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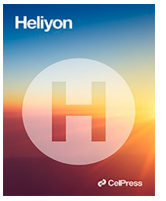

Research article

Evaluating the *Leishmania tarentolae* response to inorganic strontium-based oxyfluorides

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ABSTRACT

The increasing global prevalence of the parasitic vector-borne disease leishmaniasis combined with rising resistance to current therapeutics necessitates the search for novel approaches to combat leishmania. This study evaluates the effects of novel strontium-based oxyfluorides for potential therapeutic use by testing cultures of *Leishmania tarentolae*, a species of *Leishmania* found in reptiles, as a model species. Cells were cultured with a range of mixed metal strontium oxyfluoride compounds selected to systematically test the relationship between compound structure and cell viability and enzyme activity over time.

1. Introduction

Leishmaniasis is a vector-borne parasitic disease, caused by the protozoa parasite genus *Leishmania* [1]. Both animal and human infections can be found in portions of the tropics, subtropics, southern Europe, and recent studies show a growing number of cases along the Texas-Mexico border [2]. It is anticipated that climate change may facilitate the spread northwards of leishmaniasis, increasing the ecological risk to human populations in the United States [3]. Transmission of this disease starts with a bite from an infected female phlebotomine sand fly. Once infected, victims continue to experience a decrease in the quality of their life as the therapeutics available do not completely eradicate the protozoa from the host. Most patients are left scarred and immunocompromised in addition to the medicinal side effects. There are currently several drugs used as treatment, consisting of pentavalent antimonies, miltefosine, and amphotericin B [4].

Resistance is developing quickly to existing therapeutics, and the side effects are extensive. This study aims to evaluate the effects of strontium-based compounds for potential novel therapeutic use, examining their effect on cultures of *Leishmania tarentolae*, a species of *Leishmania* that is found in reptiles and has been shown to act similarly to human strains. These protozoa secrete enzymes such as proteases and secreted acid phosphatases (SAP) which are postulated to promote communication between cells and overall growth and infectivity of the culture [4,5]. If a compound is found to affect the levels of this enzyme, it may prove useful as a component in future novel therapeutics.

Previously, it has been demonstrated that Bi-doped strontium-based oxyfluorides $Sr_{3-x}Bi_{2x/3}AlO_4F$ ($0 \leq x \leq 0.1$) [6] can have a negative effect on *Leishmania* in culture [7]. Although there was no direct cytotoxic effect, a modest decrease in SAP combined with an

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Table 1
List of compositions of $Sr_{3-x}A_xMO_4F$ (A = Ca, Sr, Ba; M = B, Al, Ga).

Compound Label	Composition
C1	$Sr_{2.5}Ba_{0.5}AlO_4F$
C2	$Sr_{2.5}Ca_{0.5}AlO_4F$
C3	Sr_3AlO_4F
C4	$Sr_{2.5}Ba_{0.5}GaO_4F$
C5	$Sr_{2.5}Ca_{0.5}GaO_4F$
C6	Sr_3GaO_4F
C7	$Sr_3Al_{0.9}B_{0.1}O_4F$
C8	$Sr_3Ga_{0.9}B_{0.1}O_4F$

approximately 50 % decrease in NO production was observed for Sr-containing samples (including the $SrCO_3$ control). Strontium is a naturally occurring alkaline earth metal which is present within the human body and is commonly stored in bones. In moderation, strontium is used in a handful of current medications and dietary supplements and has been shown to be safe for human consumption [8], therefore could form the basis of a promising low-toxicity therapeutic.

There are a vast number of compounds which are known to crystallize with perovskite-type structures and these materials have been of much interest due to their varied physical properties [9] and applications ranging from solar cells [10] to superconductors [11]. The anti-perovskite type crystal structure, which has the unusual structural feature of containing anion-centered octahedra, is less well known but equally highly tolerant of substitution by metal ions of different charge and size [12]. Commonly, this approach is used in solid state and materials chemistry to influence the structure-property relationship in crystalline materials, for example using aliovalent charges to induce desirable electromagnetic properties [13] or tuning photoluminescent emission by altering the unit cell size and/or octahedral tilts [14]. The structure-property relation is also crucial in drug discovery; for instance, pharmaceutical compounds need the optimal chemical structure to be able to interact with the target biological system [15].

