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Study of the Toxicity and Antiviral Effect of Natural Compounds Extracted from *Pseudospinx Tetrio* and *Allamanda Cathartica* on Sars-Cov-2 Infection

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Abstract

Very present in the Caribbean, the leaves of *Allamanda cathartica* L. and the caterpillar *Pseudospinx tetrio*, are unexploited resources. However, they are known to have an interesting antimicrobial activity. In this short communication, we propose a way of valorization of these resources. Indeed, the antiviral effect conferred by the molecules extracted from *Allamanda cathartica* L. leaves and caterpillars are tested against a virus that has become strongly known in 2019, the SARS-CoV-2. Preliminary results against this virus are promising and highlighted by biological tests performed on organic or aqueous extracts.

Keywords: *Pseudosphinx tetrio*; *Allamanda cathartica* L.; SARS-CoV-2; Antiviral effect.

Introduction

Pseudospinx tetrio (Linnaeus, 1771) is a caterpillar with a velvety black body sur-rounded by yellow rings on each thoracic and abdominal segment. It is a member of the family *Spingidea*. and is recognized to date as the only species of the monotypic genus *Pseudospinx* [1], [2], [3], [4], [5] . Very present in the tropical and subtropical regions of the Americas and in the Caribbean basin, it owes its development to the existence of a host plant adapted to the development of these larvae[6], [7], [8], [9], [10], [11] . The *Pseudospinx* caterpillar presents a very toxic content in its intestine, considerably reducing its number of predators. Moreover, within its organism, bioactive molecules make it interesting in different fields of application. Indeed, these molecules have been associated with antioxidant [12], [13], [14], antifungal [15], or antimicrobial [14], [16] activities. They can also have an action in the field of traditional medicine [17]. The gregarious larvae feed mainly on plants of the *Apocynaceae* family such as *Allamanda cathartica* Linn. and *Plumeria alba*. These plants, rich in toxic latex [18], [19] are native to Brazil and constitute one of the fifteen species of the *Apocynaceae* family [20], [21]. A close relationship exists between the target plant and the herbivorous insect predator. Indeed, depending on the target encountered, the direct associations with the predator are different and specific. This results in many different interactions between plants and herbivores. An example described by Suzuki et al. 2018, illustrates his points [22]. When an insect has the ability to lay eggs on one plant, its egg laying may not be possible on another plant due to the chemistry of the plants being detected by the insect. Plants can, however, to protect themselves selectively excrete defense metabolites upon ingestion by the insect (upon incorporation) [23], [24]. Following ingestion of these plants by herbivores, plant/insect interactions may be antimicrobial in character, which could be

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of interest against certain pathogenic microorganisms or viruses. It is in this context and with the aim of valorizing the natural and abundant re-sources of the Caribbean basin that we decided to study the impact of bioactive cells extracted from the feces of herbivorous caterpillars *P.tetrio* and healthy leaves of *A. cathartica* against a viral segment. A study conducted by Matignon et al. shows the antimicrobial effect of *allamanda* leaves and caterpillar feces by innovative analytical techniques [25]. This study first focused on the characterization of these different elements and then on their pharmacological activity against the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The percentage of inhibition of infection and toxicity on healthy cells were determined. In this short communication, the study of different parts of the caterpillar's body, of its feces, and healthy *Allamanda cathartica* plants against the SARS-CoV-2 virus is conducted. No treatment exists to eradicate this disease, but several have been proposed to slow its spread and decrease its virulence. Among others the use of so-called medicinal plants. These medicinal plants contain active substances that can eliminate or reduce the toxic effects of certain viruses or bacteria. Numerous trials have shown their effectiveness on a laboratory scale against the SARS-CoV-2 virus, responsible for the disease that the World Health Organization has named coronavirus disease 2019 (COVID-19) in 2019. The virus SARS-CoV-2 is an enveloped single strand (+) RNA virus belonging to the *Coronaviridae* family. Coronaviruses can infect many different animals and cause mild to severe respiratory infections in humans [26]. The virulence of the SARS-CoV-2 virus and its expansion have motivated this work with the aim of proposing a preventive treatment elaborated from abundant natural extracts and not valorized in this sense to date.

