grape leaf and stem extracts

The following compounds were found in the methanol and water extracts from the GC/MS analysis: alcohols, aldehydes, ketones, acids, esters, phenolics, furans and terpenoids. There were 44 compounds in MEL, of which furfural (9.97×10^7), 5-methyl-2- furancarboxaldehyde (3.96×10^7), benzeneacetaldehyde (2.73×10^7), acetic acid butyl ester (3.00×10^7), 2-hydroxy-cyclohexanecarboxylic acid ethyl ester (2.01×10^7), 2-methoxy-phenol (7.13×10^7), 1,2-benzenediol (29.55×10^7), 2-methoxy-4-vinylphenol (25.01×10^7), 4,4'-(1-methylethylidene) bis (2-methyl-phenol) (3.42×10^7), 2-hydroxy-5-methylisophthalaldehyde (83.89×10^7), 3,4-dihydro-2H-pyran (2.15×10^7), furyl hydroxymethyl ketone (3.79×10^7), 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (3.65×10^7) and 2,3-dihydro-benzofuran (49.91×10^7) were the main compounds. WEL contained 22 compounds, including 5 aldehydes, 3 ketones, 1 acid, 2 esters, 4 phenolics, 2 furans and 5 terpenoids. Thirty three compounds were determined in MES, of which 3 alcohols, 6 aldehydes, 1 ketone, 1 acid, 1 ester, 5 phenolics, 5 furans and 11 terpenoids were found. Nineteen compounds were determined in WES, including 2 alcohols, 3 aldehydes, 2 acids, 1 ester, 5 phenolics, 1 furan and 5 terpenoids. Among these compounds, phenolics, furans and terpenoids were the main compounds in the 4 extracts (Table 1).

Table 1. The main compounds of grape stem and leaf extracts

Main compounds		***Peak area (×10^7)***			
		MEL	***WEL***	***MES***	***WES***
Alcohols	2-Hexyn-1-ol	0.24±0.03	—	—	—
	Benzyl alcohol	1.71±0.12	—	0.83±0.05	0.47±0.02
	Phenylethyl alcohol	—	—	0.39±0.03	0.61±0.03
	1-Methyl-4-(1-methylethyl)-3-cyclohexen-1-ol	—	—	0.68±0.04	—
Aldehydes	Furfural	9.97±0.53	0.79±0.04	43.65±2.37	1.07±0.04
	2-Hexenal	0.62±0.05	—	0.72±0.04	—
	5-Methyl-2-furancarboxaldehyde	3.96±0.72	0.35±0.03	7.26±0.38	0.28±0.02
	2,4-Heptadienal	—	—	1.25±0.11	—
	1H-Pyrrole-2-carboxaldehyde	1.60±0.11	0.61±0.03	—	0.86±0.04

