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SYNTHESIS, CHARACTERIZATION AND BIOLOGICAL EVALUATION OF OCTYLTRIMETHYLAMMONIUM TETRATHIOTUNGSTATE
--Manuscript Draft--

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Corresponding Author:	Lilian Beatriz Romero Sánchez Universidad Autónoma de Baja California - Campus Tijuana tijuana, Baja California MEXICO
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Universidad Autónoma de Baja California - Campus Tijuana
Corresponding Author's Secondary Institution:	
First Author:	K. Vega-Granados
First Author Secondary Information:	
Order of Authors:	K. Vega-Granados
	J. Cruz-Reyes
	J.F. Horta-Marrón
	M. Marí-Beffa
	L.J. Díaz-Rubio
	I. Córdova-Guerrero
	D. Chávez-Velasco
	M.C. Ocaña
	M.A. Medina
	Lilian Beatriz Romero Sánchez
Order of Authors Secondary Information:	
Funding Information:	
Abstract:	Octyltrimethylammonium thiotungstate (ATT-C8) was obtained by incorporating an quaternary ammonium ion substituted by three methyl radicals and one octyl radical to the ammonium thiotungstate salt (ATT).The characterization of the materials was carried out using the following spectroscopic techniques: Ultraviolet-Visible (UV-Vis), Fourier Transform Infrared (FTIR) and proton nuclear magnetic resonance (^{1} H-NRM). The biological and toxic aspects were evaluated by in vitro and in vivo assays, using bovine aorta endothelial cells (BAEC) and zebrafish (Danio rerio) embryos. The obtained results suggest that ATT-C8 has better biocompatibility, showing a significantly lower lethal concentration 50 (LC $_{50}$) value in comparison to ATT. Zebrafish embryos assay results indicate that the both thiosalts at studied concentrations increases the hatching time. Even more, in vivo assay show that synthesized materials behave as copper antagonists and have the ability to inhibit its toxicological effects. Also, both materials were found to be active for the in vitro 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay.

Response to Reviewers:	Herein, the authors acknowledge the time taken to review our manuscript and the valuable observations of the editors and reviewers. In the following points we answer each one of the reviewers´ comments and we describe in detail the corresponding modifications of the presented manuscript.

Reviewer #1:

1.The ATT-C8 compound is a salt of a cationic hydrophobic ammonium ion, specifically octyltrimethylammonium, and an anion, specifically tetrathiotungstate.   Considering that the entire paper is about this material and its biological effects, would it not be proper to provide a figure of both ATT and ATT-C8? I recommend to use a Chem Draw figure in 3-D. The anion is also categorized as a coordination complex.

Answer: The reviewer recommends including a figure of the synthesized compounds in the manuscript. The authors agree with the importance of showing the molecular structures of the synthesized thiotungstate salts and the 2D and 3D structures have been added. The figures are now in the manuscript as Fig. 1, and in the supplementary information as Fig. S3 and S4.

2.The description of ATT-C8 as being obtained by incorporating an quaternary ammonium ion substituted by three methyl radicals and one octyl radical to the ammonium thiotungstate salt (ATT) is very poor and implies that radicals are involved. This is very bad chemistry, because ammonium salts are prepared by nucleophilic reactions and not radical reactions. I was very concerned when reading this, because this flies in the face of fundamental chemistry and is so bad that it frankly would justify rejection of this paper. However, after careful consideration, and realizing that the biology of this work is of better quality, I will upgrade that decision to major revision.

Answer: The authors are in agreement with the reviewer´s comments. In the first sentence, we described the ATT-C8 molecule, however, it was not very clear. In this regard, the sentence has now been corrected and it has been added in the experimental section: ATT-C8 was prepared by the reaction between ATT (1 equivalent) and octiltrymethylammonium bromide (2 equivalent) in aqueous solution following the method reported by Alonso et al(Alonso et al., 1998, 2000).

3.Similarly the first line of the introduction is inappropriate. This paper is about the biological effects of this salt and the fact that tetrathiotungstate readily chelate copper and thus act as a copper antagonist. This should be described in the first line of the manuscript.

Answer: We are in agreement with the reviewer and the abstract and introduction has been modified. The first line now indicates the biological study that has been made, regarding the ability of the thiotugstate salts as copper chelating agents and then the biological assays are described.

4.However, examining the paper the authors need to fix their chemistry part. Specifically they need to improve the synthesis description - one needs to use the same number of significant figures in the g and mol values. So it is not (15 g, 5.07 mmol) - but 15.x g, 5.07 mmol or 15 g, 5.1 mmol. This needs to be fixed through-out the experimental.

Answer: We acknowledged the suggestion and the quantities have been corrected having the same significant figures.

5.The NMR and other data needs to be added after the syntheses in the experimental section and not as Table 1.

Answer: We are in agreement with this comment, and the spectroscopic results have been added right after the experimental part and Table 1 has been eliminated.

6.Generally, to sure that the proper material is prepared it would be desirable if an elemental analysis or a mass spectrum was obtained; the data provided is not generally enough to characterize new compounds.

Answer: The authors thank the reviewer for this valuable suggestion, however, the synthesis of tetraalkylammonium thiometallates has been widely studied in the field of catalysis and we consider that the employed characterization techniques allow us to make a proper identification.

7.The reaction shown as
$(NH_4)_2WS_4 + 2[CH_3-(CH_2)_7-N(CH_3)_3]Br \rightarrow [CH_3-(CH_2)_7-N(CH_3)_3]_2WS_4 + 2(NH_4)Br$

is a simple cation exchange reaction and not a metathesis reaction. This is VERY WRONG.
Answer: The authors have taken into account the suggestion of the reviewer.

8.Fig. 1 should be removed, it is of substandard quality for the manuscript and frankly does not belong there but could be placed as is in the supplemental material. Something is wrong with the baseline of Figure 1. Similar recommendation for Figure 2, although those are better spectra. The data should be listed after the experimental synthesis.
Answer: The authors agree with the reviewer and we have added the spectroscopic results right after the experimental synthesis section. The spectra (Figures 1 and 2) have been added into the supplementary information section.

9.In the conclusion - ATT-C8 is described as a new bioinorganic material - when in fact it is simple a new salt of tetrathiotungstate. This should be fixed.
Answer: We agree with the reviewer and the suggested modifications have been taken into account.

10.The statement in the conclusion: "Our results show that ATT and ATT-C8 neutralize free radicals, a property useful for treatments of oxidative stress-induced degenerative diseases." Is really misleading. There are no free radical formation and it is very poor science to suggest this. The authors should focus on the ability to chelate the copper

Answer: Regarding the comment of the reviewer, we have corrected the mentioned sentence and we have improved the rest of the conclusions.

Finally, we thank the reviewers and editors for the evaluation of our manuscript. We considered all of the reviewers' comments and suggestions and we think our work has improved substantially. We look forward for your feedback.

SYNTHESIS, CHARACTERIZATION AND BIOLOGICAL EVALUATION OF OCTYLTRIMETHYLAMMONIUM TETRATHIOTUNGSTATE

Karla Vega-Granados[a], Juan Cruz-Reyes[a], José F. Horta-Marrón[a], Manuel Marí-Beffa[b], Laura J. Díaz-Rubio[a], Ivan Córdova-Guerrero[a], Daniel Chávez-Velasco[c], M. Carmen Ocaña[d], Miguel A. Medina[d], Lilian.B. Romero-Sánchez[a*].

[a] Faculty of Chemical Sciences and Engineering, Autonomous University of Baja California, 22390 Tijuana, B.C., Mexico.

[b] Department of Cell Biology, Genetics and Physiology, University of Malaga, 29071 Malaga, Spain.

[c] Center for Graduates and Research in Chemistry, National Technological Institute of Mexico/ Technological Institute of Tijuana, 22510 Tijuana, B.C., Mexico.