Material and Methods

Materials

Plant material

Allamanda cathartica leaves and *Pseudosphinx tetrio* caterpillar were collected in Guadeloupe archipelago specially in “Le Gosier” city (16°13'00.5"N 61°31'09.9"W).

Biological materials

***Cell Lines and Culture*:** Vero E6 cells (African green monkey kidney cells) were obtained from ECACC (SigmaAldrich, Merck, Darmstadt, Germany) and maintained in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS, Thermo Fisher, Waltham, MA, USA) 50 U/mL of penicillin (Ozyme, Saint-Cyr-l’école, France), 50 µg/mL of streptomycin (Ozyme, France) and 25 mM of HEPES at 37°C with 5% CO_2.

***SARS-CoV-2 Virus*:** SARS-CoV-2/CHU Montpellier/France was isolated from CPP Ile de France III, n ◦2020-A00935−34 and “Centre de Ressources Biologiques” collection of the University Hospital of Montpellier (France). The virus was propagated in Vero E6 cells with DMEM containing 2.5% FBS and 25 mM HEPES at 37°C with 5% CO_2 and was harvested 72h post inoculation. Virus stocks were stored at −80°C. All work with infectious SARS-CoV-2 was performed in an approved biosafety level 3 (BSL3) facilities by trained personnel at the CEMIPAI UAR3725.

Virus Titration: Virus titration from infected cell culture supernatant was monitored using plaque assays on a monolayer of Vero E6 cells, as previously described [27]. Briefly, Vero E6 cells were inoculated with 10-fold serial dilutions (10−1, 10−2, 10−3, 10−4, 10−5) of a SARS-CoV-2 stock and incubated for one hour at 37°C with regular rocking. Inoculum was removed and replaced with 500 µL/well of agar overlay (MEM; 0.1% sodium bicarbonate, 0.2% BSA, 20 mM HEPES, 0.6% oxoid agar) (Fisher Scientific, France). Once the overlay had solidified, plates were incubated at 37°C for 72h prior to fixing with 4% PFA (Euromedex, Souffelweyersheim, France) and staining with crystal violet (Sigma, France) to visualize plaques. Plaques were quantified and viral titer of the stock sample was determined as plaque-forming units (PFU)/mL by taking the average number of plaques for a dilution and the inverse of the total dilution factor.

Assessment of Antiviral Activity and cytotoxicity: Remdesivir (#282T7766) was purchased from Tebu (France) and prepared at a con-centration of 10 mM in DMSO (Sigma, France). Antiviral activity was assessed by a cy-topathic effect (CPE) reduction assay on Vero E6 cells. Briefly, 30,000 cells per well were cultured in 96-well culture plates (Dutscher, BERNOLSHEIM, France) for 24h. Com-pounds were diluted in DMEM medium at the concentration 10µM for Remdesivir and 10µg/mL for the natural extracts. Cells were incubated with 100 µL of the compounds for 2h. Subsequently, cells either were mock infected (for analysis of cytotoxicity of the compounds) or were infected with 400 PFU of virus per well (MOI of 0.01) in a total volume of 110 µL of medium with compounds. In that context, the experiments were performed in an on-going viral replication in the treated cells. Cellcytoxicity was assessed 3 days post-treatment by MTS assay using a Cell Titer 96 aqueous cell proliferation kit (Promega, Charbonières-les-Bains, France). CPE was assessed 3 days post-infection using Viral ToxGloTM (ToxGlo assay, Promega, France). Absorption at 495nm (MTS) or luminescence (ToxGlo) were measured with an EnVision multilabel plate reader (PerkinElmer, Villebon S/Yvette, France). The percentage of Inhibition of Infection and the percentage of Toxicity were calculated according to the following equations (1) and (2):

$$\%\ Inhibition\ of\ Infection = \left(\frac{signal\ infected\ cells\ treated - signal\ infected\ cells}{signal\ uninfected\ cells - signal\ infected\ cells}\right) * 100 \quad (1)$$

$$\%Toxicity = 100 - \left[\left(\frac{signal\ treated\ cells}{signal\ untreated\ cells}\right) * 100\right] \quad (2)$$

Microscopic Observation: Cells were observed at the magnification 4X in BrightField on a Evos® microscope.