This study aimed to systematically determine whether the previously observed inhibition of the *Leishmania tarentolae* protozoa could be affected by substituting the larger Ga or small B atom for Al, and whether incorporating other alkaline earth metals such as smaller Ca and larger Ba enhanced or hindered *Leishmania tarentolae* inhibition.

2. Methodology

The $Sr_{3-x}A_xMO_4F$ family of compositions (A = Ca, Sr, Ba; M = B, Al, Ga) are known to exhibit tetragonally-distorted anti-perovskite type structures showing strong crystallographic site preference. Substitution of the smaller Ca cation preferentially occupies the smaller 8-coordinate equatorial Sr(2) site, which is observed as a contraction in the a lattice parameter from 6.7822(1) Å to 6.6517(5) Å (≈1.9 % decrease) accompanied by a similar contraction in the c lattice parameter from 11.1437(2) Å to 10.9443(9) Å (≈1.8 % decrease). The larger Ba cation preferentially occupies the larger 10-coordinate apical Sr(1) site, which is exhibited as an expansion in the a parameter to 6.9192(2) Å (≈2.0 % increase) with a smaller increase in the c parameter to 11.2072(4) Å (≈0.6 % increase) [16]. Similarly, the tilting distortion of the FSr_6 corner-linked octahedra ($a^0a^0c^-$ in Glazer notation) is driven by the orientation of the MO_4 tetrahedra. Substitution of Ga for Al has been shown to increase the octahedral tilt angle from 17.3° for M = Al to 18.0° for M = Ga [14] (≈4.0 % increase) and incorporation of the smaller B atom for Al also increases the octahedral tilt angle to 18.0° [17], presumably as it is too small to fit comfortably on the tetrahedral M lattice site.

In order to systematically investigate the effect of A cation (A = Ca, Sr, Ba) and M cation (M = B, Al, Ga) substitutions, a selection of compositions (Table 1.) were chosen to isolate the effects of lattice expansion (A = Ba) and contraction (A = Ca) and increasing the FSr_6 octahedra tilt angle (M = B, Ga).

Compounds 1–8 were synthesized *via* the ceramic method from stoichiometric quantities of $SrCO_3$ (Sigma Aldrich, ≥99.9 %), SrF_2 (Sigma Aldrich, 99 %), $BaCO_3$ (Sigma Aldrich, ≥99 %), $CaCO_3$ (Sigma Aldrich, ≥99.0 %), Al_2O_3 (Sigma Aldrich, 99.8 %), Ga_2O_3 (Bayville Chemical, 99.9 %), and B_2O_3 (Alfa Aesar, 99.98 %). The reagents were ground and heated for 12 h at 700 °C, 800 °C, 900 °C, then 72 h at 1000 °C and 1050 °C using a Thermolyne FD1545 M 1200 °C muffle furnace equipped with a Eurotherm 8 segment programmable controller. The final heating was repeated until powder X-ray diffraction (PXRD) showed pure single-phase $Sr_{3-x}A_xMO_4F$ compounds were formed [16]. In addition to the $Sr_{3-x}A_xMO_4F$ anti-perovskite oxyfluorides, the simple compounds $SrCO_3$ (Sigma Aldrich, 99.9 %), SrF_2 (Sigma Aldrich, 99 %), and H_3BO_3 (boric acid, USB Corporation, Cleveland Ohio) were tested to determine if any effect on *Leishmania tarentolae* is due to biochemical action of isolated Sr, F, or B regardless of local bonding environment.

