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Contribution

Nahid Awad and Samuel Vega-Estévez undertook the practical work on the growth of algae treated with phenolics, assays on MDA and fatty acid profiling. Gareth Griffiths undertook the lipoxygenase inhibitor work and was responsible for the overall direction the research.

Salicylic acid and aspirin stimulate growth of *Chlamydomonas* and inhibit lipoxygenase and chloroplast desaturase pathways

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Running title: Stimulation of algal growth by salicylic acid and aspirin

Highlight

Salicylic acid and aspirin stimulated algal cell proliferation while inhibiting lipoxygenase and chloroplast desaturases leading to an increase in biomass for enhanced bio-energy/product applications.

Abstract

Chemical stimulants, used to enhance biomass yield, are highly desirable for the commercialisation of algal products for a wide range of applications in the food, pharma and biofuels sectors. In the present study, phenolic compounds, varying in substituents and positional isomers on the arene ring have been evaluated to determine structure-activity relationship and growth. The phenols, catechol, 4-methylcatechol and 2, 4-dimethyl phenol were generally inhibitory to growth as were the compounds containing an aldehyde function. By contrast, the phenolic acids, salicylic acid, aspirin and 4-hydroxybenzoate markedly stimulated cell proliferation enhancing cell numbers by 20-45% at mid-log phase. The order of growth stimulation was ortho > para > meta with respect to the position of the OH group. Both SA and aspirin reduced 16:3 in chloroplast galactolipids. In addition, both compounds inhibited lipoxygenase activity and lowered the levels of lipid hydroperoxides and malondialdehydes in the cells. The present study has demonstrated the possibility of using SA or aspirin to promote algal growth through the manipulation of lipid metabolising enzymes.

Keywords: Chlamydomonas reinhardtii, Phenolic compounds, Salicylic acid, Cell division, Lipoxygenase, Polyunsaturated fatty acids.

Abbreviations: Malondialdehyde (MDA), Thiobarbituric acid reactive substances (TBARS), fatty acid methyl esters (FAMES), Lipoxygenase (LOX),3-hydroxybenzoic acid (3-HBA),4-hydroxybenzoic acid (4-HBA), salicylic acid (SA), 4-methyl catechol (4-MC), 2,4dimethyl phenol (2,4-DMP).

Introduction

Algal growth is controlled by a number of key environmental factors that include light intensity, nutrient availability, temperature and pH (Chapra *et al.*, 2017; Endo *et al.*, 2017; Mondal *et al.*, 2017). Enhancing and optimising algal biomass production in a cost-effective manner is a key commercial incentive for the development of the emerging algal technologies for nutraceutical, pharmaceutical and biofuel applications (Ambati *et al.*, 2014; Mondal *et al.*, 2017; Scranton *et al.*, 2015). One of the promising approaches to enhance algal biomass involves applying chemical triggers or enhancers to improve cell growth and accumulation of high value bio-products (Shah *et al.* 2016). One such group of compounds are the phenolics which are among the most ubiquitous group of secondary plant metabolites produced in the shikimic acid and pentose phosphate pathways through phenylpropanoid metabolism (Mhlongo *et al.*, 2016) and that play key roles in lignin biosynthesis, pigment biosynthesis and plant defense mechanisms in higher plants (Kumar *et al.*, 2016; Shahidi and Ambigaipalan, 2015). A wide range of phenolics have also been identified in algae (Onofrejova *et al.*, 2010) although their roles are less well established than those in terrestrial higher plants. For example, in the red alga, *Ganiorrichum alsidii,* (Fries, 1974) reported a strong growth stimulation *with p*-hydroxybenzaldehyde, vanillin, syringaldehyde, and veratraldehyde while the corresponding acids gave a lower response. The study also reported that 3, 4-dihydroxy benzoic acid, 3-methyl-catechol, 4-methylcatechol and *p*-hydroxyphenyl pyruvic acid also stimulated growth. Further studies revealed that *p*-hydroxyphenylacetic acid and phenylacetic acid also enhanced the growth of the red alga *Porphyra tenera* and suggested auxin-like effects as observed in higher plants. In the green alga, *Pseudokirchneriella subcapitata*, low concentrations of 4-hydroxybenzoic acid (4-HBA) stimulated growth whereas other positional analogues such as 2-HBA and 3-HBA did not (Kamaya *et al.*2006). Probably the most widely studied phenolic is salicylic acid (SA) which functions as a plant hormone regulating many physiological properties in higher plants, particularly in response to pathogen attack. Interestingly, this signalling molecule is also present in microalgae suggesting a long evolutionary conservation for signal transduction pathways. In algae, Czerpak *et al.* (2002) reported that incubation of *Chlorella vulgaris* in media containing SA (10^{-4} M)

resulted in a 40 % increase in cell numbers. SA was also found to enhance the production of the valuable carotenoid, astaxanthin, in the green alga *Haematococcus pluvialis* (Gao *et al*. 2012) while (Raman and Ravi, 2011) showed that at high concentrations, SA increased superoxide dismutase activity and ascorbate peroxidase suggesting roles in free radical scavenging. SA has also been shown to stimulate lipid accumulation almost two-fold in *Chlorella vulgaris* (Wu *et al.*2018) while Lee *et al.* (2016) reported that SA plays a role in the down regulation of the unsaturated fatty acid in *C. reinhardtii* and significantly reduced both linoleic and linolenic acids.

In order to shed further light on how phenolics manipulate cell growth in algae, we have undertaken a systematic study of the effects of a range of phenolic derivatives substituted at various positions on the arene ring in order to investigate if there is a structure-activity relationship between growth and type of substitution. Since many phenolics exhibit antioxidant activity we have determined the changes in lipid peroxidation and lipoxygenase activity following treatment with these compounds as this has received relatively little attention to do date. Since high rates of lipid peroxidation have been associated with higher rates of cell division in cancer cells (Hoque *et al.*, 2005) we would anticipate that lowering peroxidation by addition of an antioxidant would slow down cell division. To facilitate this study we have used the model species *Chlamydomonas reinhardtii* and in particular the cell wall mutant strain CC-406 cw 15 mt$^-$. In the wild type, the cell walls are multilayered and composed of a hydroxyproline-rich glycoprotein framework with mannose, galactose and arabinose as the main carbohydrates. Other components include uronic acids, glucosamine and proteins (Blumreisinger *et al.*, 1983; Popper *et al.*, 2011). This mutant is described as cell wall deficient based on the minimal amounts of cell walls produced, the incorrect formation of proteins and extracellular cross linking (Harris, 2001). The principal advantage of using this mutant is the relative ease with which exogenous compounds can be readily taken up, as recently demonstrated by us (Tassoni *et al.*, 2018). This study aims to reveal the role of lipid oxidation during the phase of rapid cell division and to identify potential chemical triggers for optimizing enhanced growth of microalgae.

Materials and methods

Algal growth

C. reinhardtii cultures of the cell-wall mutant [CC-406 cw 15 mt$^-$] were maintained in Tris-acetate-phosphate (TAP) agar medium incubated in a plant growth chamber at 25 ºC under 12 h of light and 12 h of dark per day. Liquid cultures incubated in an Innova 44 orbital shaker incubator with continuous rotary agitation at 200 rpm at 25 °C under continuous cool white 12V, 10 Watt halogen lighting. At 24h intervals, aliquots were removed from the culture wells and cell number was determined, daily up to 144 h in triplicate, using a Neubauer improved bright line haemocytometer. Phenolic compounds were dissolved in ethanol and added to the growth medium at a final concentration of 1% ethanol in the medium with appropriate controls containing the same final concentration of ethanol.

Two kinetic parameters were evaluated, growth rate (μ, hours^{-1}) and duplication time (dt, hours). These parameters were calculated according to:

$$\mu = \frac{\ln(N) - \ln(N_0)}{\Delta t}$$

$$dt = \frac{\ln(2)}{\mu}$$

Where Δt is the difference of time between the two measurements, N is the number of cells per ml at the end of the interval, N_0 is the number of cells at the beginning of the interval.