Methods

Preparation of healthy Allamanda cathartica leaves

After cleaning the leaves carefully with distilled water, they were freeze-dried and powdered. Then, 50g of the powdered leaves were macerated for 48h at room temperature in a ternary mixture of dichloromethane/methanol/distilled water solvent (1:1:1 v/v, ca 200 mL). Finally, the macerate was filtered and purified by liquid-liquid extraction. Organic and liquid phases were obtained, and each was dried with a rotary evaporator.

Preparation of the caterpillar's body and feces

In order to examine the body and the feces of the caterpillar, the caterpillar was left 24 hours in a cage in the presence of *Allamanda* leaves. At the end of these 24 hours, the feces were collected. After that, the caterpillar was left 48h without food to collect a body completely emptied of all its digestive material. Then, the feces and the body were freeze-dried and reduced to powder. 50g were macerated in a ternary mixture of di-chloromethane/methanol/distilled water solvent (1:1:1 v/v, ca 200 mL). Finally, the macerate was recovered and purified by liquid-liquid extraction. The two phases, organic and liquid, obtained were put to dry by rotary evaporation. The different extracts obtained after extraction and purification are presented in Table 1 below.

Bioassays of caterpillar's feces and body and healthy leaves

The 6 samples were solubilized in DMSO with a concentration of 1mg/mL.

The screening of the molecules was done in several steps. The first was the dilution of the molecules extracted from caterpillar's feces and body leaves in a culture medium in order to obtain a concentration of 10 µg/mL and 10µM for the Remdesivir, a molecule used as a reference. Then, a step of treatment and infection of the cells is implemented. Samples diluted in the culture medium were put in contact with VeroE6 cells for 2h at 37°C on two 96-well plates. The first plate was infected with SARS-CoV-2 virus at Multiplicity of infection (MOI) of 0.01 and contains infected (IC) and uninfected cells (UFC). This first plate allowed to have information on the possible effects of the extracted molecules on the replication of the virus. The Remdesivir molecule was also present and served as a reference.

Table 1: Phases obtained for each manipulation after liquid-liquid extraction

	Organic phase	Aqueous phase
Healthy *A. cathartica* leaves	1.1	2.1
Caterpillars's bodies (*P. tertio*)	1.2	2.2
Caterpillars's faeces (*P. tertio*)	1.3	2.3

The second 96-well plate allowed to measure the toxicity of the extracted molecules on healthy cells (no presence of the virus). This plate contained untreated cells and cells with Remdesivir which served as controls.

Then, both plates were incubated at 37°C for 72h at -5% CO_2.

Before the interpretation of the data, a reading of the screening was performed for the infection test which measures the potential antiviral effect of the extracted molecules and the toxicity test which provided information on the toxicity of the natural compounds extracted from the body and feces of the caterpillar but also from healthy *Allamanda* leaves. The antiviral effect of the cells infected with the different extracts on the SARS-CoV-2 virus was observed under an Evos® microscope. Then, cell viability was quantified using the Viral ToxGloTM Assay kit (Promega). To report on the toxicity of the compounds on virus-uninfected cells after observation under an Evos® microscope (the magnification is 4X and the scale is 1000µm), viability was quantified with a Promega Cell Titer 96® AQueous One Solution Assay. The signals resulting from the infection and toxicity assays were read through an EnVision® microplate reader (Perkin Elmer).

Characterizations

^{1}H NMR spectra were realized with a Brunker Avance 300 MHz spectrometer equipped with a BBO probe and automatic tube changer. 15mg of each organic extract was solubilized in DMSO-d_6. All spectra were processed using Topspin 2.1.

Results and Discussion

Preparation of each sample

First, the molecules were solubilized in DMSO at a concentration of 1mg/mL. The molecules extracted in the organic phase from *Allamanda* leaves and caterpillar's feces were partially soluble in DMSO as well as the caterpillar's body extracts from the aqueous phase. The other samples were completely soluble in DMSO (Table 2).

