

Effect of the ethanolic extract of *Randia aculeata*'s fruit in heart and skeletal muscle oxidative stress caused by snake venom

Carlos Ángel Gallardo Casas,^{1*} María del Carmen Castillo Hernández,²
Marcos Ayala Hernández,² Hugo Alberto Sánchez Maldonado,¹
Edgar Cano Europa²

Abstract

The purpose of this investigation was to prove if *Randia aculeata* is able to protect against oxidative stress generated by snake's venom. Forty-eight male CD1 mice were used divided in groups of 8. 1) control group, 2) *Randia aculeata*, 3) *Bothrops asper* venom, 4) *Randia aculeata* and *Bothrops asper*, 5) *Crotalus simus* venom and 6) *Randia aculeata* and *Crotalus simus*. Blood was obtained and then fibrinogen, Lactate Dehydrogenase (LDH) and Creatin kinase (CK) were quantified. Muscle and hearts from animals were obtained in order to analyse Lipid-peroxidation (LPX), and antioxidant enzymes (catalase, super oxide dismutase [SOD], and glutathione peroxidase, [GPX]). Mice treated with the venom had an increase in CK and LDH enzyme activity. Fibrinogen decreased. These disturbances were prevented with the administration of extract. It also prevented increasing levels of lipoperoxidation in both organs. It was found that SOD, catalase and GPX enzyme activity in skeletal muscle, were not altered. In heart tissue, SOD and GPX decreased levels were found in groups that received the venom, but ethanol extract prevented this decrease. Catalase activity increased in groups treated with snake venom, and the one that received venom from *C. simus* and ethanol extract. The plant minimizes significantly

KEY WORDS

Poison, Damage, Medicinal plant, Heart.

Efecto del extracto etanólico del fruto de *Randia aculeata* en el estrés oxidativo del corazón y del músculo esquelético causado por el veneno de serpiente

Recibido: 10 de marzo de 2022
Aceptado: 26 de marzo de 2022

Resumen

El objetivo fue comprobar si *Randia aculeata*, protege contra el estrés oxidativo generado por el veneno de serpiente. Se utilizaron 48 ratones CD1 macho divididos en grupos de ocho. 1) grupo control, 2) *Randia aculeata*, 3) veneno de *Bothrops asper*, 4) *Randia aculeata* y *Bothrops asper*, 5) veneno de *Crotalus simus*, y 6) *Randia aculeata* y *Crotalus simus*. Se obtuvo sangre y se cuantificaron fibrinógeno, lactato deshidrogenasa (LDH) y creatinina cinasa (CK). Se obtuvo el músculo y corazón, para analizar la peroxidación lipídica (LPX), y enzimas antioxidantes (catalasa, superóxido dismutasa [SOD] y glutatión peroxidasa, [GPX]). Los ratones tratados con el veneno tenían un aumento en las actividades de las enzimas CK y LDH, el fibrinógeno disminuyó. Esto fue prevenido por el extracto, también impidió el aumento de la lipoperoxidación en ambos órganos, las actividades de SOD, catalasa y GPX en el músculo esquelético no se alteraron. En corazón, SOD y GPX disminuyeron en los grupos que recibieron veneno, pero el extracto lo impidió, la actividad de catalasa aumentó en los grupos tratados con veneno y en el que recibió veneno de *C. simus* y extracto. La planta minimiza significativamente los efectos oxidativos que causan los venenos de víbora en ratones.

PALABRAS CLAVE

Veneno, Daño, Planta medicinal, Corazón..

¹Universidad de Chalcatongo, México. ²Instituto Politécnico Nacional, México.
*Autor para correspondencia: carlosg84@unicha.edu.mx

Introducción

Medicine plants, particularly those used as anthiophidic, are of special interest in Mexico, since different cultures have been using them for many years. However, information is still scarce and in most cases there is a lack of real scientific analysis. In addition, the traditional knowledge related to these plants, is getting lost due to the old age of the owners and the lack of interest by young people in learning the use of plants as anthiophidic.¹

There are people in Colombia, Brazil and Mexico, known as “Culebreros” (traditional medicine therapists, specialized in treatment for snakebites), who claim to have knowledge on the application of several plants when a venomous snake attack occurs.² In many cases, the presence of this sort of “healers” represents the only option when emergency health care is absent to provide an antivenom, and also they maintain and transmit a valuable tradition and a wide knowledge about plants and animals from each region.