	4-Heptenal	0.83±0.04	—	—	—
	Benzeneacetaldehyde	2.73±0.13	0.43±0.02	1.99±0.15	—
	cis-4-Decenal	0.73±0.09	—	—	—
	2,4-Dimethyl benzaldehyde	—	0.24±0.02	—	—
	5-Acetoxymethyl-2-furaldehyde	—	—	1.10±0.06	—
Ketones	2-Cyclopentene-1,4-dione	—	0.23±0.02	—	—
	2-Hydroxy-2-cyclopenten-1-one,	0.60±0.03	—	—	—
	1,2-Cyclopentanedione	—	0.93±0.05	—	—
	6-Methyl-5-Hepten-2-one	—	—	1.25±0.07	—
	2-Methyl-cyclohexanone	—	0.32±0.03	—	—
	2-Allylcyclohexanone	0.78±0.10	—	—	—
Acids	3-Methyl butanoic acid,	—	—	—	0.62±0.03
	2-Methyl hexanoic acid	—	0.40±0.02	—	0.52±0.03
	5-Hexenoic acid	0.77±0.06	—	—	—
	Dihydro-3-methylene-2,5-furandione	0.71±0.04	—	—	—
	8-Nonynoic acid	1.42±0.09	—	—	—
	Mesaconic acid	0.33±0.02	—	—	—
	3-Methyl-2-furoic acid	1.01±0.08	—	—	—
	3-(4-Methoxyphenyl)-beta-alanine	—	—	31.20±2.14	—
Esters	2-Oxo-propanoic acid methyl ester	0.32±0.02	—	—	—
	Acetic acid butyl ester	3.00±0.16	3.08±0.16	5.49±0.24	3.53±0.14
	2-Hydroxy-cyclohexanecarboxylic acid ethyl ester	2.01±0.13	—	—	—
	7,7-Dimethylbicyclo[2.2.1]hept-2-yl acetate	0.42±0.04	0.31±0.02	—	—
Phenolics	2-Methoxy-phenol	7.13±0.35	1.45±0.17	3.99±0.23	1.30±0.09
	2-Methoxy phenol	—	0.13±0.06	—	—
	1,2-Benzenediol	29.55±0.98	—	10.58±0.55	3.87±0.15
	2-Amino-4-methoxy-phenol	—	—	1.33±0.12	—
	2-Methoxy-4-vinylphenol	25.01±0.89	2.47±0.21	7.89±0.33	0.59±0.03
	2,6-Dimethoxy-phenol	—	—	—	0.48±.02
	4,4'-(1-Methylethylidene)bis(2-methyl-phenol)	3.42±0.14	2.20±0.18	4.08±0.21	4.43±0.17
	2-Hydroxy-5-methylisophthalaldehyde	83.89±1.73	—	—	—
Furans	3,4-Dihydro-2H-pyran	2.15±0.11	—	—	—
	1-(2-Furanyl)-ethanone	0.30±0.02	—	0.66±0.03	—
	2(5H)-Furanone	0.69±0.05	—	—	—
	3,4-Dihydro-2H-pyran	—	0.31±0.03	—	—
	3,4-Dimethyl-2,5-furandione	0.21±0.02	—	—	—
	2,5-Furandicarboxaldehyde	0.88±0.07	—	1.11±0.07	—
	Furyl hydroxymethyl ketone	3.79±0.16	—	2.12±0.11	—
	2-Furanacrolein	1.15±0.09	—	1.37±0.08	—
	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-	3.65±0.17	—	—	—
	2,3-Dihydro-benzofuran	49.91±3.87	16.56±1.14	47.71±2.16	10.66±0. 66
Terpenoids	1,4-Cineol	—	—	0.47±0.03	—
	2-Carene	0.36±0.04	—	—	—
	trans-3-Caren-2-ol	0.45±0.03	—	—	—
	Dihydrocarveol	—	—	0.52±0.02	—
	Limonene	1.54±0.55	0.31±0.02	—	—
	Eucalyptol	—	—	0.51±0.02	—
	cis-Myrtanol	0.39±0.02	—	0.52±0.03	0.32±0.02
	isocamphane	0.92±0.06	—	0.63±0.03	0.38±0.02
	cis-Linalyl oxide	0.43±0.03	—	1.30±0.08	—
	Piperitone oxide	—	0.32±0.02	—	—
	2-(Hydroxymethyl)norbornadiene	—	—	—	—
	Isopulegol	—	—	4.18±0.25	0.56±0.03
	Camphor	—	0.17±0.01	—	—
	Phellandral	0.26±0.02	—	—	—
	8-Camphenemethanol	0.41±0.02	—	—	—
	p-Menth-4(8)-en-9-ol	—	—	0.90±0.05	—
	Menthol	—	0.23±0.02	1.22±0.09	0.63±0.03
	Carveol	0.36±0.03	—	—	—
	Terpineol	0.46±0.04	0.17±0.02	1.15±0.08	—
	4-Caranol	—	—	0.62±0.03	—

MEL: Methanol extracts from grape leaves. WEL: Water extracts from grape leaves. MES: Methanol extracts from grape stems. WES: Water extracts from grape stems. —: No compound was found.