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Table 2: Solubility of different natural extracts in DMSO

	Solubility in DMSO	
	Organic phase	Aqueous phase
Healthy *A. cathartica* leaves	±	+
Caterpillars's bodies (*P. tetrio*)	+	±
Caterpillars's feces (*P. tetrio*)	±	+

± Partially soluble, + Soluble

Chemical compounds of Allamanda leaves and caterpillars

To identify bioactive molecules that could have a protective role against the virus without being toxic, 1H NMR analyses of different samples were performed (Figure 1). The results presented below seem to show the presence of alcohol, esters, aldehydes and ketone such as glycerins, fatty acids, phospholipids, phenolic molecules, flavonoids, terpens, lactone, vitamin and glucose [28]. A particular iridoid lactone, Allamandin, is known to be very present in all parts of *Allamanda cathartica*. This lactone is toxic and is partly responsible for the toxicity of *A. cathartica*. Allamandin can be considered as phytochemical market of *Allamanda cathartica* [28]. The spectrum of *allamanda* leaves is very different from the spectra of the caterpillar. Indeed, the organic extracts of the healthy *Allamanda* leaves present many protons corresponding to different organic compounds. Protons between 2.5 and 4 ppm represent CH and CHx present on alkane chains with OH functions that could be in Vitamin C and E and glucose. The protons located around 4 ppm correspond to methoxy groups and around 5 ppm to olefins. The protons around 6.5 ppm are however the protons of flavonoids and around 7.5 ppm to aromatic groups. Particular attention is paid to protons in the *Allamanda* leaf spectrum between 3.8 and 4.6 ppm corresponding to cyclic protons, with protons at 6.4 ppm corresponding to OCOCH3 groups. These different groups could belong to Allamandin, a toxic molecule specific to *Allamanda cathartica* [29].

Antiviral and toxicity assessments of natural extracts from caterpillar's feces and body and Allamanda cathartica leaves

To determine the antiviral activity of the body of the caterpillar emptied of its digestive material, the caterpillar feces and the leaves of *Allamanda cathartica* on the SARS-CoV-2 virus, a cytopathic effect (CPE) reduction assay was performed on African green monkey kidney-derived cell line Vero E6. Cells were pretreated with the molecules at the concentration 10µg/mL, then infected by SARS-CoV-2 and incubated for 72 hours. In parallel, cytotoxicity of the