One of the damage mechanisms of the venom is the generation of oxidative damage. The factor responsible for this event is the phospholipase A₂ toxin, which increases arachidonic acid levels, and at the same time, ROS levels and lipoperoxides,³ resulting in an oxidative stress process and death by necrosis. Regarding oxidative stress, a direct correlation was found between the antioxidative capacity of Colombian plants and their inhibitory capability for phospholipase A₂, suggesting a possible mechanism of action.⁴

In Mexico, *Brongniartia podalyrioides* Kunth and *B. intermedia* Moric have been evaluated. It has been found that they contain edunol, a substance with a chemical structure that allows neutralizing the cardiotoxic effects of the venom.⁵ Similarly, in recent years it has been documented that healers from Acayucan, in the state of Veracruz, use different plants against snake bites, among which *Randia aculeata* (Crucecillo) is reported, in which case the whole plant is applied.² At the central region of Veracruz, however, in the Jamapa municipality, this knowledge has become popular and people do not attend to the healer, but they make an alcoholic preparation with the fruit of this plant, just in case that they are bitten by a snake, and they also apply this product in household animals. The cytoprotective effect on skeletal muscle, myocardium and blood has also been shown.⁶

In light of what is mentioned above, it was our interest to assess whether the protective effect on skeletal muscle and heart is due to the modification, by the plant, of the oxidative stress parameters generated by the snake’s venom, and if it contributes to a lower degree of cell death.

Materials and methods

Preparation of the plant

The plants were collected in rural communities from Jamapa, Veracruz, Mexico, identified by specialist in plants Abigail Aguilar-Contreras as *Randia aculeata* and deposited in the Herbarium of medicinal plants of the Centro Medico Siglo XXI IMSS and were registered for its traditional medical use. One kg of fruit was smashed and submerged in 5 liters of 96° alcohol for 8 days, afterwards, it was filtered, the alcohol was evaporated and solids were concentrated in a 0.09 % saline solution.

Venom

Crotalus simus and *Bothrops asper* venom was kindly provided and donated by Edgar Reina-Ponce and Orlando Reina-Ponce, directors of “Museo Viviente Veracruz Salvaje”, in Veracruz, Mexico. The quantification of proteins in the venom was made using the Bradford method, and after that LD₅₀ was made using the Lorke’s method.

Animals

Forty-eight male CD1 mice were used. Animals weighed between 20 and 25 g. They were housed in groups of six or eight in a Plexiglas cage, with food and water ad libitum, in a room with constant temperature (21 ± 2 °C) and a 12-h light:12-h dark cycle (lights on

at 0800). All experimental procedures described in this study are in accordance with the guidelines of the Laws and Codes of Mexico in The Seventh Title of the Regulations of the General Law of Health Regarding Health Research and the Mexican Official Standard NOM-062-ZOO-1999 of the technical specifications for production, care, and use of laboratory animals, and also the 2010/63/ue european parliament's directive regarding protection of animals used in scientific investigations. The animals were randomly assigned into six groups, 1) a control group that received 0.9% saline ig and 0.9% saline im (n = 8), 2) 250 mg/kg R. aculeata ig + 0.9% saline im (n = 8), 3) 0.9% saline ig + 1mg/kg B. asper venom im (n = 8), 4) 250 mg/kg R. aculeata ig + 1mg/kg B. asper venom im (n = 8), 5) 0.9% saline ig + 5mg/kg C. simus venom im (n = 8), and 6) 250 mg/kg R. aculeata ig + 5mg/kg C. simus venom im (n = 8). The administration of saline or R. aculeata ig was made immediately before saline or venom administration im. 24 hours after administration, the mice were killed by decapitation.

Blood was obtained and fibrinogen, Lactate Dehydrogenase (LDH), and Creatine kinase (CK) enzymes were quantified by commercial kits. Moreover, quadriceps muscle and hearts from animals were obtained, which were stored at ultra freezing conditions (-70° C), to perform further analysis for oxidative stress markers, such as Lipid-peroxidation (LPX), and antioxidant enzymes (catalase, super oxide dismutase [SOD], and glutathione peroxidase, [GPX]).