3.2. Effects of grape extracts on C. reinhardtii cell multiplication

C. reinhardtii cell multiplication was markedly inhibited by MEL at 1 mg·mL^{-1}, 4 mg·mL^{-1}, 8 mg·mL^{-1} and 12 mg·mL^{-1}, and the RI was -0.06, -0.08 (P < 0.05), -0.14 (P < 0.01) and -0.59 (P < 0.01), respectively, after 12 h treatment. After 24 h treatment, the inhibition was enhanced, and the RI was -0.09 (P < 0.05), -0.14 (P < 0.05), -0.23 (P < 0.01) and -0.84 (P < 0.01), respectively (Fig. 1A). After 48 h treatment, the cells were killed by the extracts at 12 mg·mL^{-1} (data not show). The cell density was also significantly reduced by WEL at 8 mg·mL^{-1} and 12 mg·mL^{-1}, with RI of -0.15 (P < 0.05) and -0.29 (P < 0.01), respectively, after 12 h, and -0.16 (P < 0.05) and -0.41 (P < 0.01), respectively, after 24 h (Fig. 1B).

When the cells were treated by the extracts from grape stems, the cell density was significantly reduced by MES at 8 mg·mL^{-1} (P < 0.05) and 12 mg·mL^{-1} (P < 0.01) (Fig. 1C), and by WES only at 12 mg·mL^{-1} (P < 0.05) (Fig. 1D).

3.3. Effects of grape extracts on chlorophyll content in C. reinhardtii cells

The content of chlorophyll a in *C. reinhardtii* cells was significantly decreased by MEL at 4 mg·mL^{-1} (P < 0.05), 8 mg·mL^{-1} (P < 0.01) and 12 mg·mL^{-1} (P < 0.01) (Fig. 2A). The similar results were also found in the content of chlorophyll b (Fig. 2B). WEL at 12 mg·mL^{-1} had the strongest inhibition on the content of chlorophyll a among the 4 concentrations, with the RI of -0.32 (P < 0.01) and -0.38 (P < 0.01), respectively, after 12 h and 24 h treatment (Fig. 2C). There was a similar effect on chlorophyll b in the treatment with WEL (Fig. 2D). Chlorophyll content was significantly reduced by MES at 8 mg·mL^{-1} (P < 0.05) and 12 mg·mL^{-1} (P < 0.01) (Fig. 2 E, F), and by WES only at 12 mg·mL^{-1} (P < 0.05) (Fig. 2G, H).

3.4. Effects of grape extracts on Fv/Fm in C. reinhardtii cells

When C. reinhardtii cells were treated with MEL at 4 mg•mL^{-1}, 8 mg•mL^{-1} and 12 mg•mL^{-1}, the Fv/Fm was significantly reduced, and the RI was -0.18 (P < 0.01), -0.29 (P < 0.01) and -0.88 (P < 0.01), respectively, after 12 h treatment. After 24 h treatment, the inhibition was enhanced, with the RI of -0.21 (P < 0.01), -0.34 (P < 0.01) and -0.96 (P < 0.01), respectively (Fig. 3A). Fv/Fm was significantly inhibited by WEL and MES at 8 mg•mL^{-1} (P < 0.05) and 12 mg•mL^{-1} (P < 0.01) (Fig. 3B, C), and it was significantly inhibited by WES only at 12 mg•mL^{-1} (P < 0.05) (Fig. 3D).

