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Metabolites from invasive pests inhibit mitochondrial complex II: A potential strategy for the treatment of human ovarian carcinoma?

Alessandra Ferramosca [a, *], Annalea Conte [a, 1], Flora Guerra [a, 1], Serena Felline [a], Maria Grazia Rimoli [b], Ernesto Mollo [c], Vincenzo Zara [a], Antonio Terlizzi [a, d]

[a] Dipartimento di Scienze e Tecnologie Biologiche ed Ambientali, Università del Salento, Lecce, Italy
[b] Dipartimento di Farmacia, Università di Napoli Federico II, Napoli, Italy
[c] Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Pozzuoli, Italy
[d] Stazione Zoologica Anton Dohrn, Napoli, Italy

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ABSTRACT

The red pigment caulerpin, a secondary metabolite from the marine invasive green algae *Caulerpa cylindracea* can be accumulated and transferred along the trophic chain, with detrimental consequences on biodiversity and ecosystem functioning. Despite increasing research efforts to understand how caulerpin modifies fish physiology, little is known on the effects of algal metabolites on mammalian cells.

Here we report for the first time the mitochondrial targeting activity of both caulerpin, and its closely related derivative caulerpinic acid, by using as experimental model rat liver mitochondria, a system in which bioenergetics mechanisms are not altered. Mitochondrial function was tested by polarographic and spectrophotometric methods.

Both compounds were found to selectively inhibit respiratory complex II activity, while complexes I, III, and IV remained functional. These results led us to hypothesize that both algal metabolites could be used as antitumor agents in cell lines with defects in mitochondrial complex I. Ovarian cancer cisplatin-resistant cells are a good example of cell lines with a defective complex I function on which these molecules seem to have a toxic effect on proliferation. This provided novel insight toward the potential use of metabolites from invasive *Caulerpa* species for the treatment of human ovarian carcinoma cisplatin-resistant cells.

1. Introduction

Recent researches suggest that the study of well-defined chemical entities contained by invasive species can contribute both to a better understanding of the biological processes associated with marine invasions and to propose a possible exploitation of vast invasive biomasses to obtain valuable chemicals of interest in pharmacology [1]. Some of those studies concerned about the compound caulerpin (CAU), a bioactive bisindolic alkaloid (Fig. 1) introduced in the Mediterranean along with both the invasive alga *Caulerpa taxifolia*, a species included in the list of the 100 world's worst invasive species listed by the International Union for Conservation of Nature (IUCN), and its congeneric *Caulerpa cylindracea* (previously known as *Caulerpa racemosa* var. *cylindracea*). *C. cylindracea*, in particular, has become the favorite food of a native fish species of considerable commercial importance, the edible white sea bream *Diplodus sargus*. It is noteworthy that, as a consequence of the *C. cylindracea*-based diet, the fish accumulates CAU in its tissues [2–5].

Although CAU, which is one of the main lipophilic components of the above invasive algae, already showed a panel of biological activities [[1], and references therein], there are still no clear evidence of a direct impact of CAU on the fish health, and/or potential risks for the human health as a result of fish consumption. More specifically, it has been observed that, in *D. sargus*, the exposure to *C. cylindracea* modulates the activity of peroxisomal Acyl-CoA oxidase, the first and rate-determining step of the peroxisomal β-oxidation of fatty acids, causing an alteration in lipid metabolism [6]. The increased activity of Acyl-CoA oxidase, observed in fish with medium and high content of CAU, could also mechanistically explain changes observed in the fatty acids levels of fish naturally

* Corresponding author.
E-mail address: alessandra.ferramosca@unisalento.it (A. Ferramosca).
[1] These authors contributed equally to this work.