Several tests were conducted to evaluate the effects of each compound on a culture of cells. Each compound was dissolved in a milliliter of sterile dimethyl sulfoxide (DMSO, Fisher Scientific, autoclaved in-house); however, these cells do not survive well in concentrations >1 % DMSO, so these solutions were then diluted in brain heart infusion broth (BHI). Each compound had a control without cells and a flask with cells. There was also a control of just BHI, BHI and 1 % DMSO (no cells), and BHI, 1 % DMSO, and cells to account for any growth or absorbance readings not attributed to the compounds' effects on the cells themselves. Starting from day 0, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was conducted to observe cell viability. Towards the end of the life span when SAP levels were at the highest, a SAP assay was conducted.

As a strategy to maintain uniformity among test cultures, 60 mL of BHI broth containing streptomycin, penicillin, and hemin (following the methodology of Apuzzo et al., 2021 [7]) were added to a 250 mL CELLTREAT suspension culture flask (ThermoFisher

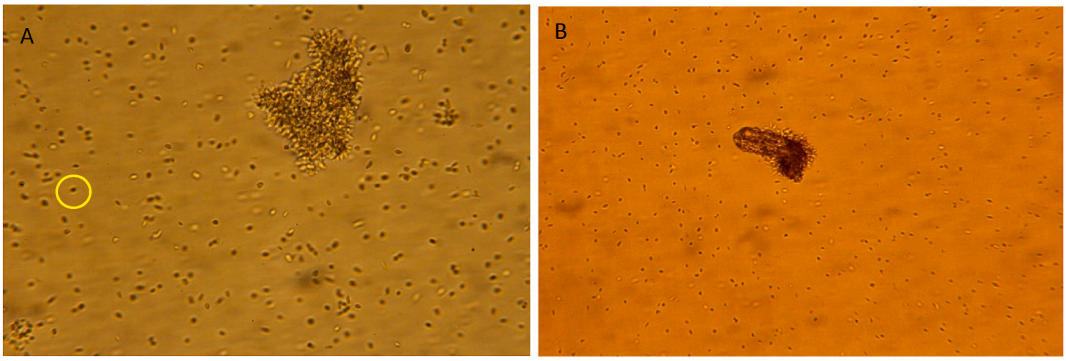

Fig. 1. Image A shows a microscopy control culture with no added compound or DMSO. Individual cells (circled) average 10 μm in size. Image B shows microscopy of a culture incubated with C5 ($Sr_{2.5}Ca_{0.5}GaO_4F$) dissolved in DMSO. Note the cell clustering around an apparent crystal of the compound.