Toxicity assays (IC_{50} determination)

To overcome the varying solubility of the compounds in aqueous solutions all compounds were dissolved in ethanol and then diluted down in the growth medium to a final concentration of 1% ethanol and screened at increasing concentrations from 10μM

up to 5mM. The half-maximal inhibitory concentration (IC_{50}) was calculated according to recommendations for acute toxicology analysis by the US Environmental Protection Agency (EPA) (EPA, 2002) by the graphical method, representing the percentage of growth inhibition versus the logarithmic concentration of the compound.

Extraction and purification of lipids

Lipids were extracted according to a modified Bligh/Dyer method (Kebelmann *et al.*, 2013). 0.2 g of the wet weight of *Chlamydomonas reinhardtii* cell wall mutant (CC-406 cw 15 mt^-) was transferred into a Pyrex tube and acidified with 1 ml of 0.15 M acetic acid and homogenised with chloroform/methanol (1:2 v/v; 7.5 ml) for approximately 2 mins. 2.25 ml chloroform was combined with the extract to which was added distilled water (2.25 ml). The lower chloroform ($CHCl_3$) phase containing the lipids was removed into a Pyrex tube and evaporated to dryness under N_2. Samples were re-suspended in 500 µl chloroform. Lipids were separated and purified by thin-layer chromatography (TLC) using silica gel 60 (Merck). Two different separation systems were used depending on the polarity of the separated lipids. Non-polar lipids were separated using hexane /diethyl ether/ acetic acid (70:30:1, by vol.) and the polar lipids were separated using chloroform/methanol/acetic acid/water (85:15:10:3, by vol.). Lipids were generally located on TLC plates by lightly staining with iodine vapour and the presence of phospholipids confirmed by spraying the TLC plates with Dittmer's reagent which stains the phospholipids with a blue colour and was used to distinguish from glycolipids in complex chromatograms.

Lipid derivatisation and Gas chromatography/Mass spectrometry (GC-MS) analyses.

Fatty acid methyl esters (FAMES) were prepared by refluxing the purified lipids *in situ* on the silica gel after TLC separation in 2.5% H_2SO_4 in dry methanol at 70°C for 90 min containing heptadecanoic acid as an internal standard. The FAMES were extracted using hexane partitioned against water. The FAMES were concentrated by evaporation

under N_2 and the composition analysed using an Agilent GC-MS (6890) on a DB-23 capillary column (length 30 m, id 0.25 mm, film thickness 0.25 µm). The GC injector was operated in split mode (50:1) with an inlet temperature of 250 ºC. The column temperature was held at 50 ºC for 1 minute, and then increased at 25 ºC/min to 175 ºC and followed by a second ramp with heating of 4 ºC/min up to 235 ºC maintained for 15 minutes. Assignment of FAME was made by using a NIST08 MS library.

Determination of lipid hydroperoxides

Aliquots of the lipid chloroform phase were dried under N_2 and re-suspended in HPLC grade methanol in the presence or absence of triphenylphosphine (TPP, 25 mM in methanol). Samples were incubated in the dark for 30 min and for a further 30 min following the addition of working FOX2 reagent. The absorbance was determined spectrophotometrically at 560 nm and the concentration of lipid hydroperoxides determined using a molar absorption coefficient derived from standard linoleate hydroperoxide (Griffiths *et al.*, 2000).

Thiobarbituric acid-reactive substances (TBARS) essay

The TBARS assay was used to estimate the amount of malondialdehydes (MDAs), a secondary end-product of polyunsaturated fatty acid oxidation as an index of general lipid peroxidation. The content of MDA was determined at intervals following the incubation with selected phenolic compounds. A stock of two reagents were prepared; Thiobarbituric acid (TBA) reagent [0.6 % (w/v) TBA was dissolved in 0.05 M sodium hydroxide (NaOH)] and the acid buffer with a pH 3.7 [90 ml of 0.2 M acetic acid was mixed with 0.2 M sodium acetate trihydrate]. For each TBARS assay, 0.2 g of *C. reinhardtii* cell wall mutant (CC-406) was homogenised with 0.5 ml trichloroacetic acid (TCA) (10 % w/v). The homogenate was washed twice with 2.5 ml acetone and centrifuged (4000 g. 10 min).The pellet was incubated at 100 ºC for 30 min with 1.5 ml of (1 %) phosphoric acid (H_3PO_3) and 0.5 ml of (0.6 %) thiobarbituric acid (TBA), then cooled on ice. 1.5 ml n-butanol was added and the mixture was agitated and centrifuged

(4000 g. 10 min). The absorbance of the coloured supernatant was measured at 532 nm and corrected for unspecific turbidity by subtracting the value of absorbance at 590 nm (Cherif *et al.*, 1996). The concentration of the MDA was measured using a standard calibration curve.

Lipoxygenase assays

Chlamydomonas cells were recovered by low speed centrifugation and homogenised in ice-cold potassium phosphate buffer (KPi, 50mM), pH7.0 containing polyvinylpyrolidone PVP (1%), Triton x-100 (0.1%) and sodium metabisulphite (0.04%). Samples were centrifuged at 10,000 x g in Eppendorf tubes and the supernatant was passed down a PD10 column equilibrated with 50mM KPi buffer and assays performed immediately on the eluate. Lipoxygenase assays were performed by monitoring the formation of conjugated dienes at 234 nm at 25° C using 30 µg of cell extract protein suspended in either Tris-HCL buffer or KPi buffer with fatty acid substrates (50nmol) dissolved in ethanol (1%) final concentration. Cell free extracts were stored at -80° C under N_2 in buffer containing 20% glycerol. Addition of glycerol maintained the LOX activity to >90% after defrosting whereas in the absence of glycerol the activity was typically < 25%. LOX inhibitors were dissolved in ethanol with 0.25% dimethyl sulphoxide (DMSO).

Protein assays

Protein assays were performed using the Pierce™ BCA Protein assay kit in triplicate.

Statistical analyses of the results

The results obtained are represented as the mean ± standard deviation (SD) of triplicate samples in all the experiments. The statistical analyses of the results were carried out using SPSS (IBM, Armonk, NY). ANOVA tests were used to compare the statistical significance of the differences and equalities between the treatments compared to the controls. Tukey's test was used to compare pairs of mean when ANOVA identified significant differences. The student‘s t-test using SPSS (IBM, Armonk, NY) was used to

compare the statistical significance of the differences and equalities between lipid peroxidation levels between the different treatments. In all analyses, a 95% confidence interval was used, and the results were considered significant at $p<0.05$.

Results and discussion

Half-maximal inhibitory concentration (IC_{50}) of the tested phenolic compounds

In order to systematically evaluate the influence of phenolic compounds on the growth of *C.reinhardtii* a range of compounds were selected with varying substituents and positional isomers on the benzene ring (Fig.1). These can be conveniently classified into 3 groups.

Group 1 included the phenol derivatives, the parent compound of the group, phenol (hydroxybenzene), catechol (1, 2-dihydroxybenzene) and the methylated substituted forms 4-methyl catechol (4-MC) and 2, 4-dimethyl phenol (2, 4-DMC).

Group 2 contained the carboxylic acid group (-COOH). We tested the parent compound of the group, benzoic acid (BA) and it's hydroxylated isomeric derivatives, 2-hydroxybenzoic acid (salicylic acid, SA), 3-hydroxybenzoic acid (3-HBA) and 4-hydroxybenzoic acid (4-HBA). A derivative of SA, aspirin, (acetylsalicylic acid or 2-acetoxybenzoic acid) was also tested to determine the effect of acetylation of the –OH group. The effects of multiple hydroxyl substitutions on the benzene ring were investigated using gallic acid (3, 4, 5-trihydroxybenzoic acid).

Group 3, for further comparative studies, we included the aldehyde function of salicylaldehyde (2-hydroxybenzaldehyde) for direct comparison with 2-hydroxybenzoic acid) and vanillin (4-hydroxy-3-methoxybenzaldehyde) in which the OH group is methylated.