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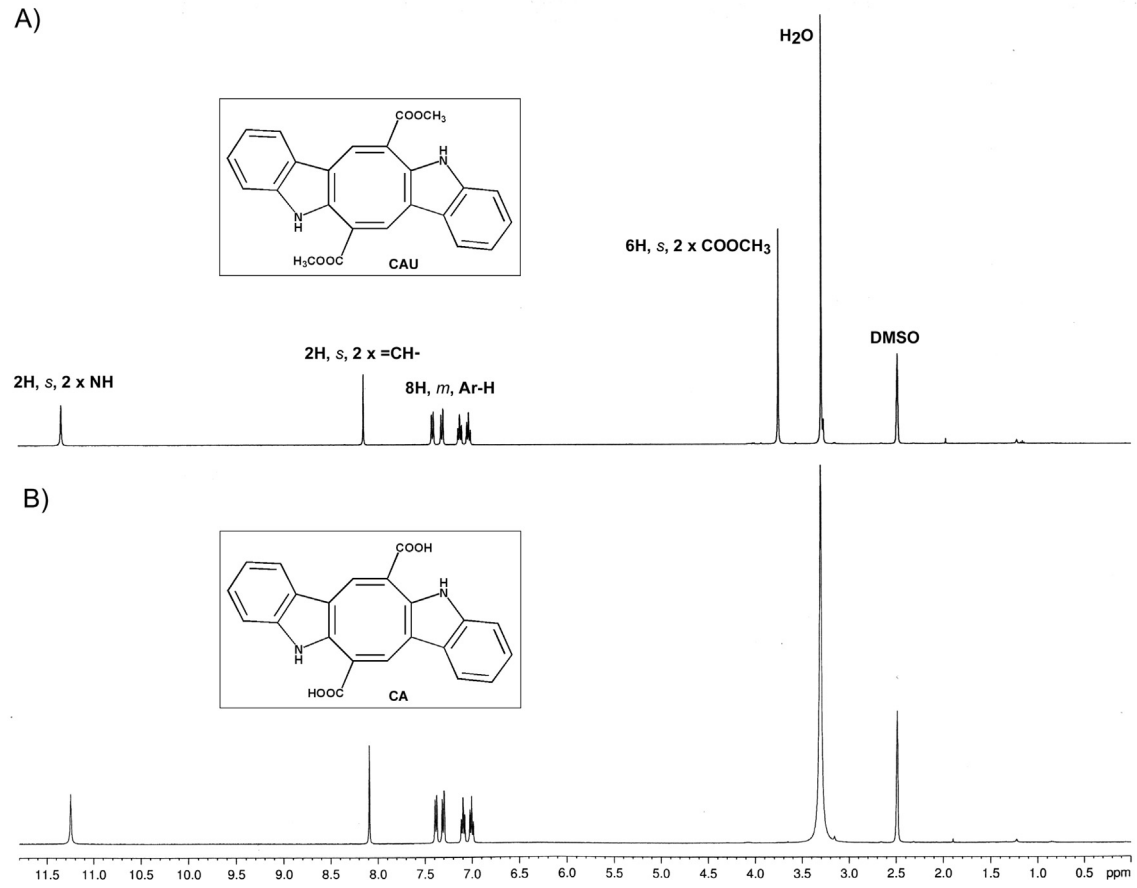

Fig. 1. Structures and ^{1}H NMR spectra in DMSO-d6 of (A) CAU and (B) its hydrolysis product CA.

feeding on the alien algae [4]. Interestingly, the increased hepatic lipid catabolism was reflected by the catalytic induction of cytochrome P450 (CYP450) [5], a condition which is often associated to elevated oxidative stress and reactive oxygen species (ROS) production [7].

Noteworthy, the hypolipidemic activity exerted on rats by *C. cylindracea* extracts [8] suggests that algal metabolites might also produce changes in the lipid metabolism of lower vertebrates. On the other hand, mammalian mitochondria are considered the principal target for nutritional and pharmacological control of lipid metabolism [9,10]. In this regard, mitochondrial and peroxisomal metabolisms of fatty acids were regarded as closely linked. These organelles are both able to degrade fatty acids, but only mitochondrial metabolism is closely linked to energy production [11]. Moreover, an adverse effect of drugs used to control lipid metabolism is the dysfunction of mitochondria, caused by an overproduction of ROS [12].