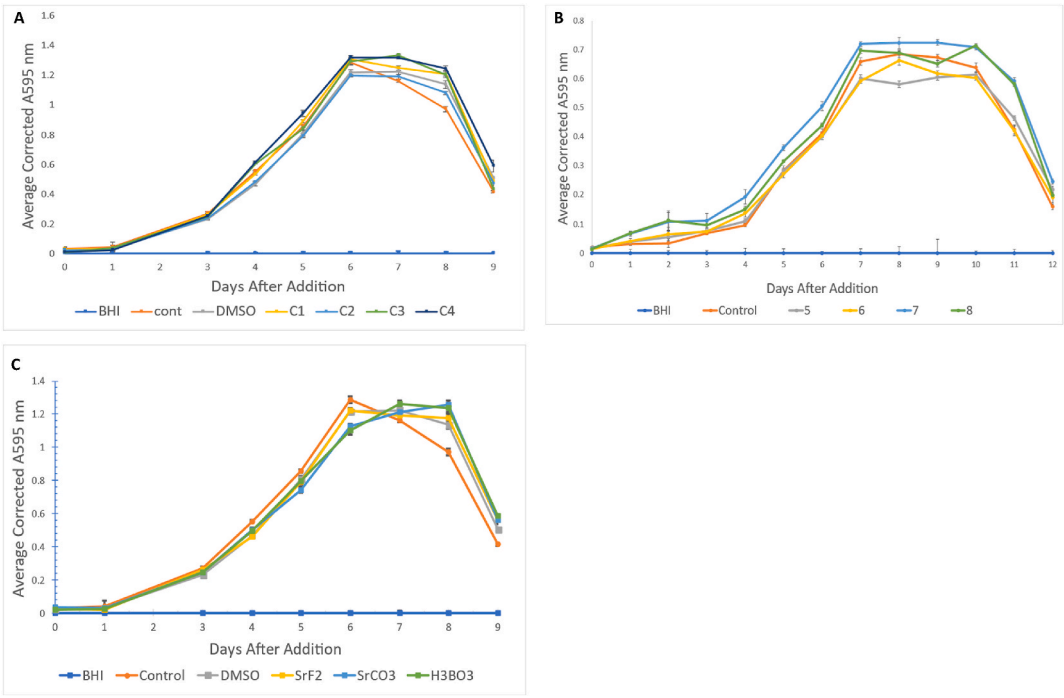

Fig. 2. The MTT viability values, for each day in culture post addition of compounds 1–8, SrF_2, $SrCO_3$, and H_3BO_3 are shown as mean ± standard deviation (SD) for n = 3 replicates (C1: $Sr_{2.5}Ba_{0.5}AlO_4F$, C2: $Sr_{2.5}Ca_{0.5}AlO_4F$, C3: Sr_3AlO_4F, C4: $Sr_{2.5}Ba_{0.5}GaO_4F$, C5: $Sr_{2.5}Ca_{0.5}GaO_4F$, C6: Sr_3GaO_4F, C7: $Sr_3Al_{0.9}B_{0.1}O_4F$, C8: $Sr_3Ga_{0.9}B_{0.1}O_4F$).

Scientific, Waltham, MA, USA). Then 10 mL was removed and placed in a 50 mL culture flask to serve as a cell free background control, and the rest of the medium was inoculated with *Leishmania tarentolae*. The flask was left to incubate in the dark at 22 °C for three days. The stock culture was mixed, then divided into five 10 mL aliquots, which were placed in 50 mL culture flasks. Samples were removed from these flasks (450 μL) and placed in separate polypropylene microcentrifuge tubes. An MTT viability assay [18] was done prior to compound addition. Test compounds, including $Sr_{2.5}Ca_{0.5}GaO_4F$, Sr_3GaO_4F, $Sr_3Al_{0.9}B_{0.1}O_4F$, and $Sr_3Ga_{0.9}B_{0.1}O_4F$, $Sr_{2.5}Ba_{0.5}AlO_4F$, $Sr_{2.5}Ca_{0.5}AlO_4F$, Sr_3AlO_4F, $Sr_{2.5}Ba_{0.5}GaO_4F$, and controls, including DMSO only, SrF_2, $SrCO_3$, and H_3BO_3 were dissolved in DMSO, to yield 1 mL of 5 mM stock solutions for each compound. Then, 100 μL of compound stock was added to their respective culture flasks; one culture did not receive any compound to serve as an additive-free cell control. The 595 nm values from the MTT assay were obtained using the Bio-rad iMark microplate Reader plate reader. Absorbance was normalized both for background using cell-free BHI and reported as an hourly average (n = 3 replicates). An MTT viability assay was performed daily for 12 days post compound addition.

On the ninth day of culture incubation, an SAP assay was conducted to measure the effects of the strontium compounds on the cells' enzyme secretion. Following the method of Apuzzo et al., 2021 [7], samples (1.5 mL) were extracted from the culture flasks and placed into 2 mL microcentrifuge tubes; Samples were centrifuged (Eppendorf 5415C centrifuge) at 10,000 rpm for 30 s. The supernatants