To estimate the level of inhibition of the phenolic compounds (Fig 2), the half-maximal inhibitory concentration (IC_{50}) was calculated. Three broad categories of response can be observed. In category 1, salicylaldehyde (group 3) was the most toxic phenolic compound to *C. reinhardtii* with strong inhibition also observed for the group 1

phenolics, catechol, 4-MC and 2, 4-DMP. The exception was the parent, non-substituted compound, phenol. Intermediate toxicity was observed for the poly-ol, gallic acid (GA) (group 2) and the aldehyde, vanillin (group 3) while there was generally low toxicity for members of the group 2 series (containing a carboxylic acid group) from benzoic acid (BA) through SA, 3-HBA and 4-HBA, with the latter being the least toxic compound tested.

Effect of phenolic compounds on growth.

Algal cultures were incubated with the compounds given in Fig 1 at concentrations ranging from 10µM up to 5mM and cell numbers were determined at regular intervals.

Group 1 compounds

Phenol represents the parent compound and showed significant stimulatory effect on algal growth at 100 µM and 1mM (Fig. 3a and Tables 1). In contrast, catechol, 4-MC and 2, 4-DMP failed to stimulate culture growth and were highly inhibitory to the cultures at 100 µM (77%, 60% and 62% respectively, data not shown). Thus the presence of an additional OH and /or methyl group to the ring has negative consequences for Chlamydomonas cultures. By contrast (Megharaj *et al.*, 1986) found that catechol concentration up to 1.0 kg ha^{-1} led to significant initial enhancement of the large scale production of algae. These contradicting findings suggest that phenolic compounds vary in their effect on the various algal species and this may reflect uptake or metabolic turnover differences of these compounds.

It has been reported that bacteria and some algal species are able to degrade phenol and to utilise it as the main source of carbon for growth e.g. the golden brown alga, *Ochromonas danica*, was able to utilise [U-^{14}C] phenol completely as a result of inducing the enzymes involved in phenol catabolism (Semple and Cain, 1996). However, although this might be the reason behind the stimulatory effect of phenol reported in this study, catechol which is one of the products of phenol catabolism was not utilised by *C. reinhardtii* showing no stimulatory effect; instead, catechol was inhibitory to the growth. This suggests that while algae are able to utilise catechol as

part of phenol metabolism; catechol added exogenously to *C. reinhardtii* cultures was not readily utilised and was instead toxic to this alga. In addition, the decomposition of aromatic compounds is comparatively slower because of the high energy required for the cleavage of the benzene ring and therefore, this is unlikely to be the reason behind the stimulatory effect of all the phenolic compounds tested here.

Group 2 compounds

Benzoic acid represents the parent compound of the tested phenolic acids but had no significant effect on the growth of cultures incubated in up to 1mM. However, higher concentrations (2 mM) were significantly inhibitory (17 % at 96 h) while 5 mM resulted in complete inhibition of the growth. In sharp contrast, SA significantly stimulated cell division at 100µM up to 2mM (Fig. 3b) with optimal stimulation noted at mid-log phase of almost 45% at 1mM with a significant corresponding reduction in doubling times (Table 1).

Aspirin, a derivative of SA, also resulted in an even more significant stimulation of growth than observed for SA (63% at 72h at 100µM Fig 3c). However, 3-HBA with an OH group at meta position had no effect on growth up to 2mM and was inhibitory at 5mM (Fig. 3d) while again 4-HBA (Fig.3f) (OH at the para position) was also stimulatory (almost 30% at 72h with either 100µM or 1mM). Unlike SA and aspirin, however, 4-HBA at 5 mM was not completely inhibitory (60% inhibition, Tables 2) and that required concentrations up to 10 mM to achieve (data not shown). Aspirin can be hydrolysed to SA and acetic acid and so the similarity in response to these compounds could in part be explained by this. The results indicate that the order of growth stimulation is ortho > para > meta with respect to the position of the OH group. SA has been reported to stimulate cell proliferation in *Chlorella vulgaris* (Czerpak *et al.*, 2002) although at levels lower than reported by us here (0.1mM).

In order to determine whether multiple OH groups (poly-hydroxy) also influenced culture growth, we examined the effects of gallic acid (3, 4, 5-trihydroxybenzoic acid) (Fig.3e). At low concentrations (10 µM and 100 µM) cell division was significantly stimulated

(20% at 10µM) and doubling times reduced by almost the same amount. However, concentrations of 1 mM inhibited growth by 96 %. Thus the cells have a lower tolerance to high concentrations of the poly-hydroxy function but these functionalities are stimulatory at lower concentrations. Low concentrations of gallic acid [10 µM] also stimulated growth in the diatom *Cyclotella* and these organisms were able to tolerate higher GA concentrations compared to *C. reinhardtii* studied here. This could be attributed to the presence of the silica shell in *Cyclotella* that may provide extra protection against chemical toxification whereas the *Chlamydomonas* cell wall deficient mutant lacks such a potential protective barrier.

While 4-HBA stimulated growth, vanillic acid which only differs from it in the presence of an extra methoxy group at C3 had little effect using concentrations up to 1mM and 2mM (data not shown).

Group 3 compounds

Unlike salicylic acid, salicylaldehyde was highly toxic at all concentrations tested with even 10 µM inhibiting cell division by 98% (data not shown). Vanillin was considerably less toxic with only 7% inhibition at 10µM (data not shown) and generally inhibitory at higher concentrations (1mM, 33%; 2mM 80%).

Overall, phenol derivatives (Group 1) and phenolic aldehydes (Group 3) were more toxic to *C. reinhardtii* at high concentrations compared to phenolic acids (Group 2). However, this is not a general rule. For example, gallic acid, was more toxic at higher concentrations than the vanillin. This indicates that the presence of three hydroxyl groups (OH) substituted on the benzene ring makes it more toxic to *C. reinhardtii* at higher concentrations than the phenolic aldehyde containing a methoxy group in the meta position and a hydroxyl group in the para position. The presence of the methoxy group in vanillin made it less toxic than salicylaldehyde, which contains one hydroxyl group in the ortho position making it the most toxic to *C. reinhardtii* growth. This is in agreement with the findings of (Nakai *et al.*, 2001) who studied the inhibitory effect of polyphenols produced by plants targeting the blue green alga *Microcystis aeruginosa*

and reported that hydroxy groups substituted at ortho- and/or para-positions to another hydroxy group have a stronger effect than the inhibitory effects of other phenolics. We also found that lower concentrations of vanillin had no effect on *C. reinhardtii* growth while all tested concentrations of salicylaldehyde completely inhibited the growth. Vanillin had little inhibition when added exogenously to *Microcystis aeruginosa* (Nakai *et al.*, 2001). These findings are at odds with earlier studies (Fries, 1974) that reported that vanillin and 4-hydroxybenzaldehyde (10 µM) stimulated the growth of the red alga, *Goniointrichum alsidii*, by up to 200 % where corresponding acids gave a lower response. Furthermore, it was suggested that the presence of an OH group in the para position in the aldehyde is desirable to stimulate algal growth (Fries, 1974). Thus the effects of these phenolics on algal growth varies considerably between the different species.

The position of the hydroxyl group relative to the carboxyl group is important for producing a stimulatory effect on the growth. For example, we found that the presence of a hydroxyl in the ortho position to a carboxyl group (SA) stimulated the growth in a dose-dependent manner. Other studies reported similar findings like those of (Czerpak *et al.*, 2002) and (Kovacik *et al.*, 2010) on the green algae *Chlorella vulgaris* and *Scenedesmus quadricauda* respectively. We also found that the presence of a hydroxyl group in the para position to a carboxyl group (4-HBA) showed significant stimulation of *C. reinhardtii* growth. A study on the freshwater green alga *Pseudokirchneriella subcapitata* showed similar results to those reported here (Kamaya *et al.*, 2006) that 4-HBA stimulated algal growth at low concentrations ranging from 0.1 to 1.0 mM with a high IC_{50} value of 9.9 mM. However, unlike reported here, SA showed no growth stimulation and was highly toxic giving IC_{50} value of 0.17 mM. This shows that although *C. reinhardtii* and *Pseudokirchneriella subcapitata* are both fresh water green algae, phenolic acids might still show different modes of action to regulate their growth. It is likely that differences in solubility of the various phenolic compounds may affect uptake and subsequent metabolism both within and between species. Certainly in higher plants SA has been shown to be strongly pH dependent and increases with increasing acidity of the medium, is temperature dependent and involves an active transport mechanism (Rocher *et al*. 2014). However to establish uptake kinetics for each

compound would require the use of its radiolabelled form and this could form the basis for a separate study. In addition it could be anticipated that the cell wall mutant of *C. reinhardtii* used here could behave differently to the wild type with respect to uptake of compounds from the medium.