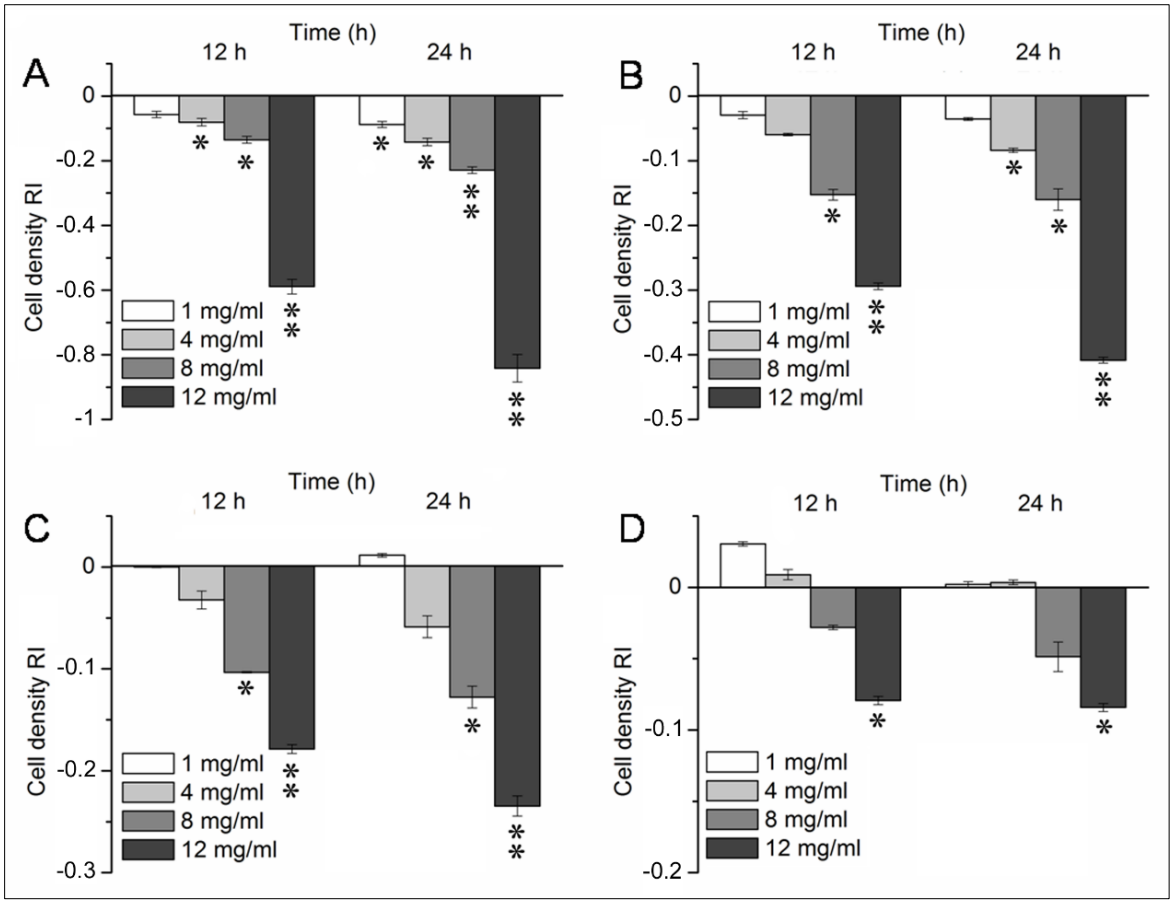

Fig. 1. Effects of extracts from grape leaves and stems on *C. reinhardtii* cell multiplication. A: MEL, B: WEL, C: MES, D: WES, *: Significant difference at P < 0.05 level; **: Significant difference at P < 0.01 level