Biochemical parameters

All of the muscles and hearts used for biochemistry assays were homogenized in 3 mL 10 mM phosphate buffer, pH 7.4

Protein quantification

Protein quantification from the homogenate was determined by the Bradford method, which foundation is the reaction between aromatic amino acids from the proteins and brilliant blue Coomassie G-250, to report results as mg of protein.⁷

Lipid peroxidation assay

A sample of 0.1 g of tissue was used, and homogenized in 5mL 0.15 M Tris-HCl buffer pH 7.4, and was mixed with 1 mL 20% Trichloroacetic acid (TCA), 2 mL 0.67% TBA, and heated during 1 hour at 100°C. Later, the mixture was placed in ice bath, and after cooling the precipitate was discarded by centrifugation for 15 minutes, 6000 rpm. Absorbance of the sample was measured at 535 nm by using a blank with all of the reagents but the homogenized tissue. As 99% of the TBARS is malodialdehyde (MDA), TBARS concentrations of the samples were calculated by using a curve of increasing concentrations of MDA.⁸

Enzymatic activities of Glutathione Peroxidase (GPX) & Superoxide Dismutase (SOD).

These enzymes were measured by following manufacturer instructions (Cayman Chemical), and by using colorimetric methods.

Catalase activity

Catalase activity was measured by monitoring the enzyme-catalyzed decomposition of H₂O₂.⁹ Briefly, 100 µL of cell extract was added to 3 mL of 100 mM phosphate buffer, pH 7.4, containing 30 mM of H₂O₂. The absorbance was recorded at 240 nm after 10 min at 37°C. The decomposition of H₂O₂ by catalase present in the samples follows first-order kinetics according to the equation $k=2.3 t \log A_0/A$, where k is the first-order reaction rate constant, t is the time over which the decrease of H₂O₂ caused by catalase activity was measured

(10 min), and Ao/A is the ratio of optical densities at times 0 and 10 min. Catalase activity is expressed as k/mg of protein.

Statistical Analysis

Data are expressed as means ± SE (standard error) and the values of all parameters were analyzed by using a one-way analysis of variance followed by the Student-Newman-Keuls test. A value of $p < 0.05$ was considered statistically significant.

Results

Plasmatic parameters

Regarding the variables measured in the plasma of mice from the different groups evaluated (Figure 1), it was found that mice treated with the venom had an increase in the CK and LDH enzyme activities, as compared to the control group (A & B), but fibrinogen decreased (C). These disturbances were prevented with the administration of ethanol extract from *R. aculeata*.

Lipoperoxidation

Lipoperoxidation, shown in Figure 2, increased in both skeletal muscle (A) and heart (B), in those groups where the venom was administered, and “crucetillo” prevented the increasing of lipoperoxidation in both organs.

SOD enzyme activity

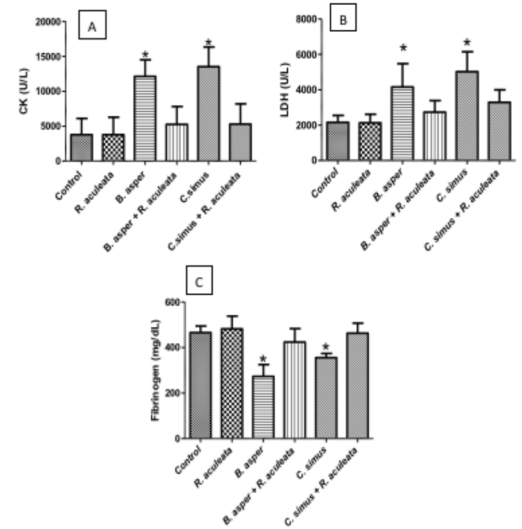
Figure 3 shows SOD enzyme activity of the different groups in this study. It is noted that in skeletal muscle (A), changes in enzyme activity are not altered, whereas it decreases in groups that received the venom. Ethanol extract prevented this decrease.

Catalase enzyme activity

The detoxifying activity of catalase in skeletal muscle (A) and heart (B) is shown in Figure 4. Only in the case of the heart, the activity increased in groups treated with snake venom, and the one that received venom from *C. simus* and ethanol extract.

Figura 1

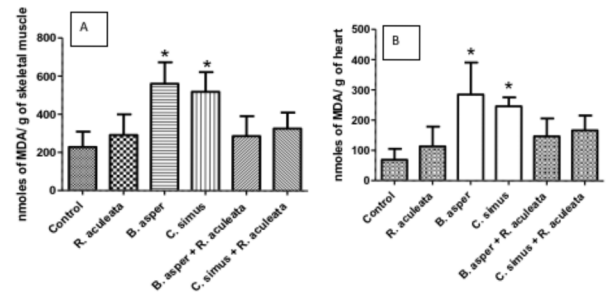
Effect of *Randia aculeata* on the enzyme activity of CK (A), LDH (B), and plasmatic fibrinogen (C) * $P < 0.05$ vs. control; the average is represented as ±EE. ANOVA one way and SNK post hoc.