A CAU-induced mitochondrial damage was first proposed by Liu et al. [13], who suggested that this metabolite is able to suppress mitochondrial respiration in breast cancer by inhibiting mitochondrial respiration at electron transport chain complex I. Although showing that CAU is also able to inhibit the growth of normal human mammary epithelial cells (HMEC), though to a lesser extent, the above research especially focuses on the mechanism of action in pathologic states of hypoxia, but does not contribute to a better understanding of the action of CAU under non-pathological/normoxic conditions. On the other hand, it has been demonstrated that energetic deficit derived from mitochondrial dysfunction correlates with low proliferative index, benign features and the inability of cancer to adapt to the surrounding microenvironment [14,15].

The main aim of the present study is to evaluate the effects of CAU on the mitochondrial respiration in normal mammalian cells. The study also includes an evaluation of the effects of caulerpinic acid (CA), which is a closely related derivative of CAU (Fig. 1) also found in *C. cylindracea* [16, as *C. racemosa*]. Mitochondria isolated from rat liver have been selected as a system in which bioenergetic mechanisms are not altered, in order to elucidate whether and how algal CAU and CA affect mitochondrial function, as well as to confirm if they are complex specific inhibitors.

The obtained results and, in particular, the specificity of CAU and CA in mitochondrial targeting, led us to investigate the role of algal metabolites on human ovary cancer cells with specific mitochondrial defects [17]. Therefore, by exploring the molecular mechanism by which CAU and CA eventually affect mitochondria function, the present study also aims to contribute to propose a novel potential use of the algal metabolites for human ovarian cancer treatment.

2. Materials and methods

2.1. Purification of CAU

C. cylindracea was collected by scuba divers, brought in the lab and extracted with acetone. The acetone extract was evaporated under reduced pressure and the residual water was extracted with diethyl ether. The ether extract was then concentrated and analyzed by thin-layer chromatography (TLC) using the mixture light petroleum ether/diethyl ether in different ratio as eluent. TLC

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spots were visualized by spraying with ceric sulphate and heating. The diethyl ether extract was then fractionated by Sephadex LH-20 chromatography eluted with chloroform/methanol in the ratio 1:1. The fraction giving a spot with Rf around 0.35 on TLC in petroleum ether/diethyl ether (1:1), producing a red colour after cerium sulphate has reacted, was further purified on preparative TLC eluted with petroleum ether/diethyl ether (4:6), affording pure CAU (red orange prisms), identified by comparison with ^{1}H and ^{13}C NMR data (proton and carbon nuclear magnetic resonance) described in the literature [16,18–20]. NMR spectra were recorded on Bruker AM400 MHz in both and DMSO-d_6 and $CDCl_3$. TLC chromatography was carried out using precoated Merck F254 plates.

2.2. Alkaline hydrolysis of CAU

Pure CAU was dissolved in methanol and NaOH 0.1 N (2:1). The reaction was stirred on water bath at 50 °C for 4 h, then at room temperature over night. The solution was then acidified to pH 5 with HCl and evaporated under reduced pressure. Extraction with diethyl ether and evaporation of the organic phase, followed by a further purification step on Sephadex LH-20 with chloroform/methanol in the ratio 1:1, gave pure CA (dark brown flakes), as confirmed by ^{1}H NMR spectroscopy (Fig. 1).

2.3. Animal studies

Male Sprague–Dawley rats were obtained from Harlan (Carezzana, Italy) and housed individually in animal cages at a temperature of 22 ± 1 °C with a 12:12 h light–dark cycle and 30–40% humidity. Animal studies were carried out in strict accordance with the European Committee Council 106 Directive (86/609/EEC) and with the Italian animal welfare legislation (art 4 and 5 of D.L. 116/92). The Italian Ministry of Health specifically approved this study.

