Research article

Determination of genetic damage induced by *Azadirachta indica* extract in human lymphocytes

Determinación del daño genético inducido por extracto de *Azadirachta indica* en linfocitos humanos

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Abstract

Objective. To evaluate the genotoxic effect of a Neem-based biopesticide (*Azadirachta indica*) on human lymphocytes, aiming to assess potential health risks for farmers and support the safe use of natural-origin compounds in pest control. **Methods.** Neem seed extract was obtained using the Soxhlet extraction method and formulated into three treatment concentrations: C1 (0.001 mM), C2 (0.0015 mM), and C3 (0.002 mM). Hydrogen peroxide was used as a positive control and phosphate-buffered saline (PBS) as a negative control. Human lymphocytes were isolated from the peripheral blood of a healthy 25-year-old volunteer. Genotoxicity was assessed using the alkaline single-cell gel electrophoresis (SCGE) or Comet Test, employing the sandwich technique for cell protection and fixation. Samples were stained with GelRed, visualized under a 40x objective using an Optika Italy B-510 series microscope, and analyzed with Comet Score™ software. The percentage of DNA in the comet tail (%DNA in tail) was used as the primary damage indicator. **Results.** The analysis showed a dose-dependent increase in DNA damage. Treatments C2 and C3 exhibited significantly higher DNA tail percentages, indicating greater genotoxicity compared to C1 and the negative control. **Conclusions.** The genotoxic effect of Azadirachta indica extract on human lymphocytes increases with concentration, suggesting a direct relationship between Neem biopesticide dosage and DNA damage. While Neem is a natural alternative, careful concentration control is essential to ensure user safety.

Keywords: Biopesticide, Comet test, Electrophoresis, Neem, Toxicity.

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Resumen

Objetivo. Evaluar el efecto genotóxico de un biopesticida elaborado a partir de semillas de Neem (Azadirachta indica) sobre linfocitos humanos, con el propósito de mitigar los posibles riesgos a la salud de los agricultores. **Métodos.** La extracción del principio activo de Azadirachta indica se realizó mediante el método Soxhlet, y se prepararon tres concentraciones de tratamiento: C1 (0.001 mM), C2 (0.0015 mM) y C3 (0.002 mM). Se utilizó peróxido de hidrógeno como control positivo y tampón fosfato (PBS) como control negativo. Los linfocitos se obtuvieron de una muestra de sangre periférica autóloga, proveniente de un individuo sano de 25 años. Posteriormente, se aplicó el ensayo Cometa (SCGE) en condiciones alcalinas, utilizando la técnica de sándwich para la protección y fijación celular. Las muestras fueron teñidas con GelRed y observadas con un microscopio Optika Italy B-510, con objetivo de 40x. Las imágenes fueron capturadas y analizadas mediante el *software Comet Score*™. Se empleó como parámetro de medición el porcentaje de ADN en la cola del cometa (%ADN en cola). **Resultados.** Los tratamientos C2 y C3 presentaron un mayor porcentaje de ADN en cola en comparación con C1 y el control negativo, evidenciando un aumento en el daño genético proporcional a la concentración del extracto. **Conclusiones.** La genotoxicidad inducida por el extracto de Azadirachta indica mostró una relación directamente proporcional con su concentración, lo que sugiere que, aunque se trata de un biopesticida de origen natural, su uso debe considerar cuidadosamente las dosis para evitar efectos adversos en la salud humana.

Palabras clave: Biopesticida, Ensayo Cometa, Electroforesis, Neem, Genotoxicidad.

Introduction

Currently, agriculture is commonly affected by various phytosanitary problems accompanied by a series of damages, such as environmental contamination and insect and pest attacks, which affect food production [1,2]. In the case of biopesticides, these have become a strategy of great interest for pest control [3]. These compounds are derived from different raw materials that show worldwide benefits, such as minimizing insect resistance and reducing the consequences that they could bring to natural enemies, so that their toxicity level is minimal for humans, animals and plants, preserving their properties and effectiveness in their application [4].