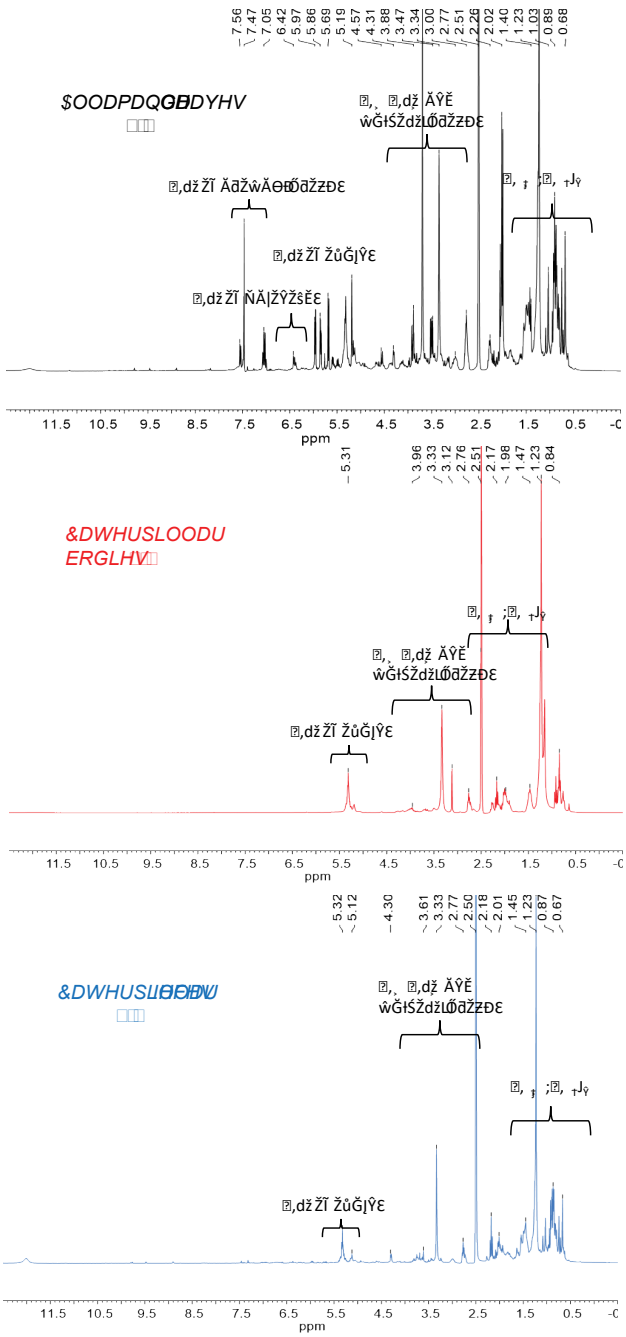

Figure 1: ^{1}H NMR spectra of organic extracts in DMSO-d_6 of Allamanda leaves, caterpillar bodies and caterpillar feces

molecules was evaluated on treated cells in absence of infection. The Table 3 recapitulates the % of Inhibition of Infection and % of Toxicity of each molecule tested. The molecule chosen as reference, Remdesivir, did not present toxicity on cells alone and showed a significant protective effect of the infection of the cells. Indeed, the percentage of inhibition of infection was 152±4%with a toxicity of 5±3% (Table 3). The results of toxicity and antiviral effect of the extracted natural compounds were therefore compared to this reference.

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Table 3: Antiviral activity and Toxicity for the different samples

Natural extracts	Inhibition of Infection		Toxicity	
	(%)		(%)	
	Organic	Aqueous	Organic	Aqueous
Healthy *A. cathartica* leaves	41±2	-2±20	11±5	14±2
Caterpillars's bodies (*P. tetrio*)	1±12	14±0.3	10±0.4	14±13
Caterpillars's feces (*P. tetrio*)	96±8	20±7	10±9	10±2
Remdesivir (control)	152±4		5±3	

The molecules present in the caterpillar's feces guarantee a greater protection of the infection for the cells. Moreover, the molecules extracted from the organic phase present a higher percentage of inhibition of infection, i.e. 96±8%, than those extracted from the aqueous phase whose percentage of inhibition was almost 5 times less important. The antiviral effect of molecules extracted from caterpillar feces from the organic phase was 2 to 90 times higher than that of the molecules present in the body of the caterpillar or extracted from the healthy leaves of *Allamanda*. Healthy *Allamanda* leaves had a percentage of inhibition of infection of 41±2% in organic medium. On the other hand, the molecules extracted from the aqueous phase showed only weak antiviral properties with a percentage of inhibition of infection of -2±20% (Table 3).

The molecules of the caterpillar body extracted in the organic phase played a minor role and had low antiviral activity compared to those extracted in the aqueous phase where the percentage of inhibition of infection is 14%.

The analyses of inhibitory effect of the different extracts were completed by observations of the cells under the Evos® microscope (Figure 2).

All natural compounds have shown no toxicity on cells in absence of infection. In-deed, the percentage of toxicity ranged from 10 to 14%. These values, although twice higher than the Remdesivir control, which was 5±3%, are still relatively low. This allows us to conclude that the natural extracts from the feces and body of the caterpillar and from the healthy leaves of Allamanda do not show any toxicity to the cells alone in the viability reading. These results were confirmed by microscopic observation of the cells (Figure 3.).