Rat liver mitochondria were prepared by standard procedures.

2.4. Cell lines

Human ovarian carcinoma cell lines, 2008 wild type and C13 cisplatin-resistant [17], were gently provided by Prof. Gaetano Marverti (University of Modena and Reggio Emilia, Italy). Cells were grown in RPMI-1640 medium, 10% fetal bovine serum (FBS), 2% glutamine and 1% pen-strep, in humidified conditions at 5% CO_2 and 37 °C. Both cell lines have a duplication time about 24 h. Cells were collected every 2 days with minimum amount of 0.25% trypsin-0.2% EDTA and seeded at 1×10^6 density in 100 mm dishes.

2.5. Mitochondrial functionality assays

Rat liver mitochondria (0.3 mg protein/ml) or digitonin-permeabilized cells (7.5×10^6 cells) were incubated for 3 min without (control) and with 10 μM CAU or CA (dissolved in DMSO).

Oxygen uptake by rat liver mitochondria or by permeabilized cells was measured by using a Clark-type oxygen probe (Hansatech oxygraph; Hansatech Pentney, King's Lynn, UK) as described in Refs. [21,22]. The addition of different substrates permitted to evaluate mitochondrial respiration when respiratory complexes I (5 mM pyruvate, 2.5 mM malate), II (5 mM succinate, 5 μM rotenone) or IV (10 mM ascorbate, 0.2 mM TMPD, 5 μM rotenone, 5 μM antimycin A) were stimulated [23]. For each substrate, after 2 min, state 3 respiration was induced by the addition of 0.3 mM ADP. Respiratory control ratio (RCR) was calculated as the ratio of the rate of oxygen uptake in the presence of added ADP (state 3) to the rate observed when added ADP had been completely phosphorylated to ATP (state 4).

Mitochondrial respiratory complexes activities were evaluated spectrophotometrically, essentially as described in Ref. [23].

Aconitase and fumarase activity were determined as described in Ref. [24] and activity ratio of aconitase to fumarase was calculated as an indicator of mitochondrial ROS production.

2.6. SRB assay

Citotoxic effect of CAU or CA on cellular vitality was evaluated by the colorimetric sulforhodamine B (SRB) assay [25]. 2008 and C13 cells were plated in 24-well plates in appropriate medium and, following overnight incubation, were treated with 10 μM of CAU or CA (in DMSO) for 72 h. After an incubation period, cell monolayers were fixed with 50% (wt/vol) trichloroacetic acid and stained with SRB for 30 min, after which the excess dye was removed by washing repeatedly with 1% (vol/vol) acetic acid. The protein-bound dye was dissolved in 10 mM Tris base solution for OD determination at 570 nm using a Victor microplate reader (Wallac Instruments, Turku, Finland).

2.7. Statistical analysis

Data were analyzed through one-way ANOVAs after ensuring homogeneity of variances by Cochran's C tests. When appropriate, SNK tests were used for multiple a posteriori comparisons of the means. ANOVAs were computed by GMAV 5 software (University of Sydney, Australia).

3. Results

The respiratory efficiency of freshly isolated rat liver mitochondria in the presence of 10 μM CAU or CA was analysed by oxygraphic methods (Table 1). When we added respiratory substrates for mitochondrial complexes I, we found that CAU and CA did not affect particularly mitochondrial respiration efficiency, as suggested by the RCR values ($F_{2,6} = 1.25$, $p > 0.05$).

Algal metabolites had a stronger effect on mitochondrial respiration efficiency after the addition of rotenone (an inhibitor of complex I) and succinate (a substrate for complex II); in this case, we found a decrease of 40% and 33% respectively in the RCR values ($F_{2,6} = 24.28$, $p < 0.01$). No effect of CAU and CA was observed after the inhibition of complex I and III (by the addition of rotenone and antimycin A) and the addition of respiratory substrates for complex IV (TMPD and ascorbate).