[d] Department of Molecular Biology and Biochemistry, Faculty of Science, University of Malaga, 29071 Malaga, Spain.

* Corresponding author: Faculty of Chemical Sciences and Engineering, Autonomous University of Baja California (UABC), Universidad 14418, UABC, Parque Internacional Industrial Tijuana, 22390 Tijuana, B.C., Mexico.
e-mail:lilian.beatriz.romero.sanchez@uabc.edu.mx (Romero-Sánchez).
**ORCID:** http://orcid.org/0000-0001-8518-5590

Abstract

Octyltrimethylammonium tetrathiotungstate salt (ATT-C8) was synthesized and its ability to chelate copper was evaluated. The biological and toxic aspects were evaluated by *in vitro* and *in vivo* assays, using bovine aorta endothelial cells (BAEC) and zebrafish (*Danio rerio*) embryos. The obtained results suggest that ATT-C8 has better biocompatibility, showing a significantly lower lethal concentration 50 (LC_{50}) value in comparison to ammonium tetrathiotungstate (ATT). Zebrafish embryos assay results indicate that both tetrathiotungstate salts at the studied concentrations increase the hatching time. Even more, an *in vivo* assay showed that synthesized materials behave as copper antagonists and have the ability to inhibit its toxicological effects. Also, both materials were found to be active for the *in vitro* 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. The characterization of the materials was carried out using the following spectroscopic techniques: Ultraviolet-Visible (UV-Vis), Fourier Transform Infrared (FTIR) and proton nuclear magnetic resonance (^{1}H-NRM).

Keywords: Tetrathiotungstate salts, Zebrafish embryo, Antioxidant activity, Anticopper therapy, Toxicity.

Highlights

- The incorporation of alkyl chains improves the *in vivo* biocompatibility of ATT.
- The ATT and ATT-C8 materials showed antioxidant activity in the DPPH assay.
- The materials have interesting properties for its use in anticopper therapies.

Graphical Abstract
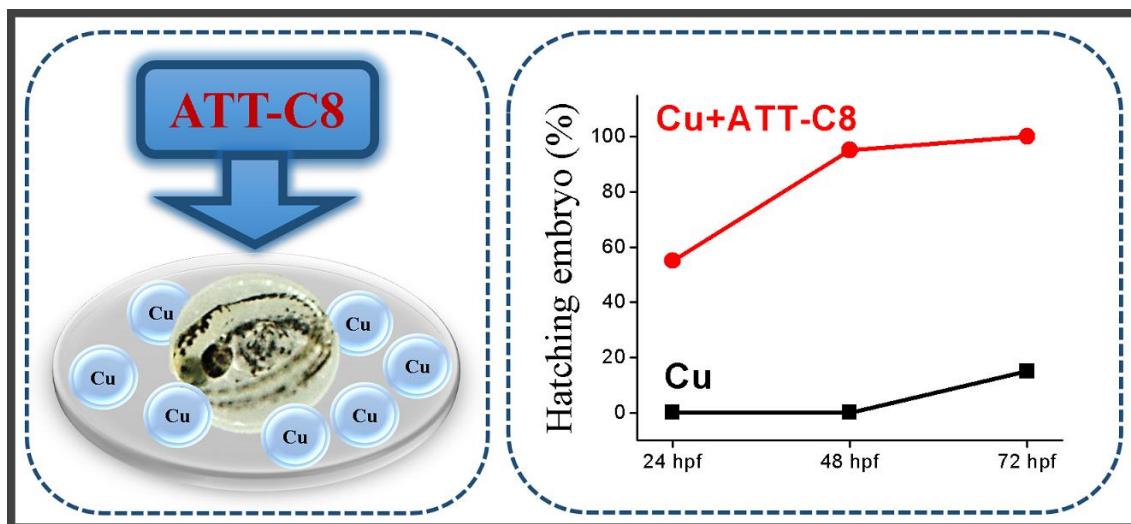
Declaration of interests

☒ 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:

Sample CRediT author statement

Karla Vega-Granados: Investigation, Writing-Reviewing and Editing.

Juan Cruz-Reyes: Conceptualization, Funding acquisition.

José F. Horta-Marron: Methodology, Data curation.

Manuel Marí-Beffa: Conceptualization, Visualization.

Laura J. Díaz-Rubio: Formal analysis.

Ivan Córdova-Guerrero: Resources.

Daniel Chavez-Velazco: Validation.

M. Carmen Ocaña: Investigation.

Miguel A. Medina: Supervision.

Lilian B. Romero-Sanchez: Writing-Original draft preparation, Project administration.

1. Introduction

Diverse studies suggest that, as ammonium tetrathiomolybdate (ATM), ammonium tetrathiotungstate (ATT) present excellent chelating properties (McQuaid et al., 1994; Young et al., 1982). ATM´s chelating properties have been thoroughly studied in the treatment of Wilson's disease (WD)(Brewer, 2009; Brewer et al., 1994, 1991). ATM forms complexes with copper and albumin that decrease the bioavailability of copper in the blood. Nowadays, ATM is in phase II and phase III of multinational clinical trials as an anticopper therapy for the treatment of neurological symptoms in patients with WD(Aggarwal & Bhatt, 2018; Brewer et al., 1996). More recently, it was found that ATM has excellent efficacy in the treatment of animal models of fibrotic, inflammatory, and autoimmune diseases, as well cancer(Brewer, 2003, 2005, 2016; Brewer et al., 2000). Nevertheless, research of the potential of ATT treatments against degenerative diseases is scarce, compared to ATM.

The catalytic activity in hydrotreatment processes of the sulfides obtained from the decomposition of these thiometallates, MoS_2 and WS_2, shows an increase when presenting superficial carbon available from the synthesis method, generating the $MoS_{2-X}C_X$ y $WS_{2-X}C_X$ species(Berhault et al., 2001; Chianelli & Pecoraro, 1981; Kelty et al., 2007; Pecoraro & Chianelli, 1985). With this knowledge, new synthesis routes have been developed, where the carbon is dosed during the process, in the form of alkyl chains, generating the tetraalkylammonium thiometallate precursors, $(NR_4)_2MS_4$, (where R=alkyl radical and M= Mo, W). There was a notable advance in the synthesis of these materials when Alonso et al. prepared the $(NR_4)_2MS_4$ precursors (where $R = H$, CH_3, C_4H_9 and M =Mo, W) in aqueous media, which afterwards were decomposed into MoS_2 and WS_2(Alonso et al., 1998, 2000). In this sense, it would be of interest to evaluate the influence of superficial carbons dosed by alkyl chains in the chelation properties of salts derived from these compounds when administered to animal models.

Zebrafish embryo is becoming a new model of choice for preclinical studies. Several interesting tests that use these embryos have been proposed as rapid, high- throughput, and cost- effective drug and chemical screens(Dooley & Zon, 2000; García-Caballero et al., 2018; Tobia et al., 2011; Tran et al., 2007; Wilkinson & Van Eeden, 2014). A number of different biological aspects such as toxicity, inflammation, organ regeneration or angiogenesis are studied in these screens to test drugs and biomaterials as chemical modulators. Recent studies with zebrafish embryos have evaluated the pro-angiogenic modulation of copper either directly administrated to water or lixiviated from biomaterials(Romero-Sánchez et al., 2018).

The objective of the present work is to explore the biological aspects and the copper chelating ability of a new tetrathiotungstate salt. The study includes the antioxidant activity and toxic effects of a modification of ATT with an alkyl group (octyltrimethylammonium tetrathiotungstate, ATT-C8). With this objective, the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, *in vitro* endothelial cell viability and migration assays, and an *in vivo* toxicity zebrafish embryo assay were used.