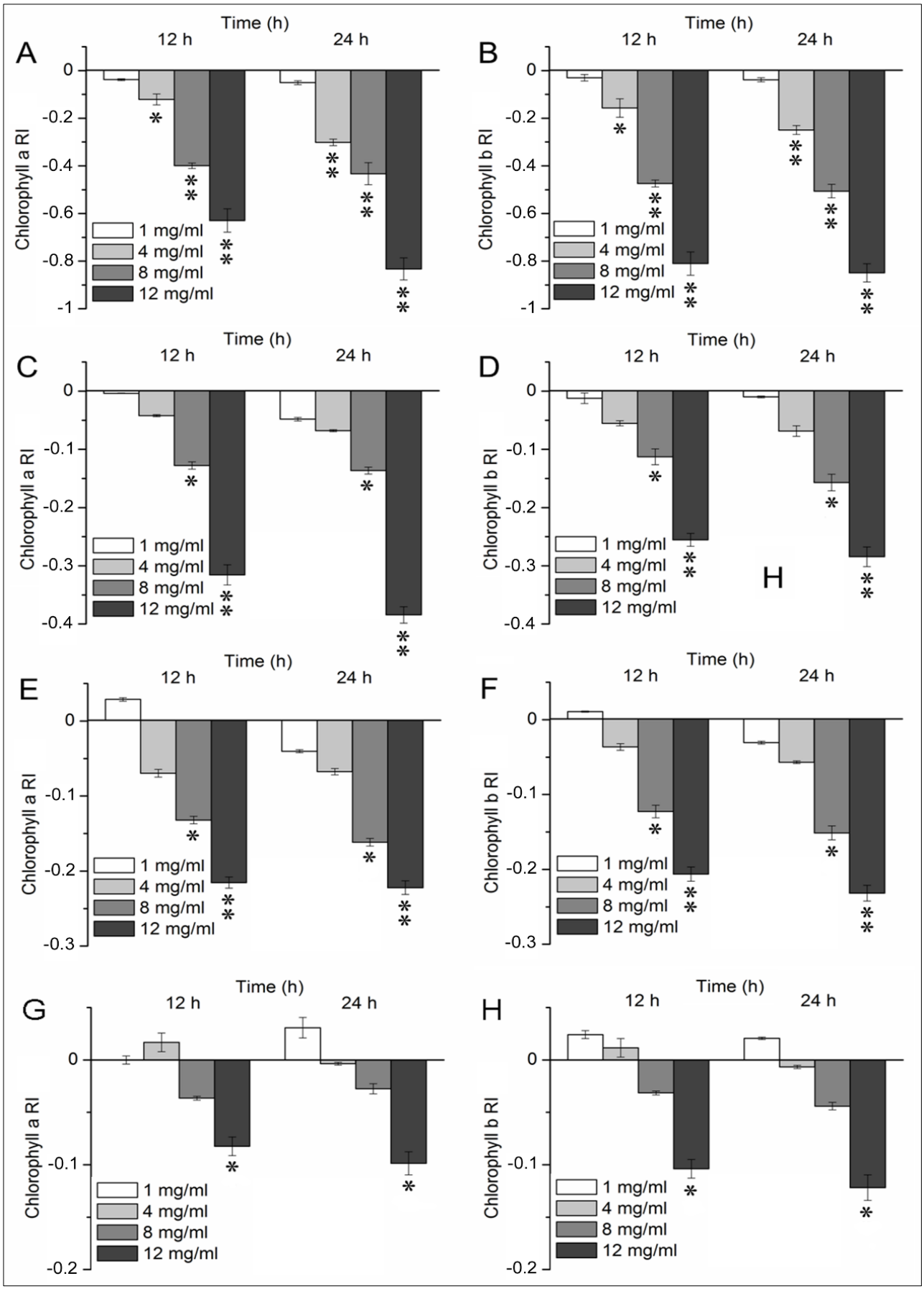

Fig. 2. Effects of extracts from grape leaves and stems on chlorophyll content in *C. reinhardtii* cells. A and B: MEL, C and D: WEL, E and F: MES, G and H: WES. A, C, E and G: Chlorophyll a. B, D, F and H: Chlorophyll b. *: Significant difference at $P < 0.05$ level; **: Significant difference at $P < 0.01$ level