Source: self made.

Figura 2

Effect of *Randia aculeata* and snake venoms on the peroxidation of lipids in skeletal muscle (A) and heart (B). The average is represented by ±EE. * $p < 0.05$ vs. control. ANOVA one way and SNK post hoc.



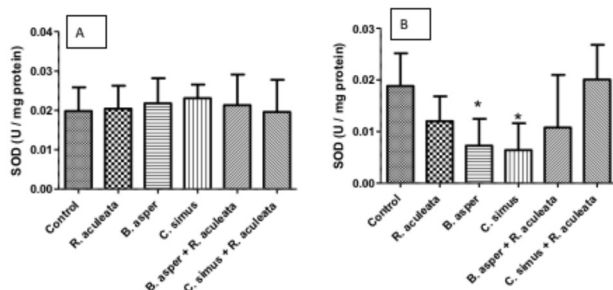
Source: self made.

Figura 4

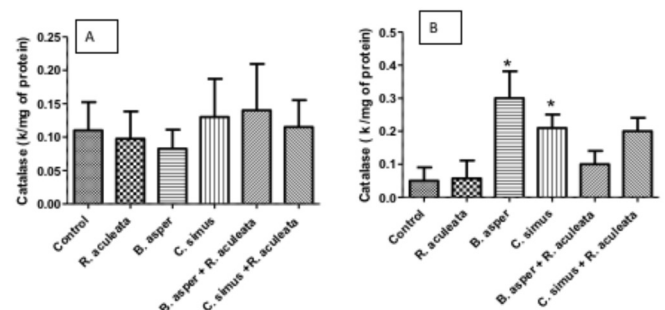
Effect of *Randia aculeata* and snake venoms on the catalase enzyme activity in skeletal muscle (A) and heart (B). Average is represented as ±EE. * $p < 0.05$ vs control A one way and SNK post hoc.

Figura 3

Effect of *Randia aculeata* and snake venoms on the SOD enzyme activity in skeletal muscle (A) and heart (B). The average is represented as ±EE. * $p < 0.05$ vs control ANOVA one way and SNK post hoc.



Source: self made.



Source: self made.

GPX enzyme activity

GPX enzyme activity in skeletal muscle (A) and heart is shown in Figure 5. Only for the heart, the enzyme activity decreased in groups treated with snake venoms, and this effect was prevented with the extract of *Randia aculeata*.

Discussion

One of the principal effects found in medicine plants with anti-phobic properties has been the partial neutralization of the myotoxic effects¹⁰, since enzymes PLA₂, metalloproteases and crotoxins are responsible for these effects. *Randia aculeata* has shown to provide a partial protective effect on skeletal muscle and myocardium, because when venom is administered, both muscles exhibit marked necrosis and these effects are partially inhibited by the administration of *Randia aculeata*, followed by notable leucocytes infiltration. Probably this event is largely due to the fact that the powerful enzymatic system that causes necrosis is inhibited by different phytochemicals, which are in the ethanol extract.⁶

Other studies have shown an enzymatic inhibition for the in vitro proteolysis by ethanol extract from *Renealmia alpinia* as proteolytic activity of venoms and is responsible for a fibrinogen decrease in plasma, in our model it can be seen that fibrinogen is not reduced in groups that received venom and later were administered with the ethanol extract, since when proteases are inhibited, fibrinogen will remain, leading to an antihemorrhagic effect.¹¹

A minor systemic damage due to the plant can be observed because CK and LDH enzymes only raise their activity in those groups that received the venom, these enzymes increase the muscular damage and when both are raised it represents a cardiac damage¹², in groups where we administered the plant plus the venom, these increase was not present, and this is consistent with a minor damage found in skeletal muscle and heart, in accordance to earlier studies.

The present results could indicate a complementary treatment for serum treatment since fabotherapics do not neutralize the effects in tissue degradation by snake venoms.¹³ In our study, this hypothesis is supported by the parameter values we obtained from oxidative stress in skeletal muscle and heart. We observed that both organs presented lipoperoxidation after 24 hours, as reported by Abdurrahmanen, in the African snake *Echis pyramidum*. Said snake includes phospholipase A₂ among its toxins.¹⁴ For this study, we found that the ethanol extract reduced significantly lipoperoxidation as phospholipase activity is inhibited, therefore there is more ROS generation, and oxidative stress will not be present.