These natural compounds are considered of great importance due to their phytochemical components, being the best alternative for

pest control [5]. In this context, the Neem tree becomes an important species of great relevance since it has been studied as a source of a variety of natural products [6]. Its seed is characterized by the presence of a high concentration of azadirachtin, so the development of a biopesticide based on the seed extract of the Neem tree (A. indica), is presented as an option for agriculture given its numerous components with insecticidal activity [7]. Efforts to protect the environment and mitigate pollution have led to the development of environmentally friendly products [8]. Therefore, agricultural biotechnology has become a strategy for crop maintenance [9], since it allows the use of a set of techniques based on living organisms for their improvement or sustainable use [10]. Likewise, the use of carbamate, organophosphate and organochlorine pesticides are widely used in fruit and vegetable crops worldwide [11, 12, 13, 14].

Research carried out by [15] identified an in vitro cytotoxicity and genotoxicity potential of methomyl (methyl-N-[(methylcarbamoyl)oxy]thioacetamidate), which is the active component of the insecticide and acaricide. On the other hand, studies conducted by [16] and [17], demonstrated a genotoxic effect on human lymphocytes, which induced chromosomal aberrations and micronuclei that were dependent on the dose applied by the insecticides. It should be noted that lymphocytes are cells of the immune system that protect against infectious diseases from pathogens such as bacteria, viruses and fungi that penetrate the body, in addition to recognizing and neutralizing harmful substances in the environment [18].

The use of chemicals, especially organochlorines, has sometimes been accompanied by risks to human health and the environment due to their toxic effect and their high persistence, bioconcentration and, above all, nonspecific toxicity [19]. The evidence of genotoxic risk is due to lack of protection, occupational hazards such as direct and indirect contact with the substances. It is important to estimate the risk to exposure [20], as it has been observed in chemicals from all major functional classes of pesticides, including insecticides, herbicides, fungicides, and fumigants, because they have significant associations with a variety of cancer sites [21].

According to [22], most farmers lack knowledge about the toxicity generated by pesticides. As well as the measures to be taken for their use. Authors such as [23], mention that farmers use mixtures of pesticides, which exceed the recommended doses.therefore, to mitigate the impact of contamination generated by pesticides, Thus, the objective of the present research is to evaluate the genotoxic incidence of Azadirachta indica in human lymphocytes. This study was based on obtaining the natural extract of the seed of the Neem tree, with

which the genotoxic risk was determined using the Comet Test as an indicator of genotoxicity, since it is a tool that establishes the damage caused to genetic material in living beings [24], being also a sensitive indicator of DNA damage caused by exposure to a wide variety of toxicants [25], which allows identifying the damage that genetic material can suffer under environmental conditions [26].

Materials and methods

Azadirachta indica oil extraction.

Two kg of *A. Indica* seeds were used. First, the external layer was removed and then the drying process was carried out in a tray oven for 72 hours. The grinding of the seed required a manual mill where the crushed material of the sample was obtained. The extraction was performed by the Soxhlet method following the methodology proposed by [27], where 10 g of *A. indica* flour were placed in the equipment cartridges, for which 200 mL of 96% ethanol were measured in a 1:20 ratio (10 g of flour and 200 mL of ethanol), placed in a balloon at a temperature of 250 °C with a total initial volume of ethanol of 400 mL in a period of 6 hours.

Lymphocytes isolation

Lymphocytes were extracted from a female individual aged 25 years in healthy condition, with a hypodermic syringe and a heparin-containing tube with 3 mL peripheral blood was used following the methodology of [28]. Subsequently, the sample was divided into 1.5 ml microtubes and taken to the centrifuge at 1000 rpm for 5 min. The plasma was collected and stored in 1.5 ml tubes. Next, different treatments were performed at different concentrations of 0.001, 0.0015, 0.002 mM respectively, to identify the genotoxic effect. As a negative control the cells were exposed to phosphate saline PBS(PBS) pH. 7.0, where exposed cells can prove cell damage and cell function [29]. For the positive control hydrogen peroxide (H202) was used, which is well known for its oxidizing power in human erythrocytes. It produces alterations in the genetic material that are expressed as single and double chain breaks, it was chosen as a positive control, since it is a compound highly used for genotoxicity studies in the Comet assay [30, 31]

Comet assay

The assay was performed following the methodology proposed by [32], with some modifications, with a 1:1 mixture ratio with the different concentrations, with 70 µl of *Azadirachta Indica* extract and 70 µl of lymphocytes from each treatment, comprising three replicates. 70 µl of PBS plus 70 µl of lymphocytes were used as negative control and 70 µl of hydrogen peroxide plus 70 µl of lymphocytes were used as positive

control, which were exposed for 30 min at room temperature. Likewise, frosted slide sheets were prepared where 140 µl of 1.5% agarose were placed and refrigerated until solidification for 5 min. Then, they were covered with a second layer of agarose until solidification, being immersed in a lysis buffer (NaCl 2.5M, EDTA 0.1M, Tris HCL 10mM, Triton X-100), for a period of four hours.