In general, the stimulatory effects are most evident during the exponential phase of growth. Typically, by 96 h of incubation, the cells are approaching the stationary phase and so there is some 'catch up' by the non-stimulated controls. However it is evident from Fig. 3 that the max cell number is generally higher in the cells treated with phenolic compounds compared with controls (except 3-HBA and the highest concentration of some of the compounds). These type of growth kinetics imply that the cells are stimulated to divide faster post the 24h induction period.

Effect of selected phenolic compounds on lipid composition

Salicyclic acid is known to bind to numerous iron containing enzymes (Ruffer *et al.*, 1995). Since desaturases are non-haem iron containing enzymes that regulate the level of unsaturation in lipids they could be a target site for this compound. The lipid composition of *C. reinhardtii* has been well documented, see review (Li-Beisson *et al.*, 2015) and contains high levels of the chloroplast glycolipids, MGDG and DGDG which constitute over 50% of the acyl lipids. DGTS (l,2-diacylglyceryl-3-O-4-(N,N,N-trimethylhomoserine) is also a major lipid in *C.reinhardtii* and contains an ether-linked betaine moiety constitutes around 15%, while the sulpholipid, SQDG constitutes 10% with phosphatidylethanolamine (PE) (9%) and phosphatidylinositol (PI) making up 5%. We examined whether the treatment with SA or aspirin (both stimulated cell proliferation) influenced the lipid composition. Lipids were purified by TLC in a non-polar solvent that separated neutral lipids (composed of triacylglycerol, diacylglycerol, sterol esters and squalene) from the galactolipids (MGDG and DGDG,) sulpholipid (SQDG), DGTS and phospholipids (PE, PG PI). GC-MS analysis revealed no significant differences in the effects of both SA and aspirin on the mass composition of the various lipid classes including triacylglycerol (a stress marker, data not shown) and visually presented in Fig 4. This is in contrast to observations with *Chlorella* in which an almost two-fold stimulation of lipid was observed following treatment with 10mgL^{-1} SA.

The major fatty acids detected in Chlamydomonas share features typical of higher plants such as Arabidopsis in that essentially all fatty acids are esterified to polar glycerolipids and are of 16 or 18 carbon chain lengths (Li-Beisson *et al.*, 2015). *C.reinhardtii* differs from other chlorophytes in that they contain Δ4 and Δ5 unsaturated PUFA with the novel PUFA 16:4 $\Delta^{4,7,10,13}$ predominantly located in chloroplast MGDG together with α-linolenic acid (18:3 $\Delta^{9,12,15}$), pinolenic acid 18:3 (Δ5,9,12) and 18:4$\Delta^{5,9,12,15}$) are predominant in diacylglycerol-N,N,N-trimethylhomoserine (DGTS). Treatment with SA or aspirin resulted in a reduction in the 16:3 content with a corresponding increase in the content of 16:0 and 18:1 (Δ^9) (Table 2). This suggests that SA and aspirin inhibit the activity of CrFAD7 and/or CrΔ4FAD which acts on chloroplast MGDG to generate 16:3 (Zauner *et al.*, 2012). α-linolenic acid can be synthesised in the extra-plastidic pathway (endoplasmic reticulum) on DGTS and thus its level would not be affected. A down regulation in unsaturated fatty acid metabolism has also been reported in metabolic profiling studies with Chlamydomonas treated with SA (Lee et al 2016) and is consistent with the effects observed here. However, why chloroplast desaturases and not those in other cellular compartments are affected by this treatment is unclear at the present time and worthy of further investigation.

Effect of phenolics on lipid peroxidation

Lipid peroxidation has been associated with the regulation of cell division and its aldehydic products have been implicated in regulating several cellular processes, such as proliferation, differentiation and apoptosis of normal and neoplastic cells (Hoque *et al*. 2005). However, its role in plant cell division has been much less studied. Since phenolics are able to act as free radical scavengers and thereby potentially reduce lipid peroxidation we investigated whether the stimulatory effects of the phenolics could be attributed to this phenomenon. The thiobarbituric acid-reactive substances (TBARS) assay was used to estimate the amount of malondialdehyde (MDA), a stable end product of the oxidation of trienoic PUFAs that are abundant in Chlamydomonas. Cells

were harvested at the most rapid phase of growth at 72h and 96h and the levels of MDA compared with corresponding controls (Table 3).

The results broadly fall into 3 categories in which there is either a decrease in MDA, an increase in MDA, or no change. Salicylic acid, aspirin and 4-HBA were the most potent stimulators of cell culture growth and all showed a marked lowering of MDA during rapid growth. Interestingly, in Chlamydomonas a down regulation in α-tocopherol was observed following SA treatment (Lee *et al*. 2016) which would be expected to result in an increase in lipid peroxidation although that was not observed here. Catechol [10µM] had no stimulatory effect on growth but also showed a lowering of MDA levels. Conversely, gallic acid [10µM] also stimulated culture growth but actually promoted lipid oxidation while 2, 4-DMP and 4-MC also promoted oxidation but not cell proliferation. Similar effects were reported for gallic acid on the growth and lipid peroxidation in the diatom, *Cyclotella caspia* (Liu *et al.*, 2013). Phenol, which was stimulatory to growth showed no effect on MDA levels.

Whilst we observed no effect of vanillin on lipid oxidation levels here, (Nguyen *et al.*, 2014) reported that it induced oxidative stress in yeast cells although another study by (Tai *et al.*, 2011) concluded that the antioxidant activity of vanillin was not consistent and varied depending on the method used to estimate lipid peroxidation. Vanillin and salicylaldehyde have been identified in algae and their possible role as antioxidants discussed (Onofrejova *et al.*, 2010). Overall, the results clearly reveal that lipid peroxidation levels are not correlated with effects on growth and that other mechanisms are likely to be involved.

Effect of SA and aspirin on lipid hydroperoxides and MDA

In order to further investigate the change in lipid peroxidation during growth, we monitored the production of the primary lipid oxidation product of PUFA, lipid hydroperoxides (LHPO), using the FOX2 assay (Griffiths *et al.*, 2000). Lipid hydroperoxides are unstable and their fragmentation yields a wide range of more stable products including MDA.

463 A comparison between cultures treated with SA (1mM), Aspirin (100µM) revealed that
464 all cultures showed the same trend in oxidation products during growth. Overall, LHPO
465 and MDA levels were lower during the lag phase and increased during the exponential
466 phase to reach their highest levels during the stationary phase of growth (Fig 5). SA
467 treated cultures showed 40-66% reduction in LHPO and aspirin from 37-54%. MDA
468 levels were also reduced with SA by 19-23% and with aspirin by 14-18%. Calculations
469 of the % total fatty acids oxidized in the control cells was 0.8 ± 0.1% (74 ± 4 µmol fatty
470 acid g^{-1} FW containing 0.602 ± 0.040 µmol LHPO g^{-1} FW). SA, Aspirin, 4-HBA, vanillin
471 and catechol reduced the levels of MDA and this is in agreement with other reports on
472 the free radicals scavenging properties of SA (Raman and Ravi, 2011). Since SA,
473 Aspirin and 4-HBA stimulated growth and lowered LHPO and MDA levels it suggests an
474 association between lipid peroxidation and the rate of cell division.