2. Methods and materials

2.1. Spectroscopy characterization and molecular modeling

The chemical structures of ATT and ATT-C8 compounds were characterized using the spectroscopic techniques of Ultraviolet-Visible (UV-Vis), Fourier Transform Infrared (FTIR) and proton nuclear magnetic resonance ([1]H-NRM). UV–Vis data was obtained using a HACH DR-6000 UV–Vis Spectrometer in the λ= 200–700 nm range. FTIR spectra were acquired in transmission configuration with a Perkin-Elmer Spectrum GX over the range of 4000–400 cm^{-1}. ^{1}H-NMR spectra were recorded on a Bruker Avance III spectrometer 400 MHz. The chemical shifts (δ) are presented with tetramethylsilane (TMS) (δ: 0.00) as the internal standard. The 2D and 3D-structure molecular modeling of the synthesized compound was plotted using ChemDraw Professional 16. The UV-Vis, FT-IR and RMN spectra with their corresponding data, as well as the 3-D molecular structure of the obtained tungsten salts are available in the supplementary information section.

2.2. Synthesis of materials

2.2.1. Synthesis of ATT

ATT ((NH_4)$_2WS_4$) was prepared according to the method described by Ramanathan(Ramanathan & Weller, 1985). Ammonium metatungstate ((NH_4)$_6H_2W_{12}O_{40}$x$18H_2O$; Sigma-Aldrich) (15.0 g, 5.1 mmol) is dissolved in 75.0 mL of distilled water containing 52.5 mL of an ammonium hydroxide solution (NH_4OH (20%); Sigma-Aldrich). The resulting ammonia solution was heated in a temperature bath to 60 °C for 6 h, while hydrogen sulfide was bubbled (H_2S (g)) into the solution. The reaction mixture was then cooled in an ice bath and allowed to stand for 12 h, yielding yellow crystals. The product was vacuum-filtered, washed with isopropyl alcohol (C_3H_8O; Sigma-Aldrich) stored under N_2 atmosphere at 15 °C and it was named as ATT. The molecular structure of ATT is shown in Fig. 1a. UV-Vis λ_{max} (nm): 216, 276, 393. FTIR (cm^{-1}): 460 (v Mo-S); 3128, 1395 (v N-H). ^{1}H-NMR ($CDCl_3$): δ 7.14 (s, 8H, (N**H$_4$**)$_2$).

2.2.2. Synthesis of ATT-C8

ATT-C8 was prepared by the reaction between ATT (1 equivalent) and octyltrimethylammonium bromide (2 equivalent) in aqueous solution following the method reported by Alonso et al(Alonso et al., 1998, 2000). Freshly prepared (NH_4)$_2WS_4$ ATT (2.0 g, 5.7 mmol) was dissolved in 25.0 mL of distilled water. While stirring, 25.0 mL of a second aqueous solution was added, containing octyltrimethylammonium bromide ($CH_3(CH_2)_7N(Br)(CH_3)_3$; Sigma-Aldrich) (2.9 g, 11.5 mmol). The reaction mixture is kept at rest for 12 h and the resulting precipitate is recovered by sedimentation using a centrifuge at 2000 rpm. Finally, the yellow product ATT-C8 (($CH_3(CH_2)_7N(CH_3)_3$)$_2WS_4$) was dried in an oven at 30 °C and stored, being named as ATT-C8. The molecular structure of ATT-C8 is show in Fig. 1b. UV-Vis λ_{max} (nm): 216, 276, 393. FTIR (cm^{-1}): 460 (v Mo-S); 3128, 1395 (v N-H); 3005, 2922, 2852 (v C-H); 1468 (δ C-H). ^{1}H-NMR ($CDCl_3$): δ 3.34 (m, 11H, **CH$_2$**-N), 3.08 (s, 18H, **CH$_3$**-N), 1.68 (m, 4H, **CH$_2$**-CH$_2$-N), 1.30 (m, 20H, (**CH$_2$**)$_5$-CH$_2$-CH$_2$-N), 0.87 (t, 6H, **CH$_3$** terminal).

2.3. Antioxidant activity

The antioxidant capacity of the synthetized materials was evaluated using the DPPH radical scavenging method proposed by Blois(Blois, 1958). In the method, the DPPH ($C_{18}H_{12}N_5O_6$; Sigma-Aldrich) free radical is dissolved showing a purple coloration, that upon reduction by an antioxidant or a radical species, changes to yellow due to an electron or proton transfer. The change in absorbance is spectrophotometrically monitored at a wavelength of 517 nm. The DPPH radical scavenging effect was calculated according the following equation:

$$DPPH\text{-}scavenging\ effect\ (\%) = \left[1 - \left(\frac{Absorbance\ sample}{Absorbance\ control} \right) \right] \times 100$$

The half maximal inhibitory concentration (IC_{50}) value was interpreted as the concentration of antioxidant material necessary to scavenge 50% of the initial DPPH radicals. The IC_{50} values were calculated by linear regression resulting from plotting the % of DPPH-scavenging activity versus the concentration of the test samples. Analyses were performed in three independent assays.

2.4. Cell assays

2.4.1. Cell culture

Bovine aortic endothelial cells (BAEC) were isolated from bovine aortic arches as previously described(Cárdenas et al., 2006) and maintained in Dulbecco's modified Eagle's medium (DMEM) containing glucose (1 g/L) and supplemented with glutamine (2 mM), penicillin (50 U/mL), streptomycin (50 U/mL) and 10% FBS. Cells were maintained at 37 ºC under a humidified 5% CO_2 atmosphere.

2.4.2. Cell viability assay

2.5×10^3 cells were seeded in 96-well microplates using a total volume of 100 µl of culture medium containing different concentrations of the ATT and ATT-C8 materials. Cells were incubated for 72 h (37 ºC, 5% CO_2 in a humid atmosphere). Then, 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the wells and incubated an additional 4 h in the dark. HCl isopropanol was used for resuspension of formazan crystals and absorbance data (550 nm) was collected using an Eon Microplate Spectrophotometer from Bio-Tek Instruments (Winooski, VT, USA). Data was collected by Gen5 software from the same manufacturers. Half-maximal inhibitory concentration (IC_{50}) values were calculated as the concentrations of compound yielding 50% cell survival, taking the values obtained for control as 100%.

2.4.3. Cell migration assay

Migration of BAEC was assessed using the so-called "wound-healing" assay. Cells were grown, and once they reached confluence, different concentrations of ATT and ATT-C8 were added with fresh medium and a cross-shaped scratch was done using a pipette tip. Wounded areas were observed under a microscope after 4, 7 and 24 h of incubation, photographs were taken from the same areas as those recorded at zero time. Images were analyzed with NIH Image J 1.6 software. The regrowth of BAEC into the cell-

free area was expressed as the percentage of the initial wounded area (time 0) recovered by cells migration to different incubation times.

2.5. Zebrafish embryo assays

2.5.1. Zebrafish collection

Zebrafish (Danio rerio) embryos were obtained from fish facilities at the University of Malaga following standard procedures(Kimmel et al., 1995; Truong et al., 2011).

A breeding stock of adult zebrafish was kept on a recirculating system at $27 \pm 1°C$. Fishes were maintained with a standard 12:12-hour light–dark photoperiod. Embryos were produced by one-to-one or one-to-two female-male mating in fish hatch box. Fertilized eggs are collected and disinfected using a 0.5% bleach solution during 1 min and washed several times with E3 embryo media (5.00 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 0.00003% $C_{16}H_{18}ClN_3S$; Sigma-Aldrich). Clean eggs were incubated in E3 medium in a Petri dish at 28 ± 0.1 °C.

Fishes were handled under National Laws (Law 9/2003, April 25, and Law 31/1995, November 8) by notification A/ES/12/I-22 and activity A/ES/12/24. The experiments were carried out under the procedures established and approved by the UMA Bioethics Commission under the BIO2014-56092-R grant.