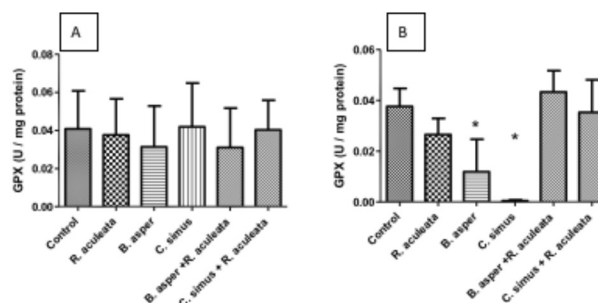
Usually, plants exhibit different antioxidant phytochemicals. It has been reported that *Randia sp.* contains several flavonoids in its fruit that confers high antioxidant capacity,¹⁵ the first mechanism of action proposed for our plant, is that its antioxidants could selectively inhibit phospholipase A₂ because it has been shown that antioxidants also selectively inhibit the site of action of this enzyme.¹⁶

The second mechanism proposed is that antioxidants from the fruit of *Randia aculeata* could neutralize ROS, acting as strong nucleophiles as shown in other models.¹⁷ Regarding antioxidant enzymes, we only observed alteration in heart, and not in skeletal muscle, since the events of oxidative stress follow a temporary course and as the administration of venoms was performed directly on skeletal muscle, possibly after 24 hours we did not find any enzyme activity due to the fact that toxins of the venom reach immediately to the cells and enzymatic disturbances can be presented during the first hours of the intoxication.¹⁸

Regarding SOD activity, it is reduced because probably this enzyme is hydrolyzed by snake proteases; however, the administration of the plant pre-

Figura 5

Effect of both *Randia aculeata* and snake venoms on the GPX enzyme activity in skeletal muscle (A) and heart (B). The average is represented as \pm EE. * $p < 0.05$ vs control ANOVA one way and SNK post hoc.



Source: self made.

vents this disturbance. On the other side, with regard to enzyme activities of catalase and GPX peroxidases, we observed an increase in catalase activity in groups where venom was administered, and this effect is counteracted by the plant, only against *B. asper* venom, this increased activity is trying to compensate the reducing observed in GPX because it is minimized with both venoms, this is the only enzyme that eliminates lipoperoxides that increased in this model due to snake toxins.

Conclusion

Randia aculeata can be a good alternative as a treatment against ophidian accidents. It showed an antihemorrhagic effect by inhibiting the decrease of plasma fibrinogen. It also provided cytoprotection of the myocardium and skeletal muscle through an antioxidant mechanism: by reducing lipid peroxidation and avoiding the reduction of the antioxidant enzyme system in the preclinical model. Therefore, it is suggested to use these results in a clinical model so they can contribute improve the health of people who suffer snake bites.

Acknowledgments

This study was partially supported by SIP-IPN, EDI, COFFA and CONACyT.

Thanks to Abigail Aguilar-Contreras for identifying the plant.

Conflicts of interest

The authors declare no conflicts of interest.