Finally, the denaturation process was performed by placing the slides in an electrophoresis buffer (Tris, borate and EDTA buffer solution) at 4°C for 20 minutes. Electrophoresis was carried out in the refrigerator maintaining a temperature of 4 °C to 15 °C, 15 V, 300 mA for 30 minutes. Red gel was used as a dye to stain the cells. Visualization was performed using a B-510 series Optika Italy microscope, with observation at 40x objective. Comet ScoreTM software was used, which allowed capturing the image, where the fluorescence intensity in the tail is compared with the full intensity of the comet to determine the %DNA degraded [33].

The three most commonly used quantitative measures in comet assay study were selected: tail length (TL), percentage of DNA in the tail (% T), and tail moment (TM). However, we only worked on the basis of tail length based on the formula of [34], (Tail length x % DNA in tail) /100. the most commonly used parameters are tail length, % tail DNA and tail moment, to quantify the magnitude of damage. The % DNA was calculated as the ratio between the total intensity of the tail and the total intensity of the comet (head and tail together); the TM parameter is calculated as the product of two factors: the % DNA in the tail and the distance between the center of mass of the head and tail [35].

Statistical analysis

An analysis of variance (ANOVA) was performed with STATGRAPHICS software for the %DNA, comparing the significant difference between the positive control, negative control and the different treatments, this was determined taking into account the P value established as a probability value, less than 5% (0.05).

Results and discussion

Lymphocytes visualization

Five cells per treatment were analyzed and the concentration damage was determined; the longest tail length found in the positive control was 28 PX (pixels) (Figure 1).

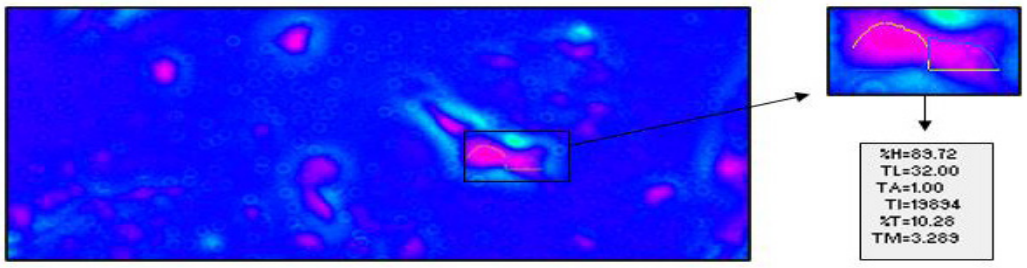


Figure 1. Positive control: hydrogen peroxide; TL: tail length, TA: tail area, %T: % DNA in tail and TM: tail moment.

According to the parameters analyzed, a percentage of 13.85 of the DNA was obtained, presenting genotoxicity or cell damage, as shown in Table 1.

Table 1. Measurement of positive and negative control lymphocytes.

POSITIVE CONTROL (+)					NEGATIVE CONTROL (-)				
% DNA of the head	Tail length (TL)	Tail área (TA)	% Tail DNA (T%)	Moment of the queue (TM)	% DNA of the head	Tail length (TL)	Tail área (TA)	% Tail DNA (T%)	Moment of the queue (TM)
89.72	32.00	1.00	10.28	3.289	99.83	00.00	1.0	**0.17**	00.00
95.87	17.00	1.00	4.13	0.702	100.0	00.00	1.0	00.00	00.00
91.13	21.00	1.00	8.87	1.863	99.98	00.00	1.0	0.02	00.00
94.96	28.00	1.00	5.04	1.499	94.57	00.00	1.0	0.43	00.00
86.15	28.00	1.00	**13.85**	3.877	99.77	00.00	1.0	0.23	00.00

For the negative control, PBS was used, lymphocytes were observed without any damage (Figure 2), where the tail length was zero, without damage or DNA degradation. According to [35], the exposed cells do not show migration and maintain their spherical shape, with an average of 0.17 % of the DNA in the tail of the lymphocytes.