We then investigated the enzymatic activity of single respiratory complexes by spectrophotometric methods (Fig. 2A). Also in this case, we found a decrease in the activity of complex II of 50% and 42% when organelles were incubated with CAU and CA, respectively ($F_{2,6} = 127.68$, $p < 0.001$). Complexes I, III, and IV remained functional (Fig. 2A).

As functional indicator of mitochondrial ROS production we calculated the activity ratio of aconitase to fumarase, because aconitase is sensitive to inactivation by superoxide, whereas fumarase is unaffected. We found that this ratio was unchanged after CAU and CA incubation (Fig. 2B).

These results lead to hypothesize an effect of algal metabolites in completely blocking mitochondria respiration in cells with defects in mitochondrial complex I, such as cisplatin-resistant C13 ovarian cancer cells. These cell lines are characterized by mtDNA mutations, reduced oxygen consumption, lower sensitivity to rotenone and increased dependence of glucose when compared to their cisplatin-sensitive and wild type counterpart, that is 2008 ovarian cancer cell lines [26,27].

We found that, also in ovarian cancer cells, CAU and CA impair mitochondrial respiration at level of complex II, both on cisplatin-sensitive (Fig. 3A; $F_{2,6} = 23.83$, $p < 0.01$) and on cisplatin-

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Table 1
Effect of algal metabolites on oxygen consumption by rat liver isolated mitochondria.

		Control	CAU	CA
Pyruvate + malate (Complex I)	V_3 (nmol $O_2 \cdot ml^{-1} \cdot min^{-1}$)	17.5 ± 0.7	13.4 ± 0.8	12.8 ± 0.9
	V_4 (nmol $O_2 \cdot ml^{-1} \cdot min^{-1}$)	5.5 ± 0.4	4.9 ± 0.4	4.7 ± 0.5
	RCR	3.2 ± 0.4	2.7 ± 0.3	2.8 ± 0.5
Succinate + rotenone (Complex II)	V_3 (nmol $O_2 \cdot ml^{-1} \cdot min^{-1}$)	59.1 ± 3.8	17.7 ± 1.1	19.2 ± 1.0
	V_4 (nmol $O_2 \cdot ml^{-1} \cdot min^{-1}$)	10.2 ± 0.9	5.1 ± 0.6	4.9 ± 0.4
	RCR	5.8 ± 0.1	3.5 ± 0.7	3.9 ± 0.2
Ascorbate + TMPD + rotenone + antimycin A (Complex IV)	V_3 (nmol $O_2 \cdot ml^{-1} \cdot min^{-1}$)	49.0 ± 2.7	47.9 ± 3.9	45.0 ± 4.2
	V_4 (nmol $O_2 \cdot ml^{-1} \cdot min^{-1}$)	8.9 ± 0.5	8.8 ± 0.5	8.9 ± 0.5
	RCR	5.5 ± 0.4	5.5 ± 0.7	5.2 ± 0.9

V_3 was the rate of oxygen uptake recorded in the presence of added ADP. V_4 was the rate of oxygen uptake observed when added ADP had been completely phosphorylated to ATP. Respiratory control ratio (RCR) was calculated as the ratios of V_3 to V_4.