475 The comparison between the analysis of the FOX2 assay and the TBARS method of
476 lipid peroxidation during *C. reinhardtii* growth revealed that both assays showed an
477 increase in lipid peroxidation during the stationary phase compared to the lag phase.
478 This indicates that the increase in MDA is the consequence of the increase in LHPO
479 peroxides formation. This is also in line with the increase observed in this study of LOX
480 activity during the exponential phase of *C. reinhardtii* growth. However, FOX2 assay
481 gave higher levels for LHPOs when compared to MDA levels measured by TBAR in all
482 tested cultures. A similar observation was reported by Hermes-Lima *et al*. (1995) who
483 found that FOX2 assay gave 6–9-fold higher values for lipid peroxidation than TBARS
484 for Cu+ induced peroxidation in liposomes. Studies on marine macroalgae showed that
485 FOX2 assay gave ≥ 20 fold higher values for LHPOs as compared to the TBARS
486 method (Kumari *et al.*, 2012). This could be due to the fact that while MDA is one of the
487 products that form mainly from fatty acids with three or more double bonds, fatty acids
488 containing two double bonds are unable to form MDA molecules. In *C. reinhardtii*, 16:3,
489 16:4 and both α- and β-18:3 could potentially give rise to MDA.

490

Lipoxygenase activity and cell division

The initiation of the oxylipin biosynthetic pathways starts with oxygen addition to the acyl chain of polyunsaturated fatty acids (PUFAs), predominantly linoleic acid and α-linolenic acid through the action of LOX (Andreou and Feussner, 2009). LOX are non-heme proteins that catalyses the regio- and stereo-specific dioxygenation of PUFAs containing the 1*Z*, 4*Z* pentadiene motif and are classified into either 9- or 13-LOX, dependent on the site of oxygenation on the hydrocarbon chain generating either 9-hydroperoxy and 13-hydroperoxy derivatives of the substrate (Griffiths, 2015).

Studies on animal cells reported the link between the inhibition of lipoxygenase (LOX) activity and cell growth (Hoque *et al.*, 2005)so we determined whether the stimulation of cell proliferation observed using SA could be attributed to an effect on LOX.

LOX activity during algal growth and effect of pH

In order to establish whether LOX plays a role in *C. reinhardtii* cell division, LOX activity was measured both during the exponential-phase growth, when cells are actively dividing and stationary-phase when net growth is zero. The pH optimum for the Chlamydomonas LOX was 7.0 but still had 90% activity at pH 5.0 at mid-log phase (Table 4). A 25% decrease was observed, however at higher pH. During the exponential phase of growth, LOX activity was consistently higher than at stationary-phase. In *Chlorella* a pH optimum of 7.5 has been reported and the LOX produced equal amounts of 9-hydroperoxylinoleic acid and 13-hydroperoxylinoleic acid (Nunez *et al.*, 2002).

In *Chlamydomonas debaryana* oxygenated derivatives of both α- and β-18:3 have been identified viz (9*E*, 11*E*, 15*Z*)-13-hydroxyoctadeca-9,11,15-trienoic acid(5*Z*, 9*Z*,11*E*)-13-hydroxyoctadeca-5,9,11-trienoic acid together with oxygenated derivatives of 16:4 namely (4*Z*, 7*Z*, 9*E*,11*S*, 13*Z*)-11-hydroxyhexadeca-4,7,9,13-tetraenoic acid, (4*Z*, 7*E*, 9*E*, 13*Z*)-11-hydroxyhexadeca-4,7,9,13-tetraenoic acid, (4*Z*, 6*E*, 10*Z*, 13*Z*)-8-hydroxyhexadeca-4,6,10,13-tetraenoic acid and (4*Z*, 8*E*, 10*Z*, 13*Z*)-7-hydroxyhexadeca-4,8,10,13-tetraenoic acid and the (5*E*, 7*Z*, 10*Z*, 13*Z*)-4-hydroxyhexadeac-5,7,10,13-

tetraenoic acid (de los Reyes *et al.*, 2014). Since 16:3 and 16:4 together with α- and β-18:3 and 18:4 are present in the *C. reinhardtii* cultures used here (Table 2) it is likely that these products would also be detected.

Fatty acids substrates utilised by LOX

Fatty acid substrate specifications of LOX revealed a preference for α-linolenic acid (α-18:3$\Delta^{9,12,15}$) although linoleic acid (18:2$\Delta^{9,12}$), γ-linolenic acid (γ-18:3$\Delta^{6,9,12}$) and arachidonic acid (20:4$\Delta^{5,8,11,14}$) were all readily utilized as substrates with 60 ± 3%, 48 ± 2% and 53 ± 2% respectively of the activity observed for α-18:3. All future assays were therefore performed using α-linolenic acid.

Effect of LOX inhibitors and SA on LOX activity

SA is a classic inhibitor of cyclooxygenase in animals and forms the basis for the control of inflammation. SA and aspirin have also been shown to inhibit LOX in human cell extracts through reduction of the ferric iron (Fe^{3+}) to Fe^{2+} located at the active site of the enzyme. In plants, SA binds to iron-containing enzymes like catalase, peroxidase and lipoxygenases resulting in the inhibition of these enzymes (Ruffer *et al.*, 1995). SA inhibited LOX activity in a dose-dependent manner (IC_{50}, 250 µM) (Fig. 6). Since SA inhibited LOX activity *in vitro* at a lower concentration (250 µM) than the observed stimulation of cell proliferation at (1 mM) *in vivo*, we considered whether (1 mM) SA suppress LOX activity *in vivo*. Cells were incubated with 1 mM SA and harvested at mid-log phase (96 h) and cell-free extracts prepared and assayed for enzyme activity. The SA treatment decreased endogenous LOX activity by 34 ± 4% (n=3).

There are a number of reported findings that the products of LOX activity are expressed and produced by cancer cells suggesting the possible role of LOX in regulating cell growth in humans (Hoque *et al.*, 2005). However, little information is available on the role LOX plays in controlling cell growth and cell division in algae. Lipoxygenases have been reported to play a possible role in “quorum sensing” a natural phenomenon which is a cell density-dependent regulatory networks of microorganisms in fungi (Brown *et al.*, 2008). In Diatoms, oxylipins were reported to mediate bloom termination (d'Ippolito *et*

549 *al.*, 2009) and showed that high expression of LOX products during the stationary phase
550 suggested that LOX products play a role as info-chemicals in mediating plankton
551 interactions (Vidoudez and Pohnert, 2008). We found that during algal growth, LOX
552 activity was markedly higher at mid-log phase (see Table 4) than at the stationary phase
553 and that LHPO levels accumulated throughout the time course which would be
554 consistent with the hypothesis that LOX products may also mediate bloom termination
555 in *C. reinhardtii*.

556

557 The phenolic compounds tested here gave a range of effects on *Chlamydomonas*
558 proliferation depending on the number and position of the functional groups on the
559 arene ring. Phenolic acids were the least toxic to *C. reinhardtii* growth when compared
560 to phenolic aldehydes and phenol derivatives and that the order of growth stimulation
561 was ortho > para > meta with respect to the position of the OH group. Salicylic acid,
562 Aspirin, 4-HBA acid stimulated cell proliferation in a dose-dependent manner and
563 significantly reduced lipid peroxidation and LOX activity. These findings demonstrated
564 that the application of these chemical enhancers could be a valuable and practical
565 approach to address low productivity issues in microalgal based processes.

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671

Table 1.

Minimum duplication time (dtmin, h) for the different compounds and concentrations tested. Values with statistical differences with respect to the controls have been marked with (*).SA (salicylic acid), 3-HBA (3-hydroxybenzoic acid), 4-HBA (4-hydroxybenzoic acid). (-) is complete inhibition. (n=3 ± SD, p>0.05)

Compound	Control	10 µM	100 µM	1 mM	2 mM	5 mM
Phenol	9.3 ± 0.3	8.9 ± 0.2	8.1 ± 0.1*	8.4 ± 1.3*	8.9 ± 1.6	-
Benzoic acid	9.8 ± 0.9	9.8 ± 0.2	9.1 ± 0.2	9.7 ± 0.4	10.7 ± 0.6*	-
SA	9.1 ± 0.4	8.6 ± 0.6	7.7 ± 0.5*	7.8 ± 1.1*	7.3 ± 0.3*	-
Aspirin	9.4 ± 0.0	9.4 ± 0.1	7.7 ± 0.1*	7.8 ± 0.3*	8.4 ± 0.4*	-
3-HBA	9.2 ± 0.4	9.2 ± 0.4	9.1 ± 0.3	9.4 ± 0.4	9.3 ± 0.3	-
4-HBA	9.2 ± 0.5	8.4 ± 0.1*	8.5 ± 0.1*	8.5 ± 1.5*	8.8 ± 0.5*	14.0 ± 0.3*
Gallic acid	8.4 ± 0.3	7.0 ± 0.7*	7.1 ± 1.1*	39.4 ± 1.7*	42.6 ± 26.5*	-
Vanillic acid	8.6 ± 0.4	8.5 ± 0.4	8.7 ± 0.2	8.5 ± 0.2	9.2 ± 0.4*	-

Table 2.