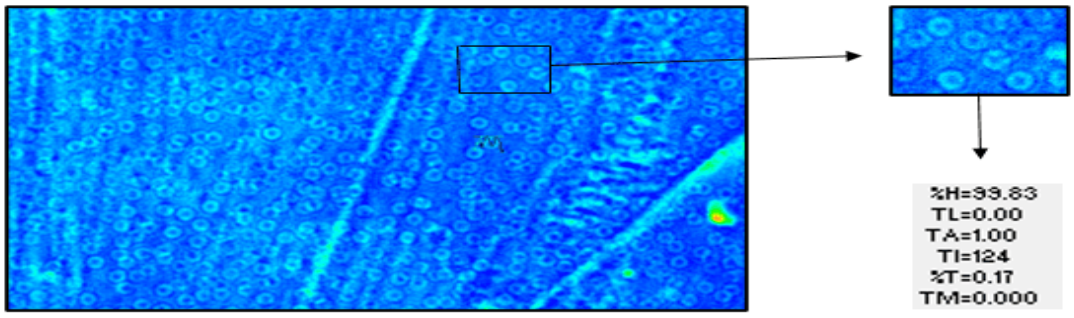


Figure 2. Negative Control: PBS; TL: tail length, TA: tail area, %T: % DNA in tail and TM: tail moment.

The treatment with 0.001 mM concentration (C1) did not register significant damage in lymphocytes. Complete spherical cells were observed without DNA degradation. It is important to highlight that the average of the percentage of tail DNA (%T) resulting with 0.3%, no complete DNA degradation was found, since the percentage of the lymphocyte head was 99.84%. DNA damage was expressed as percentage of damaged cells. [36], included all cells with low, moderate and extreme levels of DNA damage, which determined the %DNA of the comet tail as a parameter of genotoxicity.

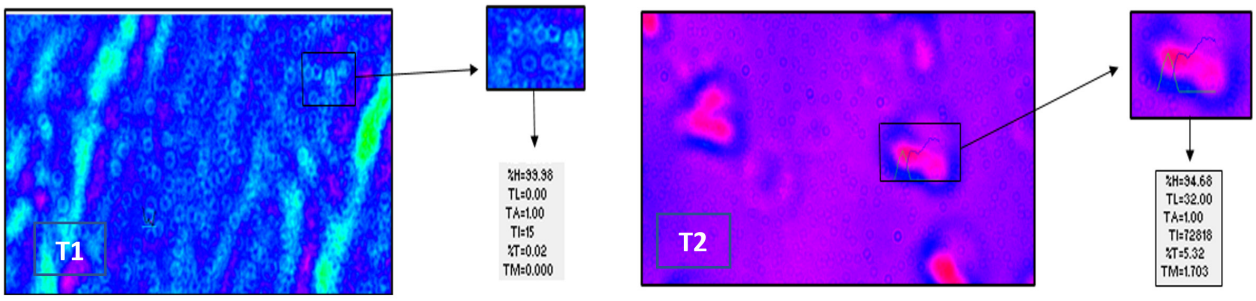


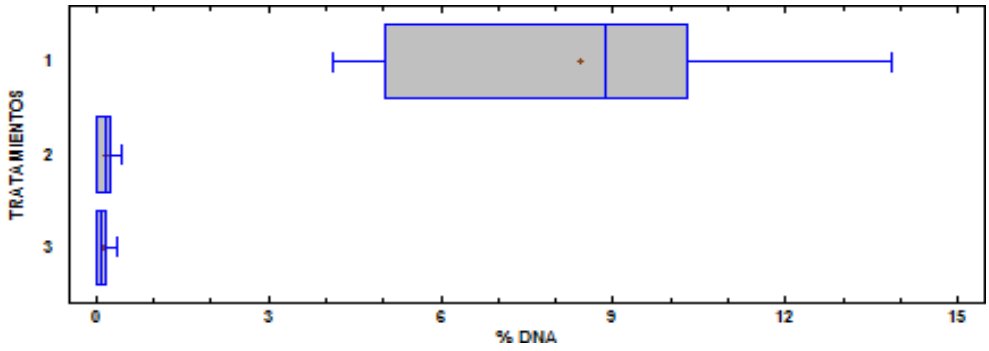
Figure 3. Treatment 1: concentration 0.001 mm. Treatment 2 concentration 0.0015 mm.