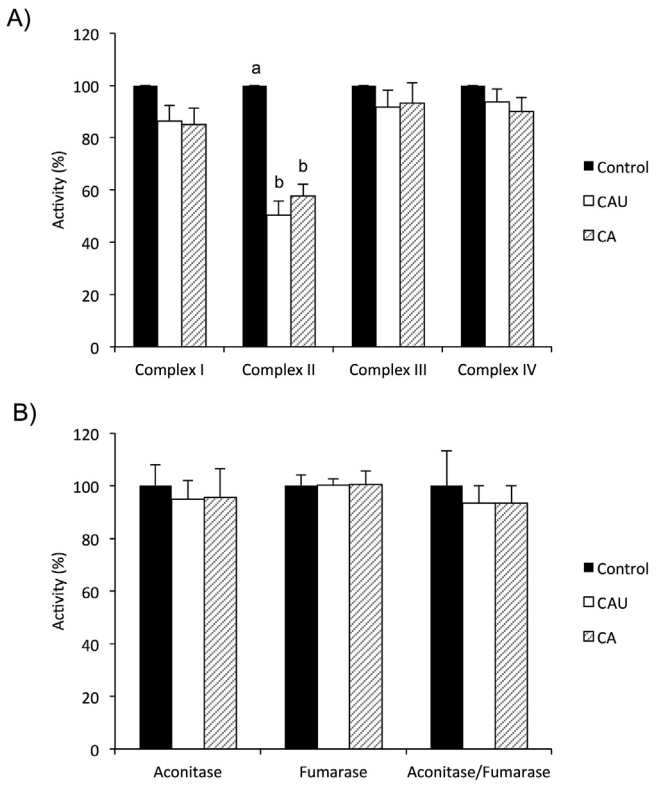

Fig. 2. Effect of algal metabolites on respiratory complexes activity (A) and on ROS production (B) in rat liver mitochondria. Rat liver mitochondria (0.3 mg of mitochondrial protein/ml) were incubated for 3 min without (control) and with 10 μM CAU or CA. Enzymatic activities were measured spectrophotometrically. Activity values recorded in the control group were set to 100%. Different letters where used to indicate, when occurring, significant differences between various exposure conditions for each mitochondrial respiratory complex ($p < 0.001$).

resistant cells (Fig. 3B; $F_{2,6} = 24.24$, $p < 0.01$). Interestingly, only C13 cells showed a defective complex I function (Fig. 3B), confirming previous observations [27] and showed a significant increase in mitochondrial ROS production (Fig. 3C; $F_{2,6} = 5.30$, $p<0.05$). Moreover, CAU and CA stimulate ROS generation (Fig. 3C) and have a toxic effect especially on proliferation of chemoresistant cells (C13; $F_{2,6} = 648.04$, $p < 0.001$) (Fig. 4). Interestingly, the effect exerted by CAU was stronger than that exerted by CA.

4. Discussions

The invasion by *C. cylindracea* and its congeneric *C. taxifolia* profoundly modified the Mediterranean marine sea-bottom landscape over the last twenty years [28]. Among the algal bioactive secondary metabolites *Caulerpa*, a variety of biological activities have been ascribed to the bisindolic red pigment CAU. In particular, it was suggested that the ability of CAU might contribute to the successful establishment of *C. taxifolia* in the Mediterranean, playing a role in defences against herbivores. Although this hypothesis supports the importance of a chemoecological approach to marine biological invasions [29], studies especially focusing on the activity of CAU on mammalian cells are limited.

In this paper we have explored the role of mitochondria as potential pharmacological targets of algal metabolites. A previous research proposed that *Caulerpa* pigment CAU inhibits mitochondrial respiration at electron transport chain complex I in breast cancer cells (T47D cells) treated with 30 μM CAU at 37 °C for 2 h, while complexes II, III, and IV remained functional [13]. Although showing that CAU is also able to slightly inhibit the growth of normal human mammary epithelial cells (HMEC), this study does not contribute to a better understanding of the action of CAU under physiological conditions. In fact, it is known that energy metabolism and ROS homeostasis in cancer cells are much different from those in normal cells [30]. Therefore, the data obtained in T47D cells could be the result of modified mitochondrial metabolism. In this regard, new evidences [31] revealed that T47D cells have a lower oxygen consumption rate, reduced ATP levels and higher proton leak, along with lower levels of cytochrome *c* oxidase, subunit IV, isoform 1.