2.5.2. Zebrafish embryo exposure assay

At 24 hours after fertilization (hpf) 15 of zebrafish embryos were transferred well of a 6-well plate. The embryos were incubated in 3 mL of culture medium with different concentrations of the ATT and ATT-C8 materials (0.1, 10, 100, 250, 750, 1000, 10000 µg/ml) for 96 h at 28 °C. All of the solutions with different material concentrations were prepared in E3 medium. The survival rate of the embryos from each treatment group was determined. Zebrafish were observed directly on the 6-well plate using a Nikon AZ100 multizoom microscope. The hatching rate and changes in morphology throughout development were also evaluated, as well as the tetrathiotungstates effect in the copper induced embryo toxicity. Zebrafish embryos were exposed to control (10 µg/mL $CuCl_2$) and two experimental (10 µg/mL $CuCl_2$, 250 µg/mL ATT; and 10 µg/mL CuCl2, 250 µg/mL ATT-C8) conditions and their hatching rate studied. All assays were repeated three times and at least 60 embryos were used per concentration. Mortality curves were generated using MS Excel 2016 and the lethal concentration 50 (LC_{50}) was calculated applying a nonlinear regression test (sigmoidal dose-response curve). LC_{50} indicates the concentration that kills half of tested population in zebrafish embryo model.

2.6. Statistical analysis

Data are shown as mean ± standard deviation (SD; 99% confidence interval) resulting from triplicate evaluations. Statistical significance was calculated using a Student t-test of control-experimental group pairs using the Statistical Package for the Social Sciences (SPSS) version 25 software (IBM, SPSS). Differences were considered significant when p-values are $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

3. Results and discussion

3.1. Antioxidant activity

The DPPH scavenging activity percentage of tetrathiotungstates at varying concentrations was measured and the results are displayed in Fig. 2.a. Significant DPPH radical scavenging activity was evident at all the tested concentrations of ATT (1.25 - 50 µg/mL) and ATT-C8 (0.63 - 30 µg/mL). It was found that the DPPH scavenging effect of tetrathiotungstates was dose dependent.

Fig. 2.b displays the DPPH IC_{50} values of ATT and ATT-C8 calculated by the regression equation of the calibration curve. The lower the IC_{50} value means higher DPPH radical scavenging of tetrathiotungstates, implying a higher antioxidant activity. ATT-C8 ($p<0.001$) was found to have better free radical scavenging ability in comparison with ATT, having an IC_{50} value of 15.04 ± 0.01 µg/mL and 46.64 ± 0.36 µg/mL, respectively. The capacity of materials to scavenging free radicals could be attributed to the electron donor nature of the substituents -NH and $-CH_3$ present in the samples, since they are known to be good hydrogen donors(Schubert et al., 1962).

Antioxidants are molecules with the ability to eliminate or neutralize the free radicals responsible for the oxidative stress associated with cancer, arteriosclerosis, inflammatory processes and degenerative diseases(Goodman et al., 2011). In fact, bioactive materials studies use antioxidant capacity as an indicator of biological activity. However, in the knowledge of the authors, there are not existent reports of the antioxidant properties of this type of coordination compounds. Thus, although the results cannot be compared with other bioinorganic materials, the IC_{50} values for ATT and ATT-C8 are important compared to some DPPH assay reference antioxidants, such as the butyrated hydroxyl toluene (BHT) (19.4-86.6 µg/mL), vitamin C (5.8-110.7 µg/mL) and tocopherol (27.1-96.0 µg/mL) (Mishra et al., 2012).

3.2. Cell assays

In this study, BAEC was incubated in the presence of ATT and ATT-C8 materials to determine their influence on cell growth and cell migration capacity. Fig. 3 displays the effects in cellular growth measured up to 72 hours-culture, in the presence of ATT and ATT-C8 solutions with concentrations of 0.1-200 µg/mL. The IC_{50} values of 1.02 ± 0.24 µg/mL and 4.27 ± 0.95 µg/mL were obtained for ATT and ATT-C8 respectively, showing that both materials have cytotoxic effects over BAEC cells; with ATT presenting more acute toxic effects with respect to ATT-C8.

The wound healing assay with BAEC was used to evaluate the inhibitory or stimulating effects on angiogenesis. At short periods (4 and 7 h), cell wound filling area is due exclusively to its migration and not to its proliferation. Fig. 4 shows cell migration after 4, 7 and 24 h of the wound healing assay compared to the control group. After 4 h, a slight promotion in cellular migration was observed for the ATT-C8 culture at low concentration (2 µg/mL), in comparison to the bare medium control culture. However, there were no significant differences between the experimental BAEC cultures, suggesting that the studied materials have no angiogenic influence at the studied concentrations (Fig. 4).

3.3. Zebrafish embryo assays

Developmental toxicity and biological responses of zebrafish embryos exposed to an ATT and ATT-C8 dissolution were evaluated. Fig. 5 shows the survival rate of zebrafish embryos treated with

8

different concentrations of tetrathiotungstates for several days. The control group, without the addition of tetrathiotungstate salts, presented a normal behavior with an overall mortality rate <1%. The tetrathiotungstate salts induced lower than 5% mortality zebrafish embryos incubated at 0.1 - 100 μg/mL concentrations.

At 72 hpf, embryos under these concentrations were anatomically indistinguishable from control (Fig. 5a and 5b). The absence of sublethal effects, such as the lack of instant motility, depigmentation and development of edemas or clots, indicate that at the studied concentrations (1-100 μg/mL), ATT and ATT C-8 do not have any toxic or teratogenic effects in zebrafish embryos. A significantly higher mortality rate than the one the measured in the control group was initially observed at a concentration of 250 μg/mL of tetrathiotungstate salts (Fig. 6), and there was a 0% survival rate when the embryos were exposed to concentrations above 1000 μg/mL of ATT for more than 48 h and above 10000 μg/mL ATT-C8 for more than 24 h.

Fig. 7 shows the mortality rate plotted for embryos exposed to ATT and ATT-C8 materials for 24, 48 and 72 h. The LC_{50} values of ATT and ATT-C8 (Table 1) were estimated based on these mortality curves. ATT shows a concentration and time dependent toxic profile with an LC_{50} of 1211 ± 84, 542 ± 57 and 316 ± 68 μg/mL at 24, 48 and 72 h of exposure, respectively. Consistent with the obtained IC_{50} values in the cell viability assay, zebrafish is more sensitive to the ATT sample at 72 h of exposure. In the case of ATT-C8, constant IC_{50} values were observed at around 700 μg/mL (p<0.001) at 24, 48 and 72 h of exposure. In this regard, the results suggest that the incorporation of carbon into the structure improves ATT biocompatibility.

Fig. 8 displays the hatching rate of zebrafish embryos incubated for 72 h to the 250 μg/mL solutions of tetrathiotungstate salts. The results show an increase in the hatching rate of zebrafish embryos of the groups exposed to tetrathiotungstate salts, compared to the control group. All of the embryos exposed to ATT and ATT-C8 hatched at 72 hpf. It is known that the activity of chorionic hatching enzymes (ZHE) and embryo movements affect their hatching time(Nechaev & Pavlov, 2004). In this case, the embryo membranes were very deteriorated just before hatching, suggesting that the studied compounds do not inactivate the involved enzymes or embryo motility. Other reports mention that the hatching of zebrafish embryos is plastic in response to potential stress changes or changes in the salinity of the environment(Ord, 2019; Warkentin, 2011). In this way, it could be inferred that an increase in the concentration of external ions causes moderate hypoxia that accelerates the hatching time. Overall, tetrathiotungstate salts were found to accelerate the hatching rate of zebrafish embryos at low concentrations and cause acute death or chronic lethal toxicity at high concentrations.