The Remdesivir molecule, the reference for cytotoxicity and antiviral effect, showed a high inhibitory effect on infected cells. Moreover, it did not present any toxicity for

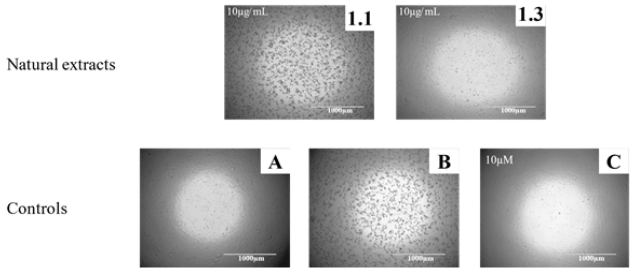

Figure 2: Microscopic observation of control samples of uninfected cells (A), infected cells (B) and infected cells pretreated by Remdesivir (C) and natural compounds extracted from the organic phase of *Allamanda cathartica* leaves (1.1) and caterpillar feces (1.3)

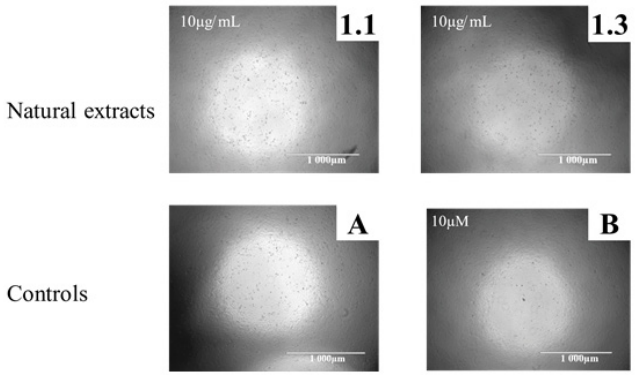

Figure 3: Cell toxicity observed by Evos® microscope of untreated (A) and Remdesivir treated (B) cell control samples and natural extracts of organic extract of *Allamanda cathartica* leaves (1.1) and caterpillar feces extracted from the organic phase (1.3).

healthy cells. Regarding natural extracts, in general, the totality of the natural extracts did not present any toxicity on cells alone. On the other hand, a difference on the antiviral effect of natural compounds on SARS-CoV-2 virus infection was observed. The bioactive molecules extracted from the aqueous or organic phases of the caterpillar feces had the highest percentage of inhibition of infection against the virus, i.e. 96%. The caterpillar body has practically no inhibitory effect on the cells when the extraction took place in organic medium. A slight increase of this effect was observed when extracting from the aqueous phase. The opposite tendency was observed for the molecules coming from healthy leaves since those extracted in the organic medium present a percentage of inhibition of 41% in organic phase and was negative in aqueous phase. However, the 41% antiviral effect in organic medium was obtained by CPE-measurement, but not confirmed by microscopic observation. This discrepancy might be due to the fact that the compound is colored and interferes with CPE reading. Therefore, the need to have a consistency between CPE measurement and microscopic observation.

Molecular modeling

Based on the experimental results, we decided to delve into the molecular basis of the potential therapeutic effects of Allamandin against SARS-CoV-2 by computational means. Because of the pharmacological relevance of the M_{pro} enzyme in this disease, protein-ligand dockings were carried out on this target. Calculations were performed using a blind docking approach on different experimental structures available in the protein data bank (ref) including ligand free forms (pdb 7lke ([30])) and ligand bound forms (7lbn ([30])), 7qt5, 7qt6, 7qt7 and 7qt9 ([31])). The entire space of the dimeric (pdb 7lke and 7lbn) or monomeric forms of the enzyme (7qt5, 7qt6, 7qt7, 7qt9) was explored with the autodock vina algorithm ([32]). Calculations setup and analysis were performed with the UCSF Chimera software ([33]) Calculations suggest good interaction between Allamandin and the SARS-CoV-2 protease. Considering the whole set of solutions, binding modes tend to present scores between -7.7 and -6.5 kcal/mol. Two main tendencies are observed (Figure 4). In all cases, solutions in the protease catalytic sites are identified. Highly exposed to the solvent, those solutions present a limited number of polar interactions of the Allamandin with the catalytic pocket, mostly with His41 and Thr45 (see Figure 5). The remaining interactions are primarily hydrophobic and involve ASP 142, CYS145, and LEU27. Other solutions are most distributed along the protein scaffold although some are often located in the intermonomeric region of the dimer. These solutions are mainly targeting the polar patch between both subunits. Depending on the experimental structures on which the calculations are performed, they could get more or less profound in this interface. The amino acids involved in those interactions are Arg4, Lys5, Glu288, Phe291, Arg131, Lys137 of both units.