For the first time, in the present study we showed a direct effect of CAU and its closely related derivative CA mainly on respiratory complex II under physiological conditions. This finding is consistent with the recent evidence that complexes I, III and IV are associated as super-complexes in the mitochondrial inner membrane [32]. Interestingly, in this experimental model, inhibition of complex II by CAU and CA did not stimulate ROS release. There is increasing evidence that complex II can be a major regulator of mitochondrial ROS production under both physiological and pathophysiological circumstances [33]. In doing so, it can adapt different roles as a producer or modulator of mitochondrial ROS depending on substrate supply and the activities of the other respiratory complexes and Krebs cycle enzymes. According to this hypothesis, an increase in ROS levels was observed in C13 ovarian cancer cells cisplatin-resistant in comparison to their cisplatin-sensitive and wild type counterpart, that is 2008 ovarian cancer cell lines.

The choice of these cell lines was based on previous observations that suggested an involvement of complex I activity in the metabolic reprogramming in ovarian cancer cells resistant to cisplatin [27]. Our results confirmed a defective complex I in C13 cells and demonstrated, for the first time, a toxic effect of CAU and CA on mitochondrial complex II and an increase of ROS production, suggesting that these metabolites could be good candidates for the treatment of cisplatin-resistant cancer. Previous

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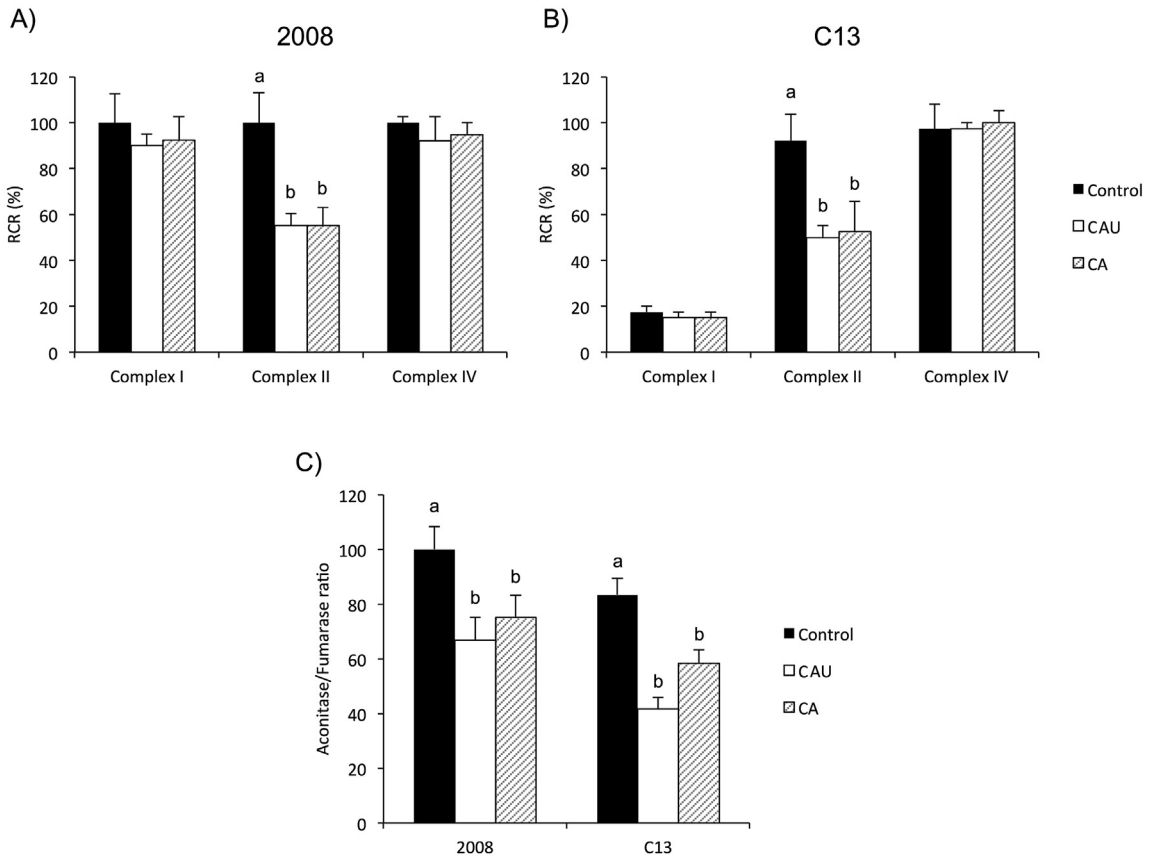