In order to evaluate whether tetrathiotungstate salts revert the copper-mediated embryo toxicity, embryos were treated with 10 μg/mL of $CuCl_2$ and a mixture of 10 μg/mL $CuCl_2$ and 250 μg/mL of ATT or ATT-C8 for 72 h. It is well documented that copper interferes with the enzymatic activity of the ZHE, delays hatching and induces abnormal morphology and mortality(Lin et al., 2013; Romero-Sánchez et al., 2018). The obtained results show that copper significantly delays hatching up to 96 hpf and 120 hpf, in good agreement with reported results. Tetrathiotungstate salts thus significantly inhibit the delaying effect of copper in the hatching process (p<0.001). Fig. 9 shows that 90% of embryos incubated into the mixture of copper and tetrathiotungstate salts hatch at 48 hpf. All these results suggest that tetrathiotungstate salts

could be effective protectors against copper toxicity. As reported for ATM and ATT, ATT-C8 also acts as a copper antagonist, becoming an alternative chelating agent in anticopper therapies.

5. Conclusions

The copper chelating abilities of ATM and its use in clinical assays have opened new research opportunities for treatments against diseases where an increase of copper levels is involved. In this work, we have studied the bioactive potentialities of a new tetrathiotungstate salt, ATT-C8 in different *in vitro* and *in vivo* assays.

Our results show that ATT and ATT-C8 behave as copper antagonists. This copper chelating ability of the tested tetrathiotungstate salts was evaluated in a zebrafish embryo hatching assay, validating their potential use in anticopper therapies. Toxicity results suggest that the materials have toxic effects in BAEC cells and zebrafish embryos over a determined concentration, which increase at higher doses and exposure time. This effect ameliorates (72 h data) with the addition of the eight carbon residues, cell migration increases and zebrafish hatching time decreases. Further studies of these promising compounds are needed to strictly define these dose-dependent effects and to analyze the stability of compounds. It is interesting to note that the results of the *in vitro* assays suggest that the tetrathiotungstate salts could neutralize free radicals (antioxidant activity), a property that is useful in the treatment of oxidative stress-induced degenerative diseases.

6. Acknowledgements

7. References

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Supplementary information

Spectroscopy characterization and molecular modeling

Fig. S1.a shows the electronic spectra obtained for dissolved thiotungstates methanol. Three main transition bands corresponding to the $(WS_4)^{2-}$ tetrahedral group at 216, 276 and 393 nm were detected, in good agreement with the literature for W complexes(Alonso et al., 1998; McDonald et al., 1983). In addition, the band broadening of the maximum wavelength at 216 nm due alkyl groups was observed.

Transmission FTIR spectra of the samples are shown in Fig. S1.b Some bands are observed in the region of 4000 - 400 cm^{-1} of the spectra attributed to the organic part of the ammonium groups(Yi et al., 2014). Both of the synthesized materials have a notable absorption band at around 460 cm^{-1} ascribed to the stretching vibration of the W-S bond, in accord with literature reports(Alonso et al., 1998). In the FTIR spectra, the N-H deformation and stretching vibrations of NH_4^+ ions were visible at 3128 and 1395 cm^{-1}. The FTIR spectrum of the ATT-C8 sample shows new absorption bands associated with the presence of C-H bonds, suggesting a successful ion exchange between the ammonium and octyltrimethylammonium ions from ammonium thiotungstate and octyltrimethylammonium bromide, respectively. The bands visible at 2922 and 2852 cm^{-1} are attributed to the asymmetrical stretching vibration of the C-H bonds of the alkyl groups ($-CH_2-$). The C-H asymmetrical bending vibration is observed at 1468 cm^{-1} and it is ascribed to the CH_3 group at the end of the alkyl chain. In the 1350-900 cm^{-1} region some weaker bands appear, which are caused by CH_2 wagging, twisting, and rocking vibrations. Finally, the band at 3005 cm^{-1} arises from the two unresolved components of the CH_3 asymmetric stretching(Senden, 1965).

The ^{1}H-NMR spectra of the ATT and ATT-C8 materials are displayed in Fig. S2. For the ATT sample, there is a singlet at 7.14 ppm, due to the integrated signal coming from the eight protons from the thiotungstate salt. In the spectrum of the ATT-C8 sample, there are signals associated with the five different types of protons present in the material. The first signal, at 3.08 ppm is attributed to the 18 protons in the three methyl groups bound to the nitrogen atom (CH_3-N); there is a multiplet at 1.68 ppm attributed to the 20 protons of the alkyl chain, and another multiplet at 0.87 ppm due to the integrated signal of 6 protons (J= 8 Hz) that correspond to the terminal methylene of the alkyl chain. Lastly, a multiplet can be observed at 3.34 ppm, due to the integrated signal of the methylene groups in the alpha position to the nitrogen (CH_2-N), in this signal in particular, the unfolding and integration could be due to a present impurity in the material.
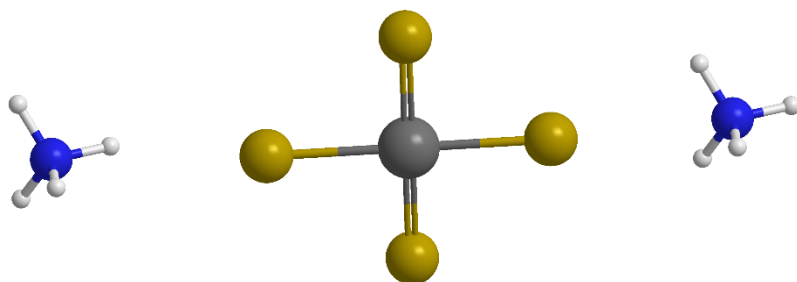
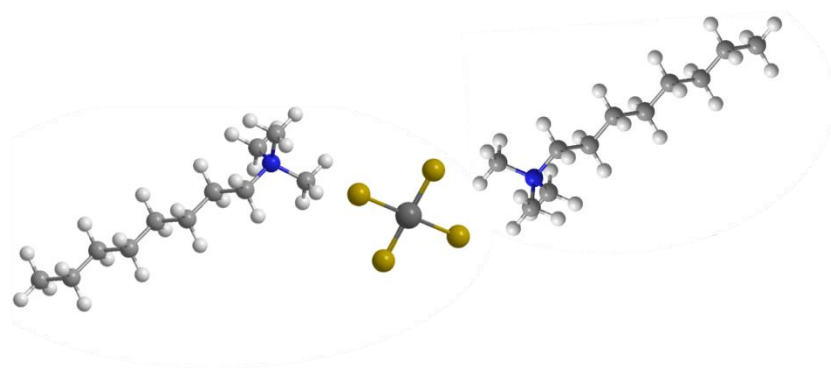The 3-D molecular structures of ATT and ATT-C8 are shown in Fig. S3 and S4.
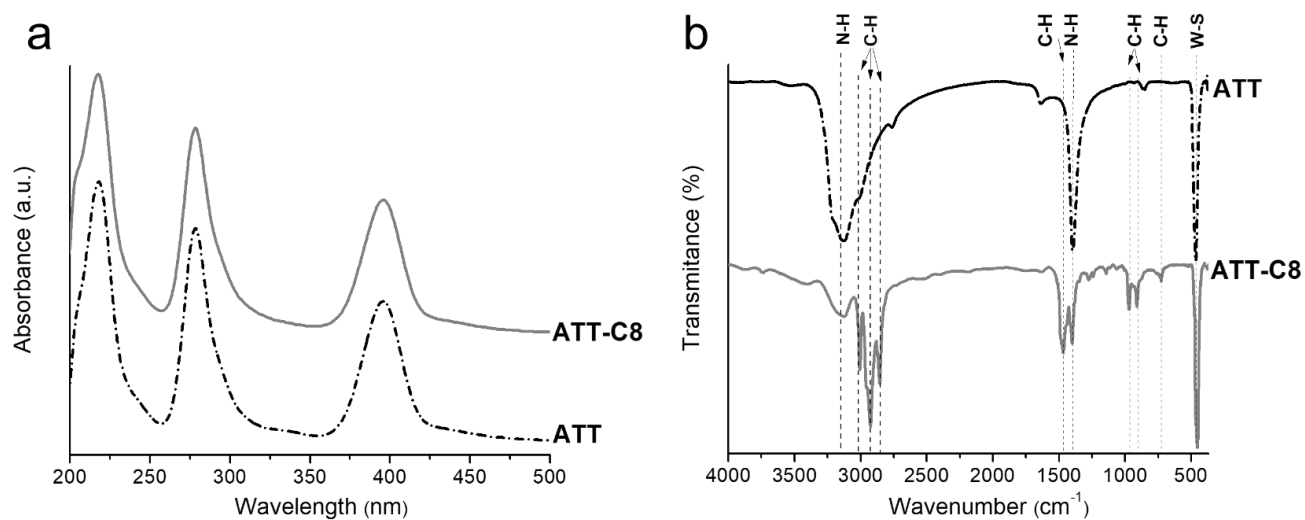
Fig. S1 Characterization of ATT and ATT-C8 materials: (a) UV-Vis analyses and (b) FT-IR
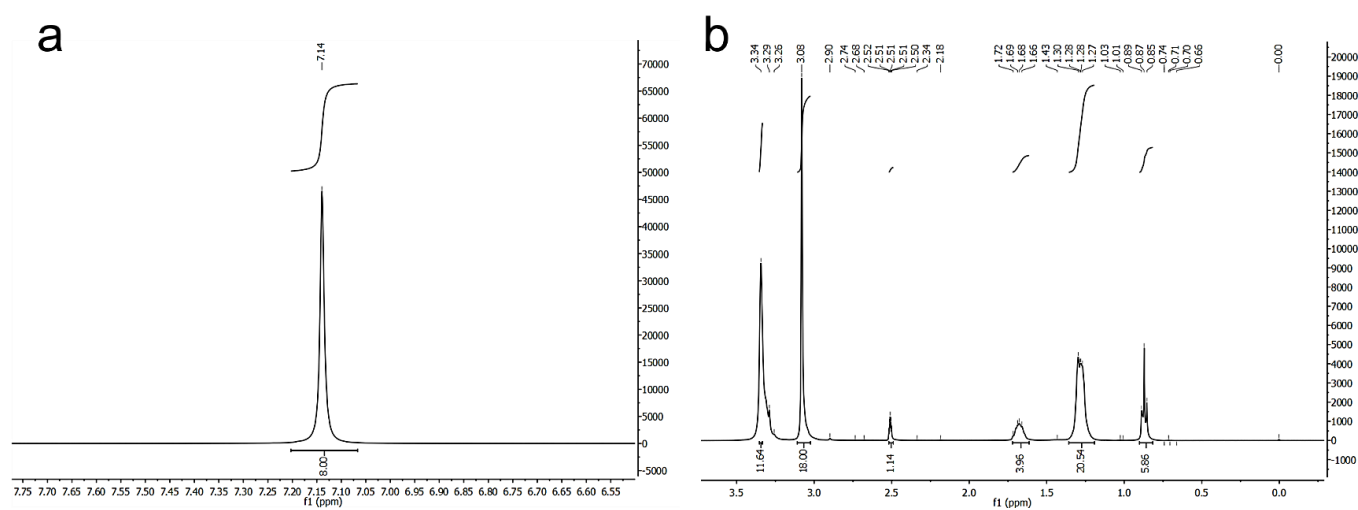
Fig. S2 ^{1}H-NMR spectra: (a) ATT and (b) ATT-C8