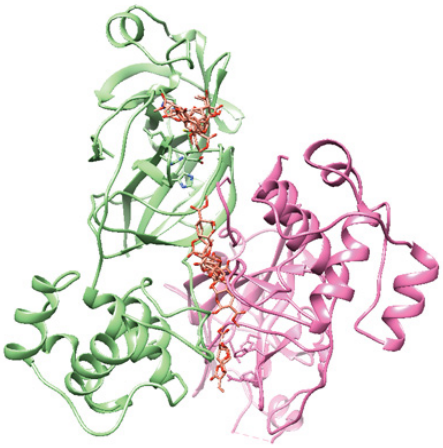

Figure 4: Docking_1. Representation of the entire set of docking solutions of Allamandin in SARS-CoV-2 dimeric ensemble. Protein atoms are represented in ribbon with pink and green color. Allamandin docking solutions are represented in red. Example taken from the structure with the pdb reference 7lbn.

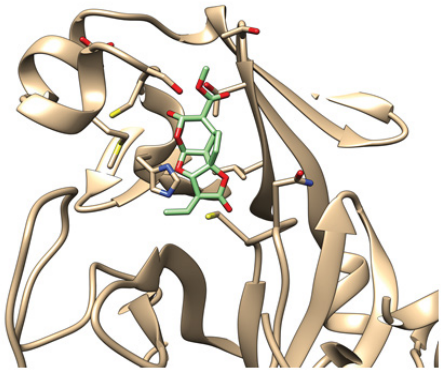

Figure 5: Docking 2. Best docking solution of Allamandin into Mpro SARS-CoV-2 binding site example taken from docking in structure with pdb reference 7lke.

The hypothesis that Allamandin could interact with the main protease of SARS-CoV-2 appears valid based on the computational modeling performed in this study. However, different interactions could be postulated, including direct inhibition of the catalytic site and possible perturbation of the inter monomeric contacts. Further experiments are therefore necessary to ascertain the robustness of the hypothesis.

Conclusion

Pseudosphinx tetrio caterpillars and *Allamanda* leaves are abundant resources in the French West Indies, for which applications against SARS-CoV-2 have not been found to date. The studies carried out during this work showed that these resources could have a significant protective effect on cell infection without being toxic to healthy cells. This protective role was demonstrated by screening measurements and microscopic observations that showed cell viability after contact with the bioactive molecules.

The *Pseudosphinx tetrio* caterpillar feces and the healthy *Allamanda cathartica* leaves played a more important protective role against SARS-CoV-2. In particular, for the natural organic phase extract of *Pseudosphinx tetrio* caterpillar feces which showed complete protection from SARS-CoV-2 infection at 10µg/mL concentration and no toxicity on cells alone in viability readings. This inhibitory effect was confirmed when cells were observed under the microscope. This can be explained by the defense process that the caterpillar sets up during digestion. Indeed, the differences observed in this study between the caterpillar feces and healthy Allamanda leaves showed that during digestion the caterpillar could have the ability to excrete molecules or to significantly modify the ingested material to confer antiviral activity. These first results are promising for a preventive treatment against the SARS-CoV-2 virus. A more detailed study of the structure of the molecules present in the different extracted phases will be envisaged, as well as an analysis of the protective and antiviral effect of the leaves herbivorated by the caterpillar.

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Patents

Author contributions

All authors have read and agreed to the published version of the manuscript. NG performed cytotoxicity and antiviral assessments. All authors have contributed to methodology, formal analysis, writing—review and editing and investigation, LM, MS and GC-T wrote original draft preparation and participate at the conceptualization

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Informed consent statement

Not applicable.

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Conflicts of interest

The authors declare no conflicts of interest.

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