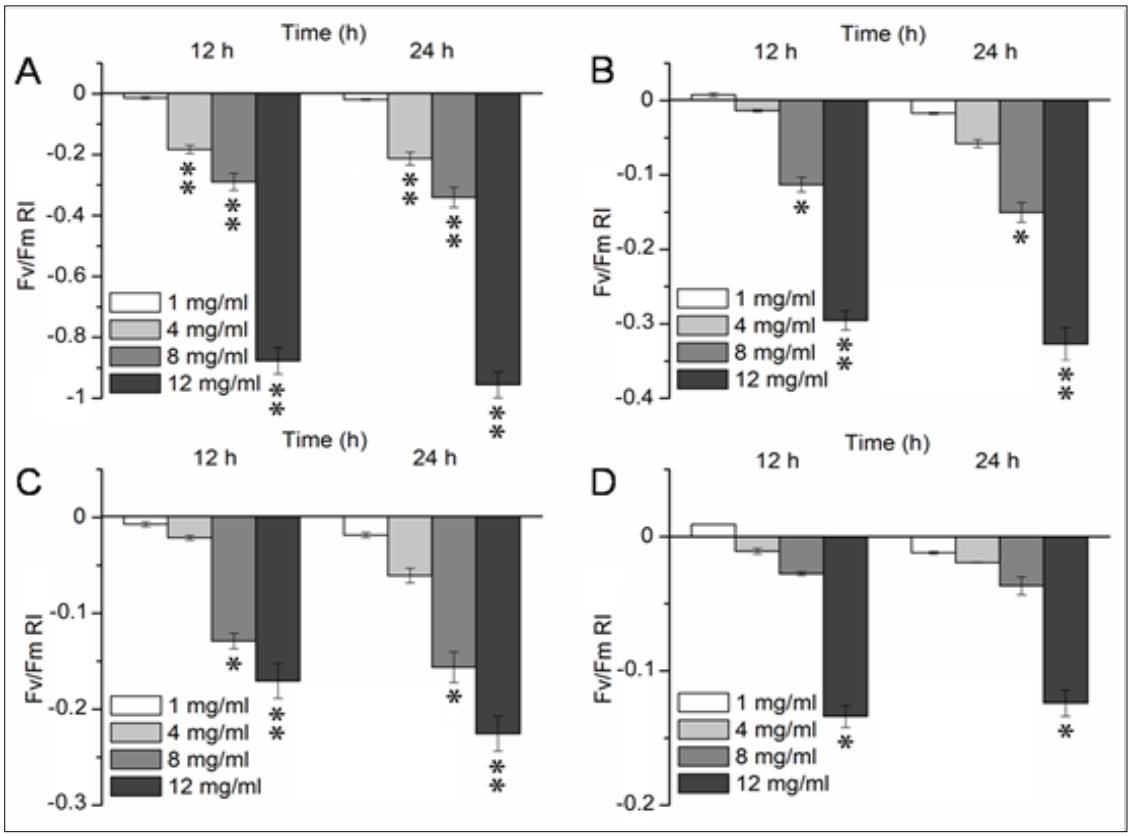

Fig. 3. Effects of extracts from grape leaves and stems on Fv/Fm in *C. reinhardtii* cells. A: MEL, B: WEL, C: MES, D: WES. *: Significant difference at $P < 0.05$ level; **: Significant difference at $P < 0.01$ level

4. Discussions

It has been reported that abundance of secondary metabolites such as organic acids, phenolics, flavonols, tannins, procyanidins, anthocyanins, lipids, carotenoids and terpenoids are found in grape leaves (Delíorman Orhan et al., 2009; Doshi et al., 2006; Felicio et al., 2001; Xia et al., 2010). Batovska et al. (2008) identified 16 secondary metabolites from grape leaves, and the major compounds were phenolics and terpenoids. Fernandesa et al. (2013) found numbers of phenolics from WEL, and the major compounds were trans-caffeoyltartaric acids, trans-coumaroyltartaric acids, myricetin-3-O-glucoside, quercetin-3-O-glucoside, quercetin-3-O-galactoside and kaempferol-3-O-glucoside. Anastasiadi et al. (2012) analyzed the content of 14 phenolics in grape stems, and found that flavonoids and stilbenes were the richest compounds. In addition, lots of phenolics are found in grape seeds, stalks and even pomace extracts (Guendez et al., 2005; Sagdic et al., 2011; Sahpazidou et al., 2014; Silván et al., 2013).

In this study, we also found lots of secondary metabolites from grape leaves and stems, including alcohols, aldehydes, ketones, acids, esters, phenolics, furans and terpenoids. Among them, phenolics were the primary type of compounds. Compared to grape stems, leaves contained more compounds, and the compounds extracted by methanol were more numerous than those extracted by water (Table 1).

Phenolics are initially identified for their antioxidant property, but many of them exhibit a wide range of biological activities such as antioxidant, antibacterial, antifungal, antiviral and therapeutic properties (Perron and Brumaghim, 2009). The extracts from grape seeds, stems and leaves contain lots of phenolics, so it is not surprised that these extracts can inhibit bacteria and fungi growth and scavenge reactive oxygen species, and their effects were strengthened with the increase of the extract concentration (Guendez et al., 2005; Sagdic et al., 2011; Silván et al., 2013). In this study, we found that the extracts from grape leaves and stems can inhibit algal growth, chlorophyll synthesis and photosynthesis (Figs. 1-3).

When cynaobacteria and eucaryotic algae were exposed to barley straw leachate, their activities were reduced remarkably, which was explained by the presence of toxicologically relevant levels of phenolics and oxidised phenolics (Everall and Lees, 1997). Nakai et al. (2000) reported that *M. aeruginosa* growth can be inhibited by four polyphenolics such as ellagic acids, gallic acids, pyrogallic acids and (+)-catechin from *Myriophyllum spicatum*, and gallic acids and pyrogallic acids had stronger inhibitory effect. When *Microcystis aeruginosa* was treated by *Iris wilsonii* fresh leaf extracts, the algal growth was inhibited markedly, and the cell density was negatively correlated with total phenolics and tannin levels in the leaves (Chen et al. 2012). Phenolics were the abundant compounds among all identified

chemicals in grape extracts (Table 1), so they might be the active ingredients to inhibit *C. reinhardtii* cell growth.

Many studies revealed the anti-algal activity of terpenoids. Ni et al. (2011) found that the extracts from *Artemisia annua*, *Conyza canadensis* and *Erigeron annuus* can inhibit *M. aeruginosa* growth, and terpenoids may be the main anti-algal active ingredients. When *M. aeruginosa* was exposed to artemisinin, a terpenoid identified from *A. annua*, its soluble protein content was decreased obviously, and SOD activity and ABA content were increased remarkably (Ni et al., 2012). In our previous studies, we found that abundant VOCs (including lots of terpenoids) released from *C. reinhardtii* cells under salt and acid stresses can affect other normal *C. reinhardtii* cell growth (Zuo et al., 2012a, 2012b). Lots of terpenoids were found in the extracts from grape leaves and stems (Table 1), which might also play an important role in the inhibition to *C. reinhardtii* cell growth.

Photosynthesis is the most fundamental biological process supporting algal growth and nutrient uptake, which can be influenced by phenolics and terpenoids due to the inhibition on chlorophyll content, quantum yield of PSII photochemistry and the accumulation of photosynthetic products (Laue et al., 2014; Liu et al., 2013; Ni et al., 2012; Yang et al., 2012; Zhang et al., 2013; Zuo et al., 2012a, 2012b). Meanwhile, those compounds can also induce the production of reactive oxygen species which cause oxidative stress and damage to algal cells (Bačkor et al., 2010, 2013; Yang et al., 2012; Zuo et al., 2012a, 2012b). The extracts from grape leaves and stems decreased the content of chlorophyll a and b (Fig. 2), and inhibited Fv/Fm (Fig. 3), which led to the reduction in photosynthesis in *C. reinhardtii* and inhibition on cell growth (Fig. 1).

C. reinhardtii is a model organism for algae with fast growth rate (Funes et al., 2007), and its family members are widely found in fresh water, damp soil, sea and sometimes even snow. The inhibitory effects of grape leaf and stem extracts on the alga indicated that they can inhibit other algal growth. However, the accurate inhibition mechanism on prokaryotic algae and practical application dose still need further study.

When *Spirogyra porticalis* (Muell.) Cleve is extracted using n-hexane, acetonitrile, methanol and water, the methanol extracts show the richest phenolic attributes and highest antioxidant capacity (Kumar et al., 2015). Methanol extracts from *Spondias mombin* leaves have highest antibacterial properties comparing to the acetone, ethanol and water extracts, due to the high content of chemicals including saponins, tannins, cardiac glycoside, terpenoids and flavonoids (Aromolaran and Badejo, 2014). Similarly, compared to water, methanol extracted more chemicals from grape (Table 1), which might result in the high inhibitory effects on *C. reinhardtii* cell growth, chlorophyll content and Fv/Fm (Figs. 1-3).

Abundance of secondary metabolites, especially phenolics, have been found in whole grape tissues, including leaves, stems, roots, seeds and skins (Anastasiadi et al., 2012; Delíorman Orhan et al., 2009; Duba et al., 2015; Eftekhari et al., 2012). Duba et al. (2015) reported that there was higher total phenolic content in grape seeds than skins. Compared to grape root extracts, higher total phenolic content is found in the leaf extracts (Eftekhari et al., 2012). In this study, more compounds were found in the grape leaf extracts in comparison with stem extracts, indicating that grape leaves might contain more compounds (Table 1), which might be the reason for leaf extracts exhibiting stronger inhibitory effects on *C. reinhardtii* cells.

5. Conclusions

In grape growing season, pruning produces massive wastes of stems and leaves which are discarded mainly in open fields and may cause environmental problems. The extracts from these wastes can inhibit the cell growth, chlorophyll content and Fv/Fm of *C. reinhardtii* cells, indicating that grape pruning wastes have a potential use value as an algaecide.

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