Fig. S3. 3D-structure molecular of ATT salt

Fig. S4. 3D-structure molecular of ATT-C8 salt

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FIGURES
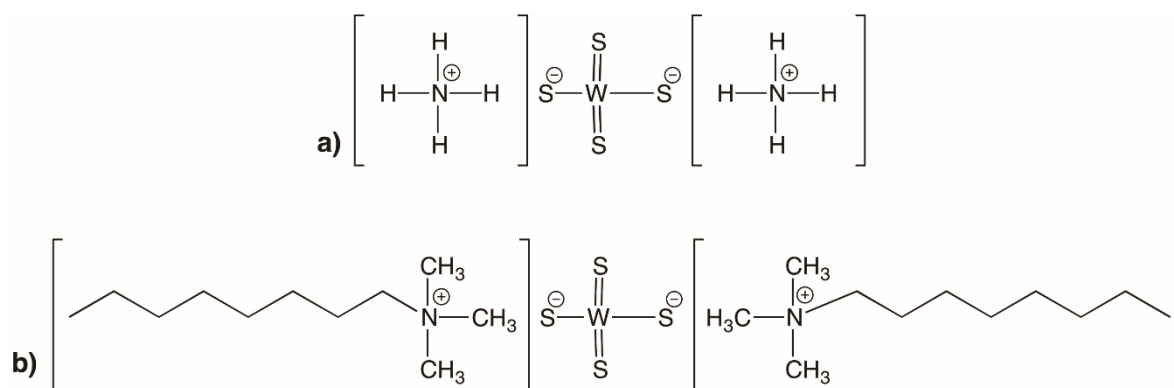
Fig. 1 Structure of the synthesized compounds **a)** ATT and **b)** ATT-C8
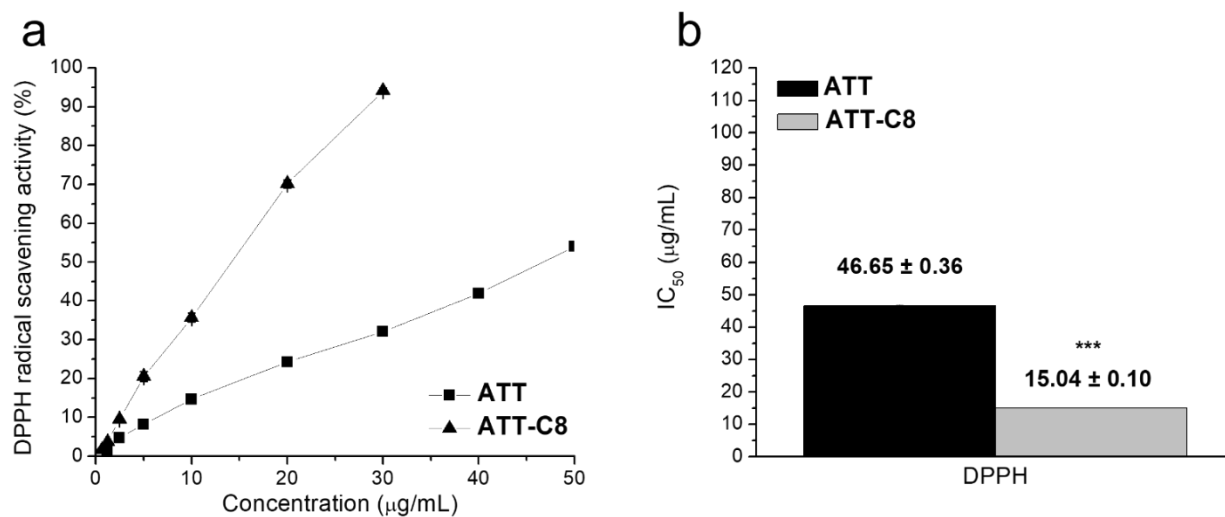
Fig. 2 DPPH free radical scavenging assay results: (a) Concentration-response curve of antioxidant activity and (b) Bar graph with IC_{50} (µg/mL) values of synthesized materials. Values represent mean ± SD of three replicates. The figure includes the statistical significance of the ATT-C8 group measured with respect to the control group (ATT); ***$p < 0.001$ by Student's t-test
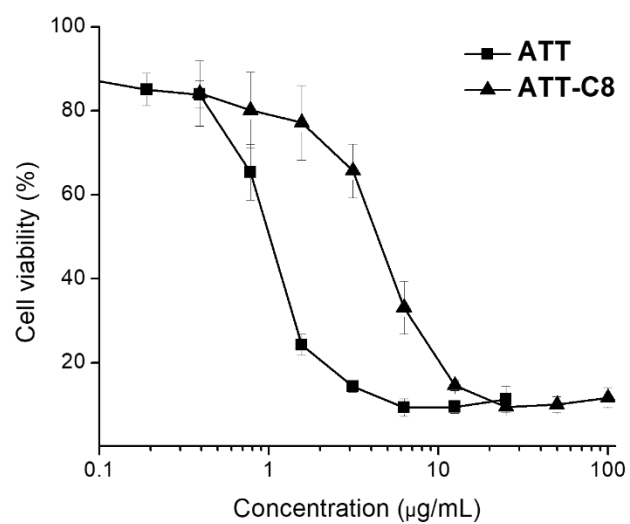
Fig. 3 *In vitro* BAEC cell growth assay obtained with the MTT assay after exposure to ATT and ATT-C8 materials at different concentrations for a 72 h incubation period. The plots represent the mean ± SD of three independent experiments. Dose-response curves were calculated using a nonlinear regression test
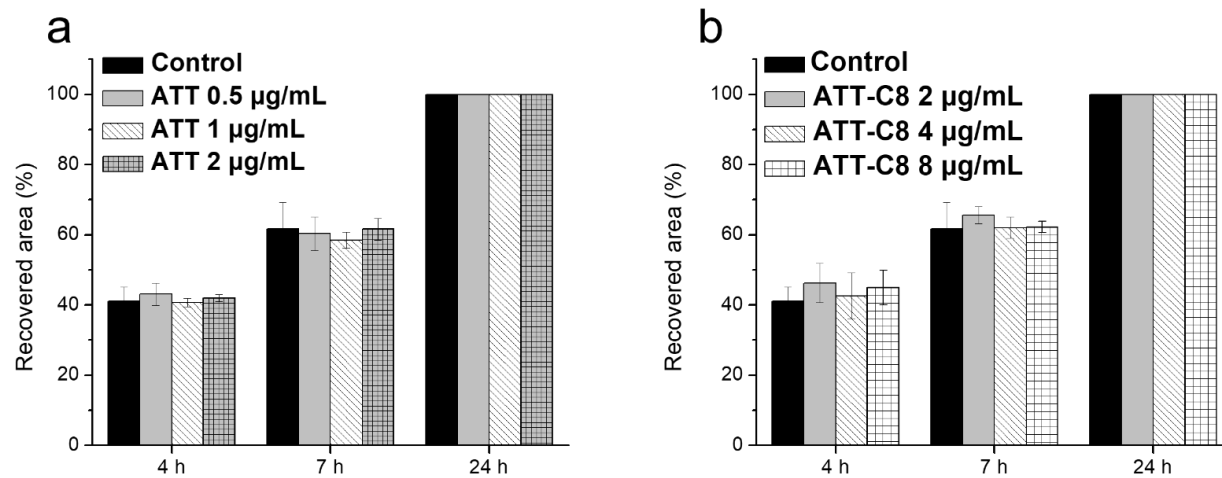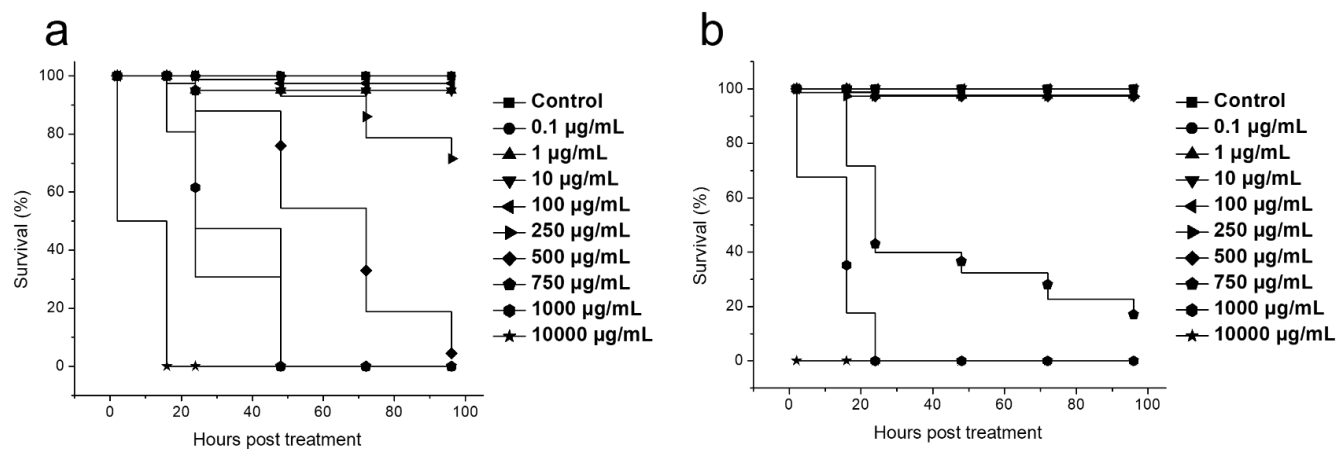
Fig. 4 Wound assay was used to assess *in vitro* cell migration of BAEC. The graphics present frequency of wound recovery in (a) ATT and (b) ATT-C8 culture cells. The values are presented as the mean ± SD of three independent experiments
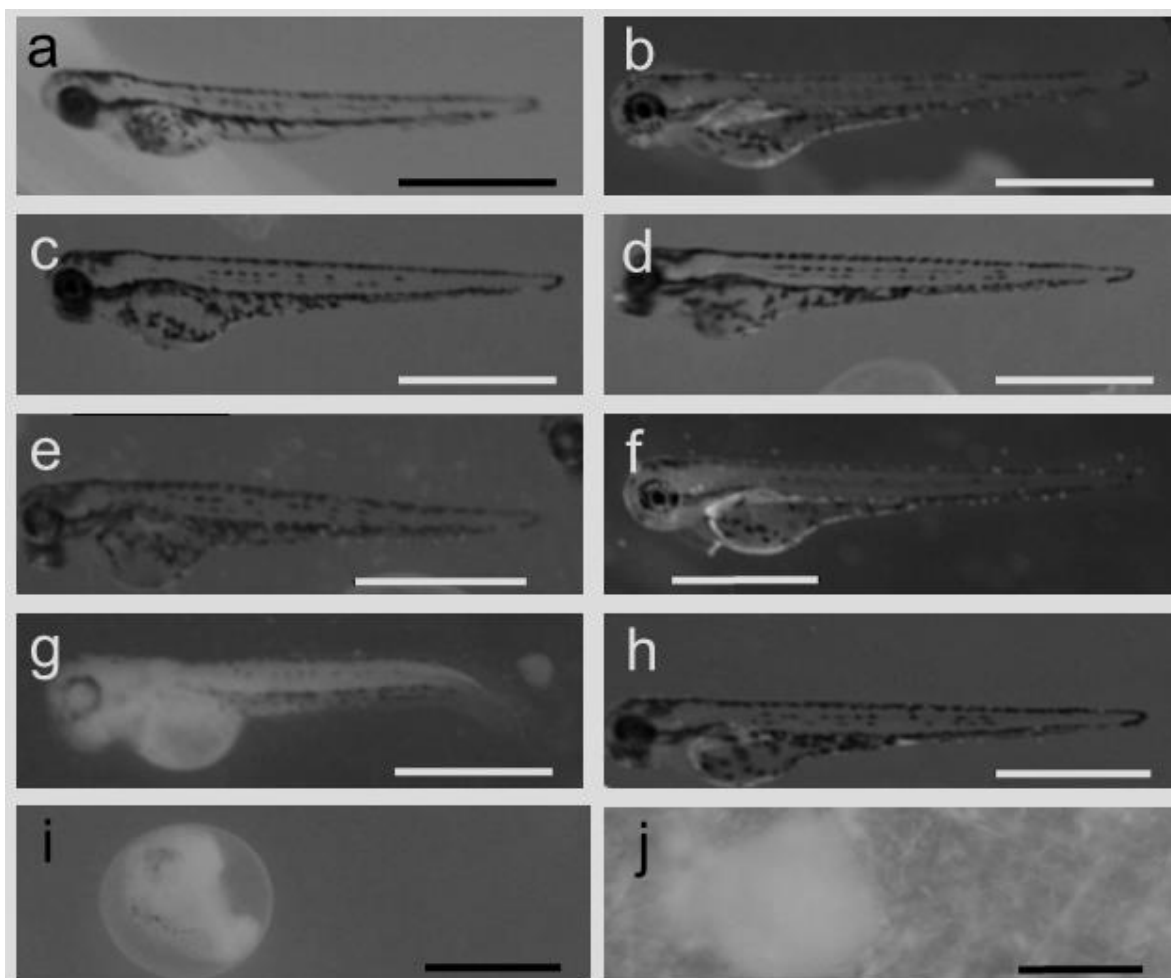
**Fig. 5** Representative images of zebrafish larvae (72 hpf) after 48 h exposure to (a) bare medium; (b) 100 µg/mL ATT; (c) 250 µg/mL ATT; (d) 250 µg/mL ATT-C8; (e) 500 µg/mL ATT; (f) 500 µg/mL ATT-C8; (g) 750 µg/mL ATT; (h) 750 µg/mL ATT-C8; (i) 1000 µg/mL ATT and (j) 1000 µg/mL ATT-C8. Scale bar: 10 µm
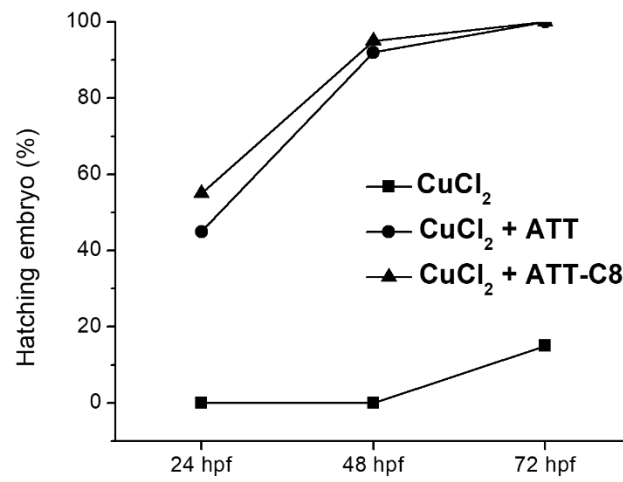
Fig. 6 Survival rate of zebrafish embryos exposed to different concentrations of (a) ATT and (b) ATT-C8 during

24, 48 and 72 h. All results are representative of three independent experiments
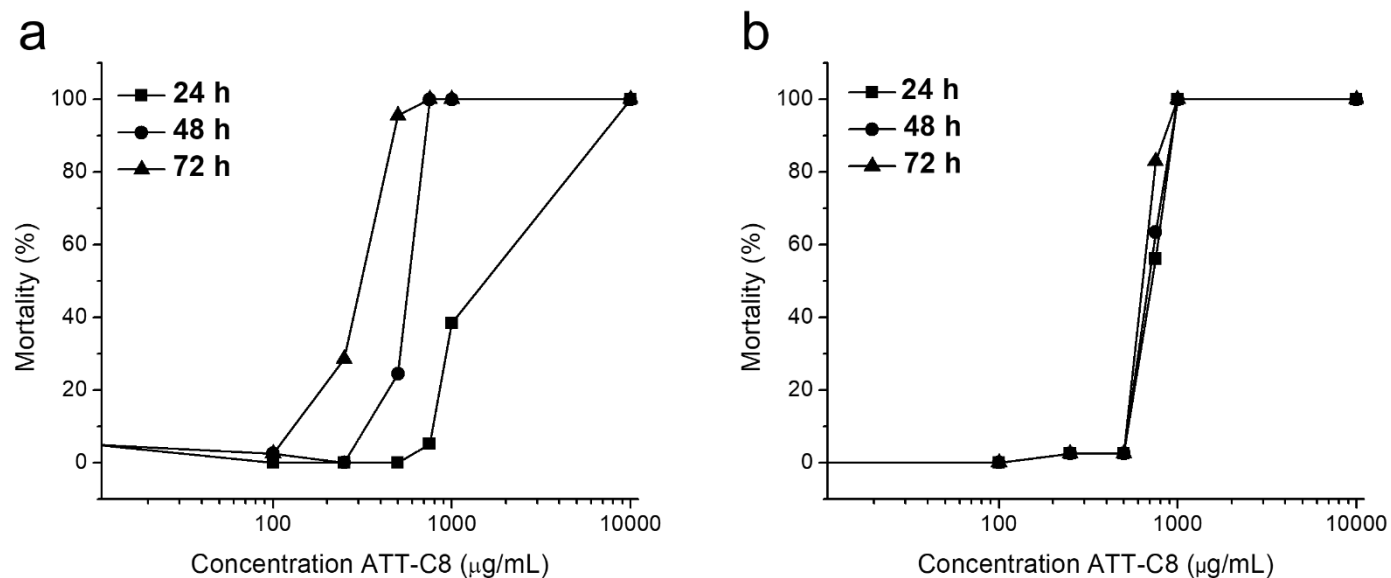
Fig. 7 Mortality curves of zebrafish embryos exposed to (a) ATT and (b) ATT-C8 materials at different concentrations during 24, 48 and 72 h exposure. All the experiments were done in triplicate. Dose-response curves were calculated using a nonlinear regression test
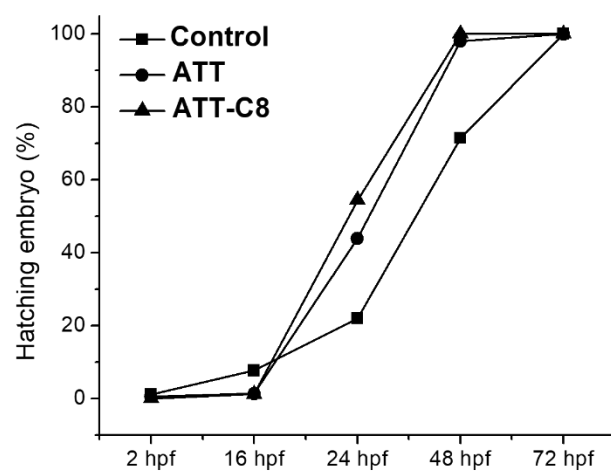
Fig. 8 Hatching rate of zebrafish embryos exposed to 250 μg/mL ATT and ATT-C8 materials during 72 h. All the experiments were done in triplicate

Fig. 9 Hatching rate of zebrafish embryos exposed to 10 μg/mL CuCl$_2$ solution (negative control), a mixture of 10 μg/mL CuCl$_2$ and 250 μg/mL ATT and a mixture of 10 μg/mL CuCl$_2$ and 250 μg/mL ATT-C8. All results are representative of three independent experiments

Tables

Table 1 Results of LC_{50} at to 24, 48 and 72 h. Values represent the mean ± SD of three independent experiments

Material	LC_{50} (µg/mL)		
	24 h	48 h	72 h
ATT	1211 ± 84	542 ± 57	316 ± 68
ATT-C8	735 ± 25	722 ± 31	689 ± 29