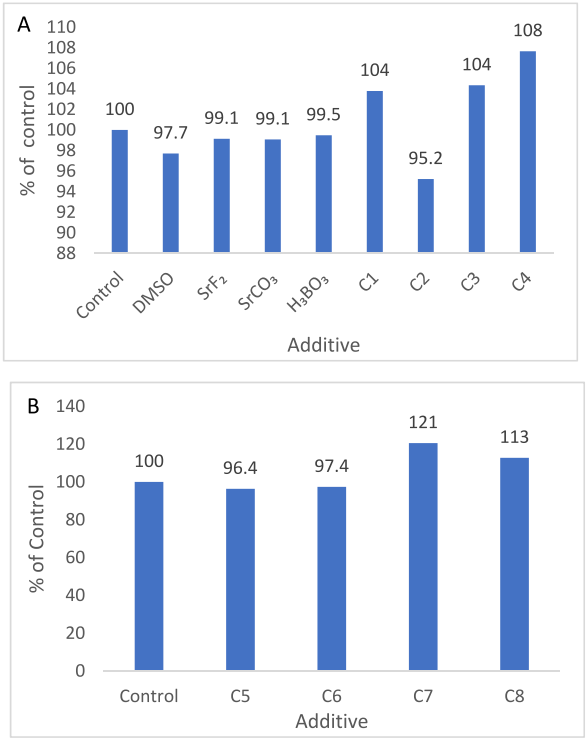

Fig. 3. The total calculated area under the growth curve for each addition is shown in A and B as a percent of the additive-free control cells (C1: $Sr_{2.5}Ba_{0.5}AlO_4F$, C2: $Sr_{2.5}Ca_{0.5}AlO_4F$, C3: Sr_3AlO_4F, C4: $Sr_{2.5}Ba_{0.5}GaO_4F$, C5: $Sr_{2.5}Ca_{0.5}GaO_4F$, C6: Sr_3GaO_4F, C7: $Sr_3Al_{0.9}B_{0.1}O_4F$, C8: $Sr_3Ga_{0.9}B_{0.1}O_4F$).

were removed using a micropipette and placed into a new microcentrifuge tube; they were then centrifuged for an additional 15 s at 10,000 rpm. This was done to fully remove cells to select only for secreted enzyme. Then 450 μL of the supernatant was then placed in each of three microcentrifuge tubes. Sodium acetate (0.5 M, pH 4.5) buffer was added to each tube (450 μL) followed by 100 μL of *para*-nitrophenylphosphate (pNpp) substrate (5 mg/mL buffer). The tubes were inverted to gently mix and placed in a dark environment to incubate at room temp for 20–24 h. The incubation was stopped using 100 μL of 10 M NaOH and vortexed to mix. The A 405 nm of each tube was obtained using an HP spectrophotometer with the BHI sample as the blank to correct for background. Results are displayed as an average of 3 replicates.

An additional analysis involving integration of the MTT assay area under the curve was performed using the formula: $A = \sum \left[(day\ y - day\ x) * \frac{MTT\ day\ x + MTT\ day\ y}{2}\right]$. The A 405 nm obtained *via* the SAP assay was also compared to the MTT values on day nine for all cultures. All statistics were analyzed using ANOVA and the Bonferroni post-tests using the One-way ANOVA with post-hoc Tukey HSD Test Calculator [19] where possible. A $p < 0.05$ is considered statistically significant when compared to the control. Further analysis, using a PerkinElmer Spectrum BX Fourier transform infrared (FTIR) spectrophotometer, to determine the stability of the compounds dissolved in DMSO was performed.

3. Results and discussion

Cell mobility and morphology were consistent among cultures, as evaluated by light microscopy (using a Jenco Light Inverted Microscope at 250× magnification). The cultures differed in cell clumping tendencies, as cells incubated with strontium compounds tended to clump around the compound crystalline structures (Fig. 1 right), as opposed to the control cells that clumped with other cells as shown in Fig. 1 (left).

The viability results for cells incubated with our various test compounds, at 50 μM final concentration, are shown in Fig. 2 A, B, and C. Addition of SrF_2, $SrCO_3$ or H_3BO_3 did not have any substantial effect on the growth curves.