Acyl composition of Chlamydomonas treated with salicylic acid (SA) and aspirin (Asp). The acyl composition was determined at mid log phase (72 h). The SD was <5% of the value stated for each fatty acid. C=control, SA; cells incubated in 1mM SA, Asp; cells incubated in 100µM aspirin. (n=3 ± SD, p>0.05)

	Acyl composition (mol %)										
	16:0	16:1	16:2	16:3	16:4	18:0	18:1	18:2	β18:3	α18:3	18:4
C	23.0	4.9	2.1	15.5	0.5	2.0	16.0	8.0	9.0	16.9	2.1
SA	39.0*	1.5*	0.5	5.7*	0.3	2.0	25.0*	5.0*	6.0*	14.0	1.0
Asp	32.0*	2.7*	0.7	10.0*	0.3	2.0	20.4*	6.4	8.4	15.1	2.0

Table 3.

Changes in the MDA content of Chlamydomonas cells following incubation with various phenolic compounds. The MDA content of control cells at the start of experiments was in the range of 47-61 ng g^{-1}. The results are expressed as a % decrease (-) or increase (+) in MDA relative to control samples at the times indicated. Results are n=3 ±SD. NC=no change. 2, 4-DMP (2, 4-dimethylphenol), 4-MC (4-methylcatechol), SA (salicylic acid), 3-HBA (3-hydroxybenzoic acid), 4-HBA (4-hydroxybenzoic acid).

	% change in MDA level	
Compound	**72 h**	**96 h**
SA	- 24.0 ± 2.0	- 21.0 ± 2.0
Aspirin	- 15.1 ± 0.6	- 18.2 ± 1.5
4-HBA	- 21.0 ± 1.5	- 15.0 ± 1.5
Catechol	- 10 ± 1.5	- 20.0 ± 3.0
Gallic acid	+ 17.1 ± 1.5	+ 11.0 ± 0.1
2, 4-DMP	+ 17.0 ± 0.9	+ 40.1 ± 2.4
4-MC	NC	+ 11.0 ± 0.2
Phenol	NC	NC
Benzoic acid	NC	NC
3-HBA	NC	NC
Vanillic acid	NC	NC
Vanillin	NC	NC

Table 4.

Effect of pH on lipoxygenase (LOX) activity at different points in the growth cycle. LOX activity was assayed using α-18:3 as a substrate. (n=3 ± SD)

	LOX activity (nmol min^{-1} mg^{-1})	
pH	**Mid log phase (72 h)**	**Stationary phase**
5.0	5.7 ± 0.3	3.8 ± 0.2
7.0	6.3 ± 0.3	4.3 ± 0.1
9.0	4.7 ± 0.2	1.7 ± 0.1

Figure legends

Fig. 1. Phenolic compounds tested on the growth of *C. reinhardtii*

Fig. 2. Half-maximal inhibitory concentration IC_{50} of the tested phenolic compounds compared to the control. The standard error is indicated by error bars. 2, 4-DMP (2,4-dimethylphenol), 4-MC (4-methylcatechol), GA (gallic acid), BA (benzoic acid), VA (vanillic acid), SA (salicylic acid), 3-HBA (3-hydroxybenzoic acid), 4-HBA (4-hydroxybenzoic acid)

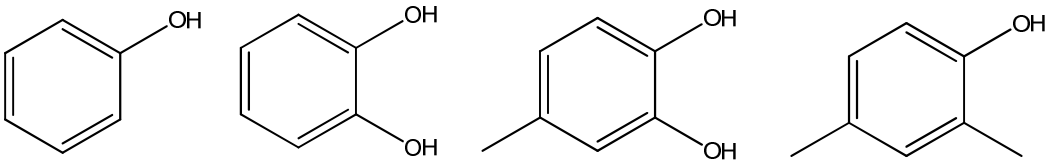
Fig. 3. Effect of various phenolic compounds on growth. Results represent mean ±SD (n=3). 3-HBA (3-hydroxybenzoic acid 4-HBA (4-hydroxybenzoic acid)

Fig. 4. Polar lipid separation by TLC. Abbreviations: MGDG: monogalactosyl-diacylglycerol; DGTS: l, 2-diacylglyceryl-3-O-4-(N,N,N-trimethyl)homoserine; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; DGDG: digalactosyldiacylglycerol; SQDG: sulfoquinovosyldiacylglycerol; PI: phosphatidylinositol. Standards were run separately.

Fig. 5. Effect of 1 mM SA and 100 µM Aspirin on (A) LHPO levels and (B) MDA levels during *C. reinhardtii* growth. (n=3 ± SD)

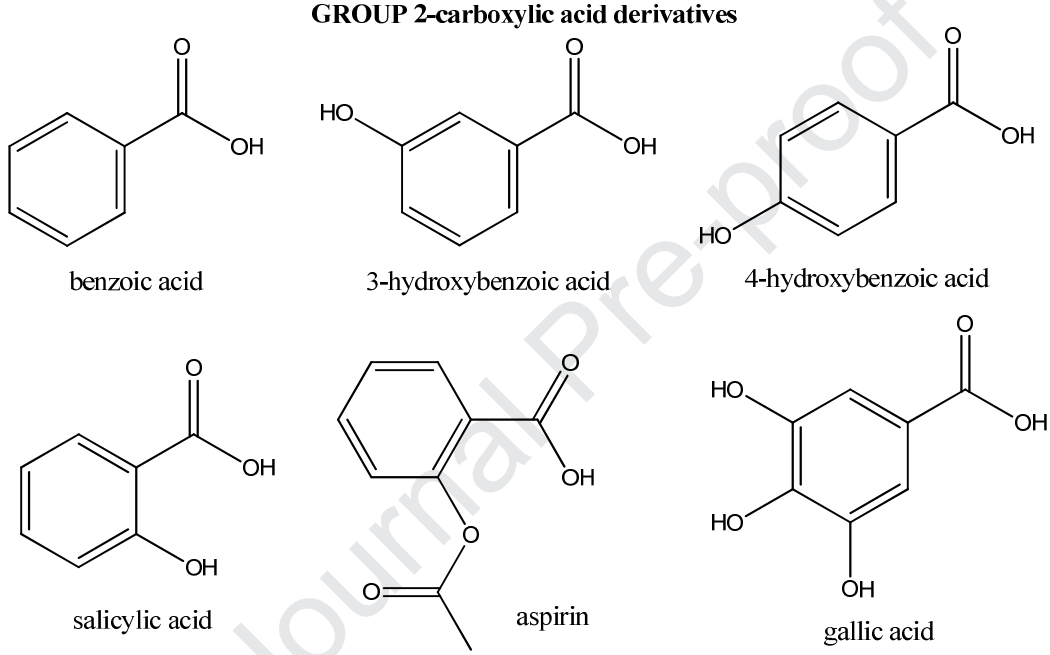
Fig. 6. Effect of increasing concentrations of SA on LOX activity.