References

1. Albalat-Botana A. Culebreros, médicos tradicionales especialistas en serpientes. *Agroentorno*. 2010; 123:32.
2. Ramos-Hernández M, Ávila-Bello CH, Morales Mávil JE. 2007. Etnobotánica y ecología de plantas utilizadas por tres Curanderos contra la mordedura de serpiente en la región de Acayucan, Veracruz, México. *Bol Soc Bot Méx*. 81, 89-100.
3. Adibhatla RM, Hatcher JF, Dempsey RJ. Phospholipase A2, hydroxyl radicals, and lipid peroxidation in transient cerebral ischemia. *Antioxid Redox Signal*. 2003 Oct;5(5):647-54. doi: 10.1089/152308603770310329. PMID: 14580322.
4. Pereañez JA, Lobo-Echeverri T, Rojano B, Vargas L, Fernandez, Gaviña CA. Correlation of the inhibitory activity of phospholipase A2 snake venom and the antioxidant activity of Colombian plant extracts. *Rev Bras Farmacogn*. 2010. 6, 910-6. <https://doi.org/10.1590/S0102-695X2010005000030>
5. Reyes-Chilpa R, Gomez-Garibay F, Quijano L, Magos-Guerrero GA, Ríos T. 1994. Preliminary results on the protective effect of (-)-edunol, a pterocarpan from *Brongniartia podalyrioides* (Leguminosae), against *Bothrops atrox* venom in mice. *J Ethnopharmacol*. 1994. 42,199-203. DOI: 10.1016/0378-8741(94)90086-8
6. Gallardo-Casas CA, Guevara-Balcázar G, Morales-Ramos E, Tadeo-Jiménez Y, Gutiérrez-Flores O, Jiménez-Sánchez N y otros. Ethnobotanic study of *Randia aculeata* (Rubiaceae) in Jamapa, Veracruz, Mexico, and its anti-snake venom effects on mouse tissue. *Journal of Venomous Animals and Toxins Including Tropical Diseases*. 2012;18(3):287-294. <https://doi.org/10.1590/S1678-91992012000300006>
7. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976. 72, 248-54.
8. Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med*. 1991;11(1):81-128. doi: 10.1016/0891-5849(91)90192-6. PMID: 1937131.
9. Aebi H. Catalase in vitro. *Methods Enzymol*. 1984. 105,121-6
10. Mors WB, Nascimento MC, Pereira BM, Pereira NA. Plant natural products active against snake bite--the molecular approach. *Phytochemistry*. 2000 Nov;55(6):627-42. doi: 10.1016/S0031-9422(00)00229-6. PMID: 11130675.
11. Fernández C.M, Ortiz C.W, Peñearez J.J, Martínez R.D. Antiophidian properties evaluation of ethanol ex-

- tract and fractions obtained from *Renealmia alpinia* (Rottb) Mass (Zingiberaceae) cultivated in vitro. *Rev Fac Quim Farm.* 2010. 17.(1). 75-82.
12. Bucarety F, Fontoura-Herrera SR, Hyslop S, Emílio Carlos Elias Baracat ECE, Vieira RS. Snakebites by *Crotalus durissus* ssp In children in Campinas, São Paulo, Brazil *Rev Inst Med Trop Sao Paulo.* 2002. 44. (3). 133-138. <https://doi.org/10.1590/S0036-46652002000300004>
 13. Rucavado A, Escalante T, Gutiérrez JM. Effect of the metalloproteinase inhibitor batimastat in the systemic toxicity induced by *Bothrops asper* snake venom: understanding the role of metalloproteinases in envenomation. *Toxicon.* 2004 Mar 15;43(4):417-24. doi: 10.1016/j.toxicon.2004.01.016. PMID: 15051405.
 14. Al Asmari A, Al Moutaery K, Manthari RA, Khan HA. Time-course of lipid peroxidation in different organs of mice treated with *Echis pyramidum* snake venom. *J Biochem Mol Toxicol.* 2006;20(2):93-5. doi: 10.1002/jbt.20121. PMID: 16615057.
 15. Santos-Cervantes ME, Ibarra-Zazueta ME, Loarca-Piña G, Paredes-López O, Delgado-Vargas F. Antioxidant and antimutagenic activities of *Randia echinocarpa* fruit. *Plant Foods Hum Nutr.* 2007 Jun;62(2):71-7. doi: 10.1007/s11130-007-0044-x. Epub 2007 Jun 19. PMID: 17577670.
 16. Chandra V, Jasti J, Kaur P, Betzel Ch, Srinivasan A, Singh TP. First structural evidence of a specific inhibition of phospholipase A2 by alpha-tocopherol (vitamin E) and its implications in inflammation: crystal structure of the complex formed between phospholipase A2 and alpha-tocopherol at 1.8 Å resolution. *J Mol Biol.* 2002 Jul 5;320(2):215-22. doi: 10.1016/S0022-2836(02)00473-4. PMID: 12079380.
 17. Gallardo CA, Cano E, López GE, Blas V, Olvera R, Franco M, Ortiz R. Phycobiliproteins from *Spirulina maxima* and *Pseudanabaena tenuis* protect against hepatic damage and oxidative stress caused by Hg²⁺ *Rev Mex de Cien Farm.* (2010). 41(2):30-35
 18. Cano-Europa E, Blas-Valdivia V, Lopez-Galindo GE, Franco-Colin M, Pineda-Reynoso M, Hernandez-Garcia A, Ortiz-Butron R. Methimazole-induced hypothyroidism causes alteration of the REDOX environment, oxidative stress, and hepatic damage; events not caused by hypothyroidism itself. *Ann Hepatol.* 2010 Jan-Mar;9(1):80-8. PMID: 20308726.