In all cases the replicates values are similar, and thus the standard deviations for a single compound are small. In general, all curves peak on either day 6 or 7 after test compound addition and have the same general curve shape; there is an apparent difference in the height of some of the peaks.

When these data were calculated as total area under the curve for each culture.

As shown in Fig. 3, only compound 7 ($Sr_3Al_{0.9}B_{0.1}O_4F$) exhibited a larger increase in cell viability (121 % of control cells). There was no substantial inhibition of cell viability for any of the test compounds, although C2 ($Sr_{2.5}Ca_{0.5}AlO_4F$), C5 ($Sr_{2.5}Ca_{0.5}GaO_4F$) and

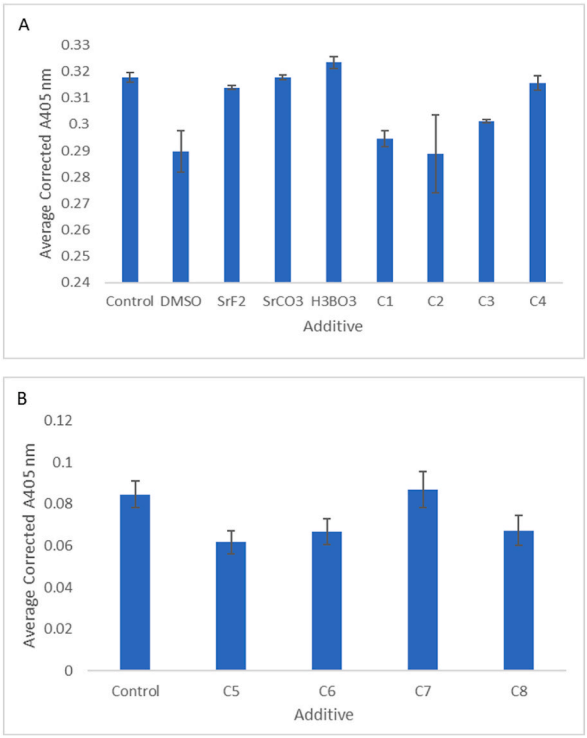

Fig. 4. The SAP detectable activity is shown for each culture as mean and SD (C1: $Sr_{2.5}Ba_{0.5}AlO_4F$, C2: $Sr_{2.5}Ca_{0.5}AlO_4F$, C3: Sr_3AlO_4F, C4: $Sr_{2.5}Ba_{0.5}GaO_4F$, C5: $Sr_{2.5}Ca_{0.5}GaO_4F$, C6: Sr_3GaO_4F, C7: $Sr_3Al_{0.9}B_{0.1}O_4F$, C8: $Sr_3Ga_{0.9}B_{0.1}O_4F$).

C6 (Sr_3GaO_4F) did yield cell viabilities less than 100 % of control cells. It is interesting to note that it is the Ca-containing structures which show lattice compression due to the smaller size of Ca compared to Sr, and the Ga-containing structures are inherently more strained due to the increased tilt angles in the Sr–F layer. It is possible that these structural differences may be the cause of these subtle differences in the area under the growth curves.

Fig. 4 A and 4 B show the SAP activity detected from each culture relative to the control cultures. Only additions C1 ($Sr_{2.5}Ba_{0.5}AlO_4F$), C2 ($Sr_{2.5}Ca_{0.5}AlO_4F$), C5 ($Sr_{2.5}Ca_{0.5}GaO_4F$), C6 (Sr_3GaO_4F), and C8 ($Sr_3Ga_{0.9}B_{0.1}O_4F$) appeared to have a modest inhibition of this enzyme. In general, the most inhibitory effects were modest (about 20–25 % inhibition) and were primarily exhibited by the Ga analogs.

In Fig. 5 A and Figure B, the ratio of SAP activity to MTT viability on day 9 of culture is shown. Only compounds C5 ($Sr_{2.5}Ca_{0.5}GaO_4F$), C6 (Sr_3GaO_4F), and C8 ($Sr_3Ga_{0.9}B_{0.1}O_4F$) have a modest decrease in this ratio relative to the control cell cultures. These are compounds all contain 100 % Ga at the center of the MO_4 tetrahedra.