In the C2 0.0015 mM treatment, five lymphocytes were recorded at different positions of each plate. Damage of the fractional %DNA of the comet tail length was evidenced with 5.32%, demonstrating greater damage to the C1 treatment with degradation in the cell, (see Figure 3) studies conducted by [37], observed that in cells in which there is greater frequency of cell damage, greater length of DNA migration degradation is also observed. On the other hand, the data obtained with the Comet Score TM software allowed providing measurement parameters of the comets formed, as shown below in Table 2.

Table 2. Measurements of C1 and C2 treatment lymphocytes.

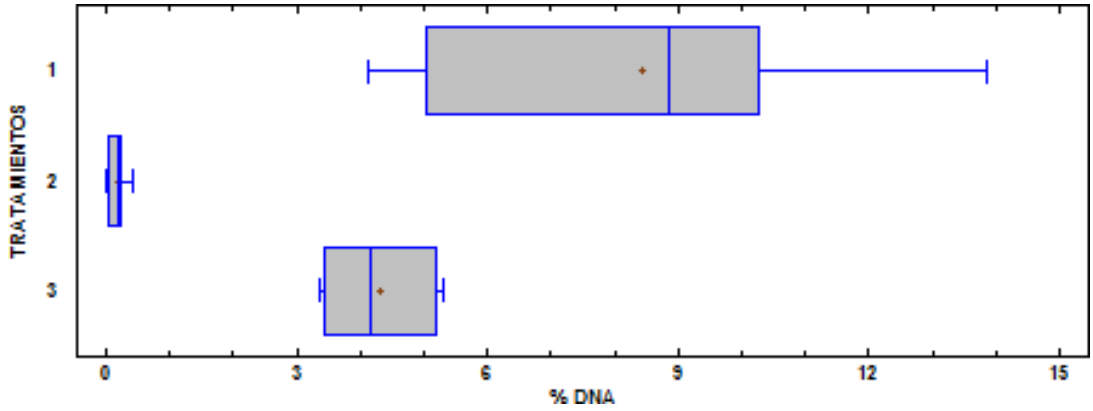
TREATMENT C1					TREATMENT C2				
% DNA of the head	Tail length (TL)	Tail área (TA)	% Tail DNA (T%)	Moment of the queue (TM)	% DNA of the head	Tail length (TL)	Tail área (TA)	% Tail DNA (T%)	Moment of the queue (TM)
99.98	00.00	1.00	0.02	3.289	99.68	32.00	1.0	**5.32**	1.703
99.63	00.00	1.00	0.37	0.702	96.55	62.00	1.0	3.45	2.139
99.99	00.00	1.00	0.01	1.863	99.83	60.00	1.0	4.17	2.500
94.84	2.00	1.00	**0.03**	1.499	94.00	11.00	1.0	5.20	05.72
99.91	0.00	1.00	0.09	3.877	96.54	7.00	1.0	3.36	0.235

When comparing the two treatments, it was observed that the %DNA of the tail from treatment C2 was 4.3 Px, compared to treatment C1, which obtained a significant difference that is directly proportional to the increase in damage and the increase in the concentration of *Azadirachta indica*, which generates an increase in DNA lesions. In research conducted by [38], they found that pesticides induce the oxidative process in DNA through reactive oxygen species. On the other hand, the probability value was determined to be less than 5%, so there is no significant difference, which, unlike what was stated by [39], if the range is greater than what was stated, it does not generate the confidence to deny the observed difference. Regarding the positive control, a value of P- 0.0016 was obtained, given that the value is lower there is a significant difference. Analysis for the C1 0.001 mM treatment showed a P-value of 0.7059, a value higher than 0.05 against the negative control, which means that the difference is not statistically significant in the %DNA of the comet tail with the mean from one level of treatment to another at a confidence level of 95.0%. Graph 1 shows the comparison of the treatments applied, for which 5 cells were analyzed in a plate of approximately 200 cells for each damage observed.



Graph 1. Comparison of %DNA means of treatment 1 and the positive and negative control. Treatment. 1 (positive control). Treatment 2 (negative control). Treatment 3 (C1 0.001 mM).

In addition, for treatment C2 0.0015 mM of *A. indica*, five lymphocytes were registered in different positions of each plate, showing damage of the fractional %DNA of the comet tail length with 5.32%, demonstrating more significant damage than treatment C1 with degradation in the cell. The average/mean %DNA of the tail for C2 treatment was 4.3 Px, compared to C1 treatment with a significant difference which is directly proportional to the increase of the damage and the increase of *Azadirachta indica* concentration, with an average of 7.23 % of the DNA, being this data higher with respect to C1 and C2 treatment (see graph 2).



Graph 2. Comparison of %DNA means of treatment 2 and the positive and negative control. Trat. 1 (positive control). Trat 2 (negative control). Trat 3 (C2 0.0015 mM).

For treatment C3 0.002 mM, a higher %DNA tail (%T) was obtained, which was 9.54% with a tail length of 86.00 Px, leaving a percentage of 90.46% in the lymphocyte´s head without degradation, as shown in Table 3.

Table 3. Lymphocyte measurements in C3 treatment.

TREATMENT C3				
% DNA of the head	**Tail length (TL)**	**Tail área (TA)**	**% Tail DNA (T%)**	**Moment of the queue (TM)**
90.46	86.00	1.00	**9.54**	8.207
93.76	28.00	1.00	6.24	1.746
91.15	60.00	1.00	8.85	5.307
94.43	50.00	1.00	5.57	2.784
94.05	77.00	1.00	5.95	4.580

Treatment three indicated damage to the tested cells, approaching the damage reported in the positive control, indicating greater damage in the 0.002 mM concentration treatment as compared to the other treatments. When comparing the %DNA data of the tail from the negative control, the difference was statistically significant with a P-value of 0.000. Regarding treatments 2 and 3 with higher concentrations of *Azadirachta indica*, the damage is directly proportional to the concentration, as shown in Table 4. [40], state that the damage generated in the DNA could present a variation depending on the type of cell used in the study, since authors such as [41], demonstrated that pulmonary epithelial cells present greater susceptibility to genetic damage with respect to other cell types.

Table 4. Mean %DNA of tail of each treatment of with intervals of *Azadirachta indica.*

TREATMENT	Quantity	Media	Lower limit	Upper limit
Positive control	5	8.434	9.72	13.85
Negative Control	5	0.17	0	0.43
T: C1 0,001	5	0.13	0.01	0.37
T: C2 0,0015	5	4.3	3.36	5.32
T: C3 0,002	5	7.23	5.57	9.54

In the statistical analysis, regarding the positive control, a value of P- 0.0016 was obtained, given that the value is lower, there is a significant difference. In the C1 0.001 mM treatment analysis, a P-value of 0.7059 was obtained, a value higher than 0.05 against the negative control, and no significant DNA degradation was found in the C1 treatment. Also, the C2 treatment was compared with the positive control which showed a P-value of 0.0531 which does not show much difference from the positive control, since the P-value must be less than 0.05 for the existence of damage. At the same time, it was compared with the negative control which presented a value of P- 0.0000.

Conclusions

Finally, it can be established that the formation of comets in human lymphocytes is a clear sign of the degradation of the genetic material produced by the *Azadirachta indica* extract, even though no significant damage was recorded in the tested cells, negative effects were observed as the concentration of the extract increases, this being a key factor in the process of genotoxicity generated in human lymphocytes, thus it is important to adjust the reference levels of *Azadirachta indica* extracts to work as a control measure for human health.

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Ethical Approval

The procedures were submitted to the criteria of the manual of procedures of the Ethics Committee office at Universidad Francisco de Paula Santander, and approved by the Ethics Committee (approval November 13, 2022).

Consent to participate

Written informed consent was obtained from individual or guardian participants.

Consent to publish

All authors have agreed with the content and all have given consent to publish.

Author Contribution

Jhossleiny Cristina Meza Ojedaj: Methodology, Writing, Original draft preparation.

Nelson Alfonso Vega Contreras: Methodology, Writing and Investigation and Data curation.

Seir Antonio Salazar Mercado: Supervision, Conceptualization.

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Competing Interests

The authors declare that they have no competing interests.

Availability of data and materials

The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

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