GROUP 1- phenol derivatives



phenol catechol 4-methyl catechol 2,4-dimethyl phenol

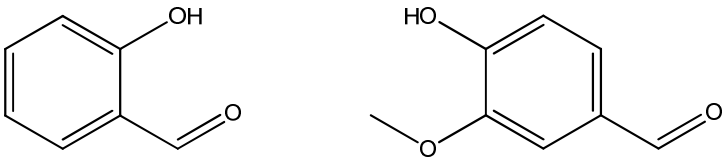
GROUP 2-carboxylic acid derivatives



benzoic acid 3-hydroxybenzoic acid 4-hydroxybenzoic acid

salicylic acid aspirin gallic acid

GROUP 3-aldehyde derivatives



salicylaldehyde* vanillin

Fig 1

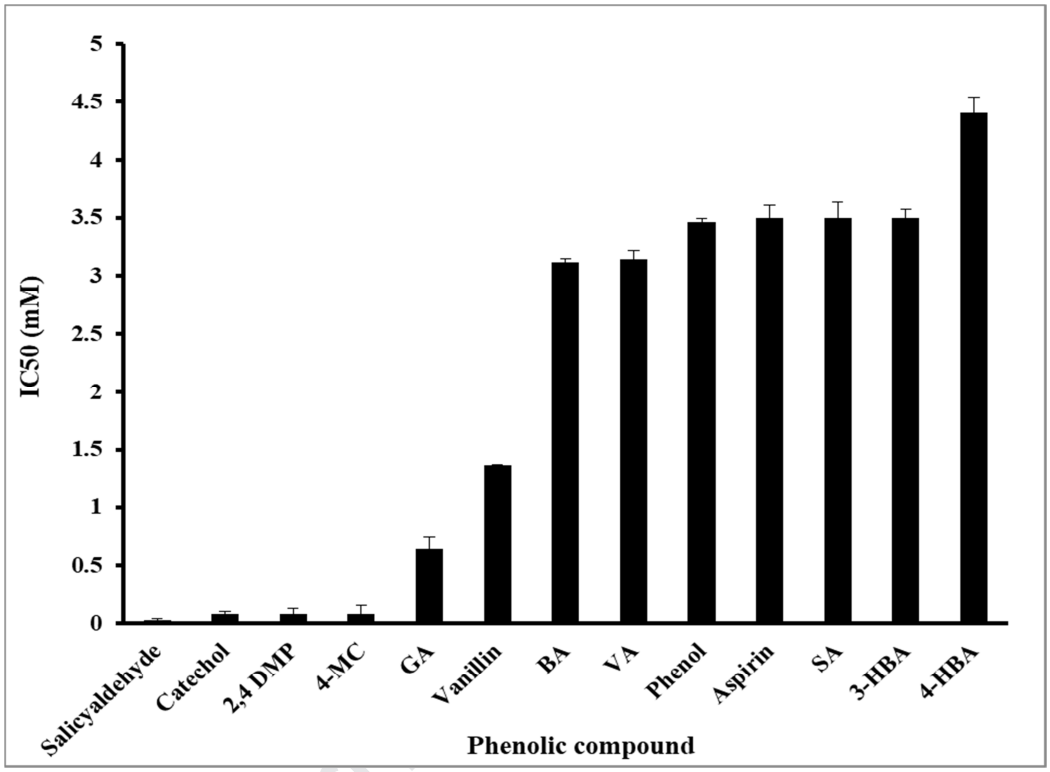

IC50 (mM)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Salicyaldehyde
Catechol
2,4 DMP
4-MC
GA
Vanillin
BA
VA
Phenol
Aspirin
SA
3-HBA
4-HBA
Phenolic compound

Fig 2

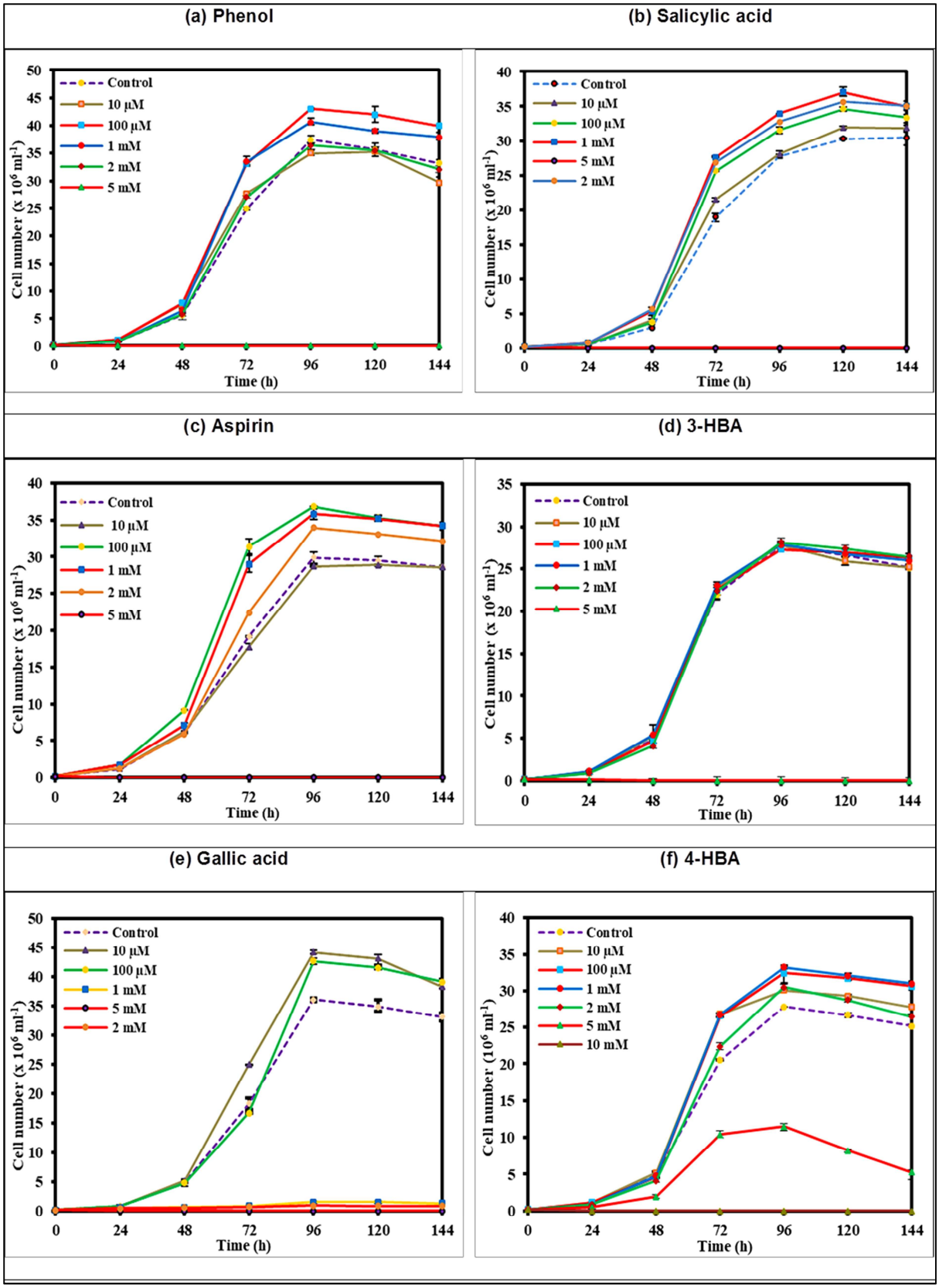

(a) Phenol
(b) Salicylic acid
(c) Aspirin
(d) 3-HBA
(e) Gallic acid
(f) 4-HBA
Control
10 µM
100 µM
1 mM
2 mM
5 mM
10 mM
Cell number (x 10⁶ ml⁻¹)
Cell number (10⁶ ml⁻¹)
Time (h)