IR spectra were obtained for each of the test compounds over time. It was shown that little to no changes were observed in IR peaks from day to day.

4. Conclusions

The *Leishmania tarentolae* growth curve results clearly indicate that these compounds, and their potential dissociated ligands have only modest effects on the cell growth implying that these compounds have similar effects on cell growth as previously reported Sr compounds [7]. These test compounds had only modest inhibitory effects on the SAP enzyme activity. When the ratio of SAP activity to cell viability (MTT) was calculated, only 3 tested compounds had a modest effect. It is of interest that all 3 contain gallium rather than aluminum as a component. More testing should be performed to ascertain whether it is the structural distortions in the Sr–F layer induced by the larger Ga atom in the MO_4 tetrahedra that increase negative interaction with *Leishmania tarentolae*, or if Ga has a biological interaction with the protozoa regardless of bonding environment. Ga is non-toxic in its elemental form and does not accumulate in the body, although halide salts and Ga_2O_3 are generally considered to be mildly toxic and prolonged chronic exposure may cause acute toxicity [20]. Ga is most commonly known for its uses in electronics and radioimaging, but there are also Ga-based medical treatments for cancers and some microbial infections [21]. There appears to be no previous report of Ga or Ga-based compounds interacting with *Leishmania* and thus this area warrants continued investigation.

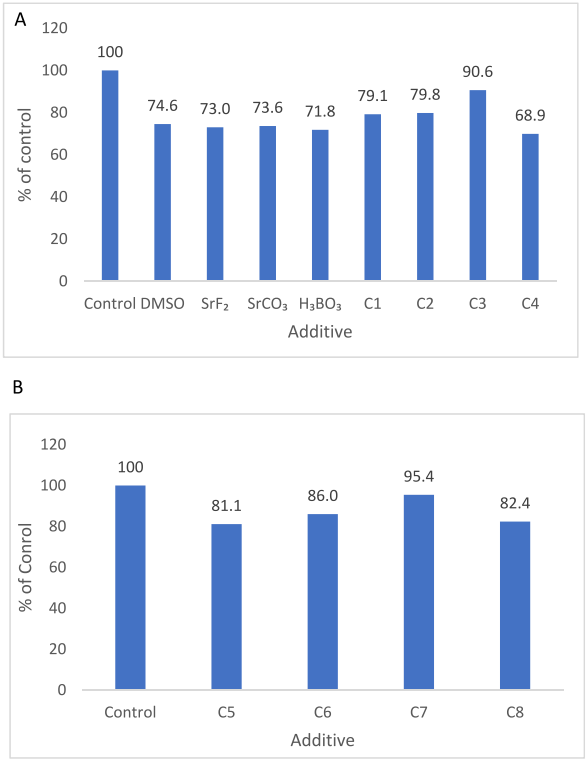

Fig. 5. The ratios of SAP activity detected divided by the detected MTT value, on day 9 of culture. All values are compared to the additive-free control. (C1: $Sr_{2.5}Ba_{0.5}AlO_4F$, C2: $Sr_{2.5}Ca_{0.5}AlO_4F$, C3: Sr_3AlO_4F, C4: $Sr_{2.5}Ba_{0.5}GaO_4F$, C5: $Sr_{2.5}Ca_{0.5}GaO_4F$, C6: Sr_3GaO_4F, C7: $Sr_3Al_{0.9}B_{0.1}O_4F$, C8: $Sr_3Ga_{0.9}B_{0.1}O_4F$).

Data availability statement

All data is included in the article or supplemental material, or is from previous work cited within the article.

CRediT authorship contribution statement

Katelyn Terry: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Joseph Drinkwater:** Investigation, Formal analysis. **Marjorie A. Jones:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Methodology, Formal analysis, Conceptualization. **Eirin Sullivan:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Formal analysis.

Declaration of competing interest

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e30634.

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