Fig. 3. Effect of algal metabolites on mitochondrial respiratory efficiency of 2008 (A) and C13 lines (B) and on ROS production by ovarian cancer cells (C). The addition of different substrates permitted to evaluate mitochondrial respiration when respiratory complexes I, II or IV were stimulated. After 2 min, state 3 respiration was induced by the addition of ADP. RCR was calculated as the ratio of the rate of oxygen uptake in the presence of added ADP (V_3) to the rate observed when added ADP had been completely phosphorylated to ATP (V_4). Aconitase and fumarase activity were determined spectrophotometrically. RCR and enzymatic activity values obtained for 2008 cells were set to 100%. Different letters indicate statistical differences, when occurring, between means of values as indicated by ANOVA and *post-hoc* comparison ($p < 0.01$).

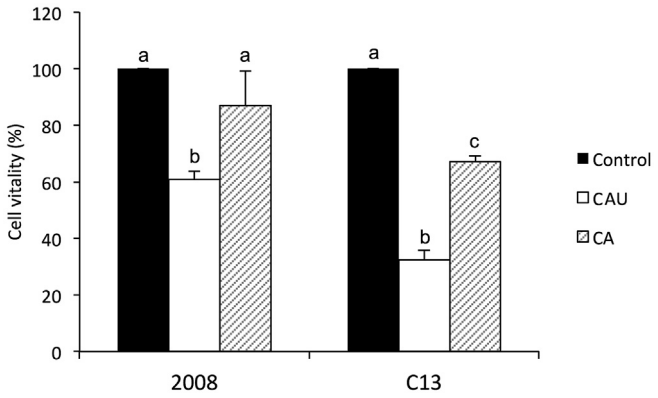

Fig. 4. Effect of algal metabolites on cell vitality of ovarian cancer cells cisplatin-sensitive (2008) and resistant (C13). Cytotoxic effect of CAU and CA on cellular vitality was evaluated by the SRB assay. Letters above columns illustrate the outcome of SNK (Student-Newman-Keuls) tests; different letters indicate significant differences at $p < 0.05$.

studies demonstrated that C13 cells have an increased glucose-uptake and consumption, increased expression and activity of the Pentose Phosphate pathway (PPP) enzyme Glucose-6-Phosphate Dehydrogenase (G6PDH) and are more sensitive to G6PDH inhibition [26]. Interestingly, recent studies on antitumor agents suggested a relationship between complex II inhibition, ROS levels increase and a suppression of PPP-derived NADPH production [34].

We are aware that more needs to be done to delineate the molecular mechanism responsible for the bioactivity of CAU and CA on ovarian carcinoma and other cancer cell lines. Nevertheless, the present study provides a preliminary insight into the potential use of algal metabolites for the prospective treatment of human cisplatin-resistant ovarian cancer. This should encourage researchers toward a possible pharmacological exploitation of high added value chemical products from invasive pests huge biomasses, paving the way for making the control of invasions profitable.

Author contributions

A.F., A.C., F.G. and A.T. conceived and designed the study; E.M. and M.G.R. purified C.A.U and C.A.; A.C. and F.C. performed the research; A.F., S.F. and V.Z. analyzed the data; A.T. coordinated the study; A.F., A.C., F.G., S.F., E.M., A.T. wrote the text.

Conflicts of interest

The authors declare no conflict of interest.

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