Fig 3

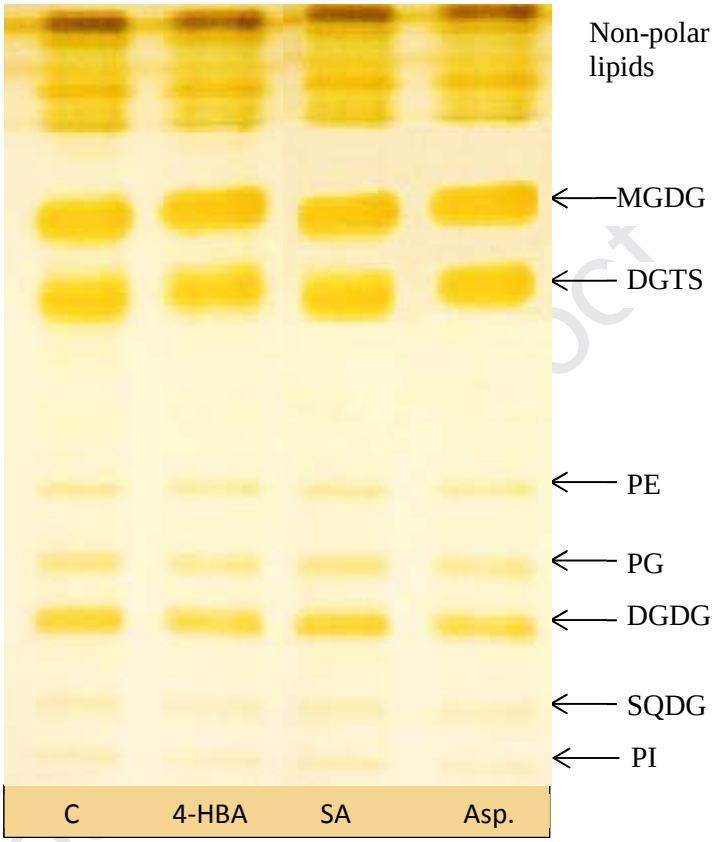


Fig 4

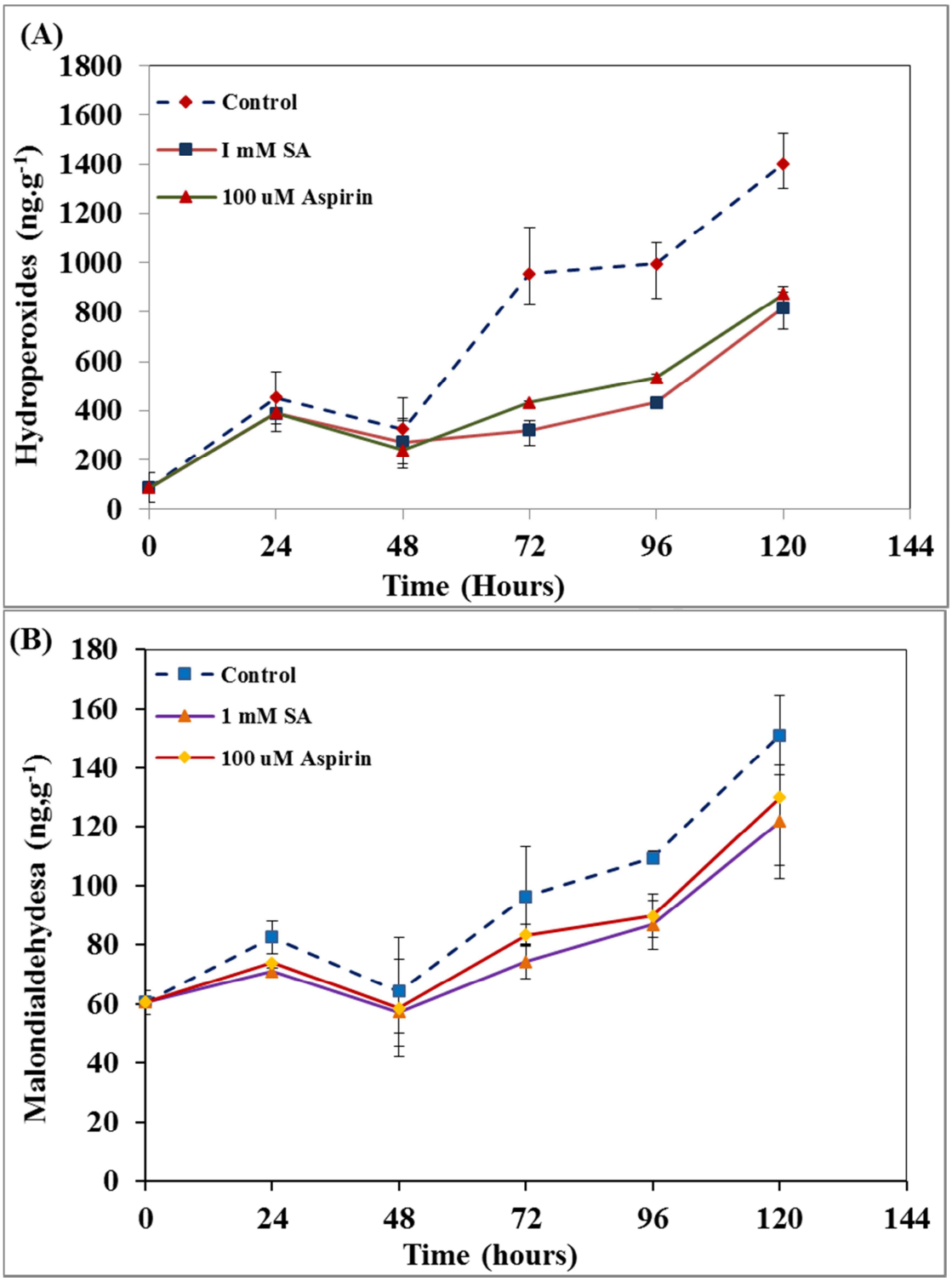

(A)
Hydroperoxides (ng.g-1)
1800
1600
1400
1200
1000
800
600
400
200
0
Control
I mM SA
100 uM Aspirin
0
24
48
72
96
120
144
Time (Hours)
(B)
Malondialdehydesa (ng,g-1)
180
160
140
120
100
80
60
40
20
0
Control
1 mM SA
100 uM Aspirin
0
24
48
72
96
120
144
Time (hours)

Fig 5

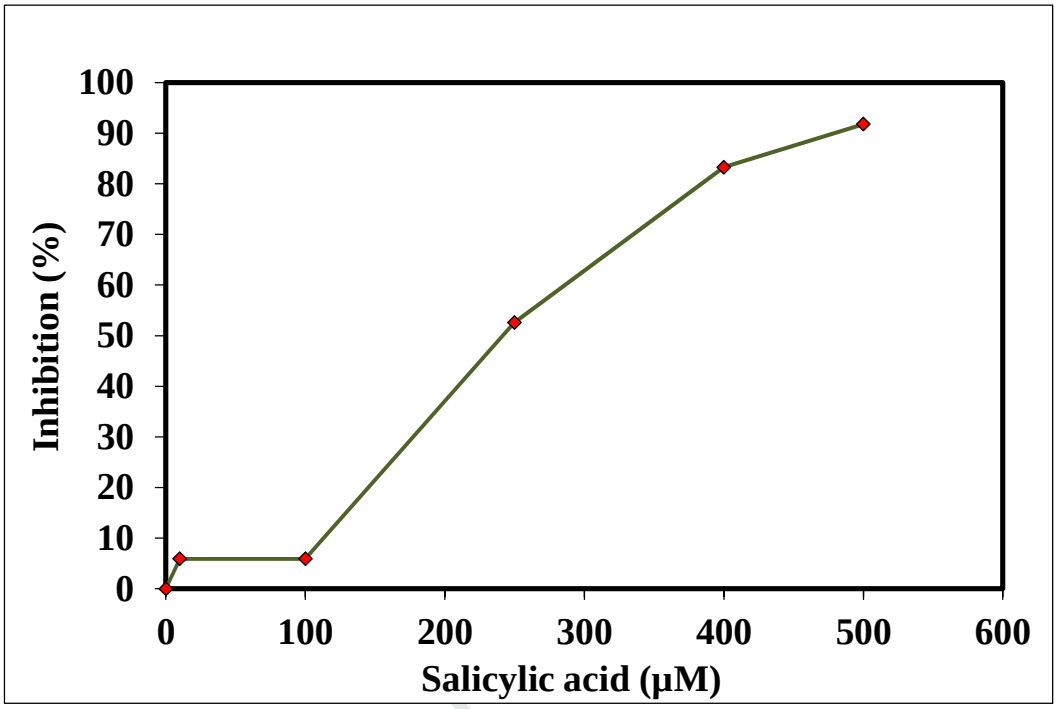


Fig 6

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: