Indian Journal of Traditional Knowledge
Vol 23(1), January 2024, pp 67-76
DOI: 10.56042/ijtk.v23i1.8147

Inhibition of *Bungarus caeruleus* snake venom toxicity by *Citrus reticulata* methanolic extract and *in-silico* analysis of possible binding modes

Srimathi Raghavan & Gurunathan Jayaraman*

School of Biosciences and Technology, Vellore Institute of Technology, Vellore 632 014, India

*E-mail: gjayaraman@vit.ac.in

Received 11 May 2021; revised 04 January 2023; accepted 04 January 2024

Snakebite envenomation has been one of the major causes of morbidity and mortality around the world. Though intravenous administration of commercial anti-venom systemically neutralizes the toxicity, adverse effects are commonly seen in the post-treatment period. Plant metabolites and their derivatives have been shown to exhibit a wide range of anti-ophidian properties. In this study, we have investigated the *Bungaruscaeruleus* venom neutralization potential of *Citrus aurantium* and *Citrus reticulata* Blanco peels in terms of phospholipase and hemolytic inhibition. Methanolic extract of *C. reticulata* exhibited the highest inhibition (93%) of PLA_2 activity. However, the extracts of both these *Citrus* species significantly inhibited the hemolytic activity of the venom (54–82%). Molecular docking indicated the binding of the citrus metabolites to catalytic site residues (TRP19, GLY30, TYR31, GLY32, and HIS48) of PLA_2. *In vivo* studies using Swiss Albino mice confirmed the neutralising capacity of the citrus peel extract, as reflected by the increase in survival time. Therefore, the study reveals that these metabolites in the methanolic extract of the citrus peel can be used as supplements for the management of snake bites.

Keywords: Snakebite, *Citrus aurantium*, *Citrus reticulata*, Venom, Neutralization, *Bungarus caeruleus*

IPC Code: Int Cl.[24]: A61K 36/752, A61K 38/17

Management of snakebites is one of the several challenges faced in medical history. The mortality rate due to snakebites is more than 100,000 per year[1], and the death due to snakebites in India is about 15,000. Of the 216 species of snakes, 52 are reported to be highly poisonous. The four major snakes responsible for accidental fatality are *Najanaja*, *Bungarus caeruleus*, *Vipera russelli*, and *E.* chiscarinatus[2]. Krait is a member of the *Elapidae* family and is of secretive habitat and docile nature. Though these snakes are less aggressive, the accidental bite is highly neurotoxic and lethal. Kraits are predominantly present in South Asian countries and are represented by various local names. The venom of the Elapidae (cobra and krait) family is known for its neurotoxicity, directly affecting the function of the central nervous system and the heart[3]. *Bungarus caeruleus* is one of the 13 species listed in the elapidae family and has five subspecies. The type of toxic proteins and their composition is not the same in all the snakes. However, these proteins constitute up to 90-95% of the dry weight of the venom. The major proteins in the venom of *B. caeruleus* contain neurotoxins, membrane toxins, phospholipase A_2 (PLA_2), and proteases[4]. In general, krait envenomation leads to abdominal pain or cramps that cause progressive paralysis and requires artificial respiratory support. Bungarotoxins affect nerve endings of the brain's synaptic cleft and lead to muscle paralysis[5]. Phospholipases hydrolyze glycerophospholipids and immobilize the prey, breakdown cells, and other tissues before they completely digest the prey[6]. Snake venom PLA_2 exhibits similarity to the PLA_2 present in the mammalian system. Along with the other venom proteins they end up in life-threatening pathophysiological manifestations.

Anti-venom/animal-derived serum contains heterologous immunoglobins, and administration of this polyvalent anti-venom is the only approved and practiced treatment for snakebite envenomation. There are about seven known anti-venom-producing laboratories in India, and the production mainly targets against big four snake species[7]. At the same time, it is necessary to find a supplementary therapeutic procedure to mitigate or delay the adverse effects caused by snakebite. One such possible

*Corresponding author

solution is to use traditional medicinal plant extracts or their decoction, which are readily accessible even in unprivileged areas[8]. In addition, such procedures may alleviate post-treatment adverse effects, such as serum sickness and anaphylaxis[9].

Plants are a potential source for snakebite treatments, especially in remote regions, when there is a limitation of anti-venom availability and considering the economic value of the drug. The use of medicinal plants against poisonous species of elapids *Naja naja* and *Bungarus caeruleus* bites were reported[10]. The knowledge of the use of medicinal plant extracts is passed orally through generations. Recently, the scientific community has taken the responsibility of providing in-depth experimental shreds of evidence for their use. *Acanthaceae*, *Amaranthaceae*, *Caesalpiniaceae*, *Euphorbiaceae*, *Lamiaceae*, *Rutaceae*, and *Verbenaceae* are reported to be the most significant families used for snakebite treatment in India[11,12].

In addition to herbal plants, some common plants have also been shown to possess pharmacological value. Several investigations are being carried out on the inhibitory potential of common plants. Functional ingredients of plants are being screened and studied for their neutralizing capacity at the molecular level. Of the several plants, citrus species are also known for their pharmaceutical and therapeutic applications[13]. Citrus belongs to the family *Rutaceae*. Among these, phytochemicals present in citrus peels exhibit anti-inflammatory, anti-oxidant, and anti-cancer activities[14]. Traditionally citrus species such as *Citrus limon*, *Citrus aurantiifolia*, and *Citrus aurantium* L., are reported for their venom-neutralization potential[14,15]. However, a detailed investigation has not yet been carried out in this direction. Hence, the current study is focused on exploring the krait venom PLA_2 inhibitory potential by *Citrus aurantium* and *Citrus reticulata* Blanco peels.

Materials and Methods

Venom and chemicals

Lyophilized *Bungarus caeruleus* venom was purchased from Irula Snake Catcher's Industrial Co-operative Society Ltd., Chennai, India. Biochemical kits were purchased from Arkray Healthcare Pvt. Ltd, Mumbai, India. All other chemicals and solvents were high-grade analytical chemicals and were purchased from SD Fine chemicals / Hi-Media, Mumbai, India.

Sample collection and extraction

Citrus fruits (*Citrus aurantium* L. and *Citrus reticulata* Blanco) were purchased from Katpadi Taluk, Vellore District. The samples were identified and authenticated by a Botanist (Dr. R. Siva) at Vellore Institute of Technology, Vellore, India, and the voucher specimens (2017/PEL/51191 and 2017/PEL/61191) are maintained. Fruit peels were washed, shade dried for 5-7 days, and powdered using mortar and pestle. 500 g of powder was used for polarity gradient solvent extraction [PGSE], using petroleum ether, chloroform, ethyl acetate, methanol, and, finally, water. The extracts were dried using a rotatory evaporator. The yield was calculated based on the obtained dried crude extract. The individual stock solution was prepared by dissolving (100 mg) the extract in 10 mM phosphate-buffered saline (pH 7.4), sonicated for 30 s 42 kHz, 135 W, and centrifuged at 25ºC (10 min at 10,000 g). The stock was stored at -4°C for further use.

Phospholipase inhibition assay

A slightly modified protocol was used to determine the PLA_2 activity of the venom[16]. Accordingly, 2% (v/v) egg yolk suspension was prepared by dissolving 2 mL of yolk in PBS (phosphate buffer saline). To the 200 µL of yolk suspension, different concentrations of venom (10-70 µg/mL*)* were incubated for 30 min at 37°C. Hydrolysis of lecithin was inferred from the optical density value at 900 nm (SHIMADZU 1280 UV-spectrophotometer). PLA_2 inhibition was inferred from the observed difference in the absorbance value for the sample treated with different extract concentrations (20, 25, 30, 35, and 40 mg).

Protease inhibition assay

The caseinolytic activity of the venom was determined using the method described by Lemos *et al.*[16]. To 0.2% (w/v) azo-casein (Tris HCl, pH 8.5), different concentrations of venom (10-70 µg/mL) was added and incubated for 1 h at 37°C. The reaction was terminated by the addition of 10% TCA (trichloroacetic acid). Further, the mixture was centrifuged at 10,000 g for 10 min. The supernatant was taken, 500 mM of sodium carbonate was added, and 0.5 N of Follin's reagent was added. The change in absorbance of the supernatant was measured at 366 nm (SHIMADZU 1280 UV-spectrophotometer).

Hemolysis inhibition activity

The hemolytic activity of the krait venom was assayed using the microtiter plate method[16]. RBC (Red Blood Cell) suspension was washed with 0.9% saline. Different concentrations of venom

(10-70 µg/mL) were then added. The final reaction was terminated by the addition of ice-cold saline, followed by centrifugation at 5,000 g, and the absorbance was measured at 540 nm (SHIMADZU 1280 UV-spectrophotometer).

Pro-coagulation activity

The pro-coagulation activity of the krait venom was assayed with plasma cells using the method[17]. Briefly, to 100 µL of plasma (separated from human citrated blood), 1 µL of venom (10-70 µg/mL) was added and incubated for 10 min at 37°C. 10 mM $CaCl_2$ (20 µL) was subsequently added, and the coagulation was monitored at 405 nm, and the time was noted. Plasma, along with only PBS, was maintained as control.

Inhibition studies

For determining the inhibition of PLA_2, protease, hemolysis, and pro-coagulation activity, 70 µg of venom was incubated with different concentrations (10-40 mg/mL) of the peel extract. Subsequently, the required substrates were added, and the reactions were monitored as described before.

Molecular docking

Sequence similarity between PLA_2 of *Bungarus caeruleus* (PDB code: 1G0Z) and *Najanaja* (PDB code: 1A3D) was analyzed using ClustalOmega (https://www.ebi.ac.uk/Tools/msa/clustalo/). AutoDock was used to infer the interaction between PLA_2 of krait venom with the plant metabolites. The protein coordinates were downloaded, whereas the ligand molecule (by GC-MS identified) was generated using ChemSketch. A grid size of 27,000 Å used for the docking study was made to cover the protein's active sites as x= 49.68, y=10.82, z=13.00. The spacing was maintained at 0.375 Å. The number of GA runs was set to 100. The ADME values of each ligand were analyzed using the online server SwissADME[18]. PyMOL and Ligplot$^+$ were used to visualize the interactions between the metabolite and protein[19]. The results were evaluated based on the predicted binding energy,root mean square deviation from the starting geometry and other non-bonded interactions. To avoid any kind of ambiguities, the statistics derived from the best five structures (represented as mean±sd) are presented.

Ethical statement

The animal study was carried out through the standard protocol as per the national and international guidelines. The study was approved by Institutional Animal Ethical Committee, followed by the Committee for Supervision of Experiments on Animals (CPCSEA), New Delhi (meeting reference number VIT/IAEC/14/NOV5/31, dated 05-11-2017). Animals 8 weeks old (male n=4) with weights 22-25 g were used for the experiment.

***In vivo* neutralization studies**

The lethal dosage of *Bungarus caeruleus* venom was determined according to the protocol described by Meier *et al*.[20]. In brief, animals were separated into six groups (n=4), and varying concentrations of venom (0.2-1.0 mg/kg) in saline (0.2 mL) were injected (negative control). The behaviour of the mice was monitored for 48 h, and the LD_{50} was calculated. The inhibition study was performed only on selected extract(s), and the selection was based on the *in-vitro* and *in-silico* studies. Different concentrations of extracts were incubated with twice the amount of the LD_{50} of venom for 1 hr at 37°C. The mixture was then administered intraperitoneally, the behavioural changes were monitored for 48 h, and ED_{50} was calculated. Higher concentrations of only MPE extract and only saline-injected groups were kept as the positive control.

Biochemical and histopathological studies

The blood serum was separated for control and test animals. Liver function was performed using Span Diagnostic Kit and as per the company specifications. For, the alkaline phosphatase level test (ALP), 50 µL of the sample was mixed with 500 µL of reagent, followed by measurement of change in absorbance at 405 nm. Aspartate aminotransferase test (AST) was performed by incubation of 50 µL of sample in 500 µL of reagent and the absorbance was measured at 340 nm after 1 min and the change in the absorbance was used for the calculation. A similar setup was maintained for the alanine aminotransferase test (ALT) by replacing AST withthe ALT reagent. All results were recorded using an ELISA plate reader (BioradxMark) and were noted as absorbance per minute (IU/I) with the extinction coefficient of 2742 and 1746 for ALP and ALT/AST, respectively. Further, the organs (heart, kidney, and liver) were used to examine the histopathological effects. The samples were processed by dehydrating with acetone (70-100%), and 100% xylene was used to remove the dehydrating solvent. Further, the samples were fixed in wax and embedded with paraffin. Thin sections

were made (4-6 µm) using Spencer '800' microtome. The sections were then allowed to dry and subsequently stained using hematoxylin and eosin and visualized under a high-power Cilika microscope for imaging.

Data processing and statistical analysis

All experiments were performed in triplicates. Values were expressed as mean±s.d. One-way ANOVA was performed using GraphPad Prism 6 software to derive the statistical significance of the data ($p<0.05$).

Results

PLA_2 inhibition

The crude extractswere of different colours (brown/orange/yellow in the case of *Citrus reticulata* Blanco and green/brown in the case of *Citrus aurantium* L.) and the yield obtained was reported earlier[26]. Previous studies indicate that *Bungarus caeruleus* venom showed 40 µg/mL of venom causes 100% PLA_2 enzyme activity on yolk suspension. Hence, 40 µg/mL venom was used for the inhibitory assay. Methanolic extract of *C. reticulata* exhibited high inhibitory activity (93%) against venom PLA_2 (Fig. 1 a, b). Besides, chloroform, methanolic, and ethyl acetate extracts of *C. aurantium* inhibited 23 – 28% of PLA_2 activity. The order of PLA_2 inhibition observed with *C. aurantium* extract was chloroform (28%) ≈ methanol (25%) ≈ ethyl acetate (23 %) > water (12%) > petroleum ether (1.2%) and in the case of *C. reticulata* the order of observed inhibition was methanol (93%) > water (77%) > chloroform (69%) > ethyl acetate (41%) > petroleum ether (22%). The results thus indicate that the extracts of *C. reticulata* exhibited higher PLA_2 inhibition potential than *C. aurantium* extracts.

Hemolytic inhibition

Maximum rupture of RBCs was found to be by 40 µg/mL of snake venom, and therefore the optical density observed with this mixture was considered to correspond to 100% hemolytic activity. Methanol and aqueous extracts exhibited 70% and 57% activity, whereas petroleum ether, chloroform, and ethyl acetate extracts of *C. aurantium* exhibited less than 5% hemolytic activity. Similarly, 82% and 74% inhibition were seen with methanolic and aqueous extracts of *C. reticulata* (Fig. 2 a,b). The extracts of *C. reticulata* exhibited higher hemolytic inhibition potential

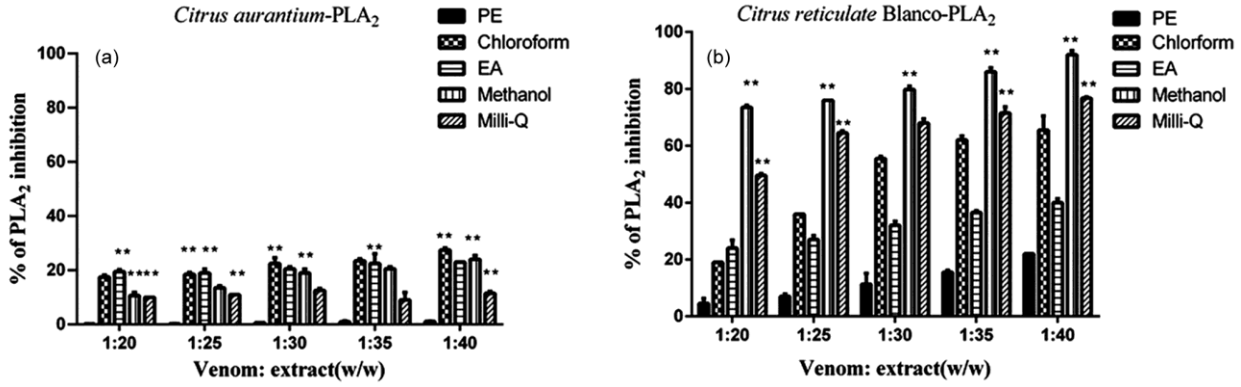

Fig. 1 — Inhibition of snake venom PLA_2 by various extracts of (a) *C. aurantium* and (b) *C. reticulata* (PE- Petroleum ether, EA- Ethyl acetate)

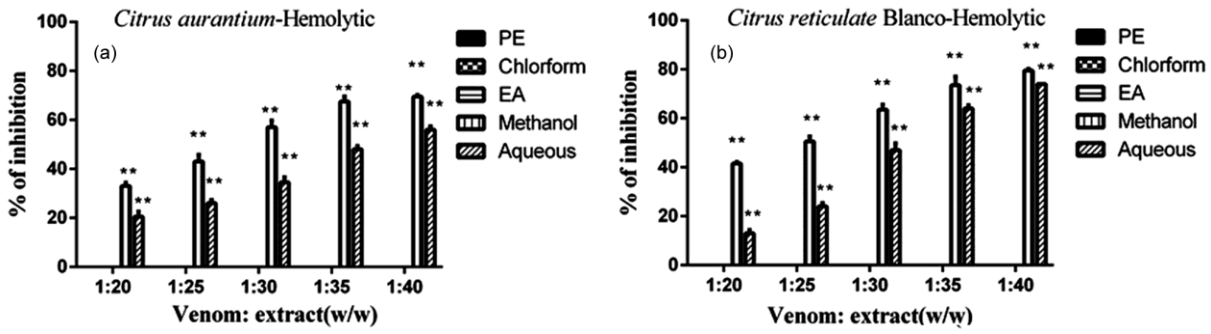

Fig. 2 — Inhibition of snake venom hemolytic activity by various extracts of (a) *C. aurantium* and (b) *C. reticulata* (PE- Petroleum ether, EA- Ethyl acetate). Methanol and aqueous extracts exhibited higher inhibition.

Molecular docking

Computation of the number of binding cavities using CASTp for krait PLA_2 indicated that the protein has 20 possible sites (with different physicochemical properties) to which the metabolites can bind. The amino acid residues present in the putative ligand-binding site are LEU2, PHE5, LYS6, ILE9, TRP19, TYR22, ILE23, TYR28, CYS29, GLY30, TYR31, GLY32, CYS45, HIS48, ASP49, TYR52, ASN62, LEU64, ILE65, PHE101. The 24 compounds in the methanolic extract of *C. reticulata*, identified earlier using GC-MS, were used to study the plausible molecular interactions[21]. Among these 24 compounds, interactions observed between the topfive ligands and PLA_2 are depicted in Figure 3, and the non-bonded interactions and binding affinity are given in Table 1.

Venom neutralization by *C. reticulata* extracts

The LD_{50} of *Bungarus caeruleus* venom for albino mice was found to be 0.4 mg/kg of animal weight, based on the dose-response study (Fig. 4). Mice showed sequential changes in behaviour, like isolation and heavy breathing, when treated only with venom. Survival time was monitored by injecting mice with venom (2 x LD_{50}) pre-incubated with extracts (Supplementary Table S1 and S2). Mice injected only with venom survived for 7.5±1.5 h. In contrast, mice injected with pre-incubated methanolic peel extract (MPE) survived for more than 24 h, indicating the extract's neutralization potential even under *in vivo* conditions. Mice treated only with peel extract did not show any changes in survival time.

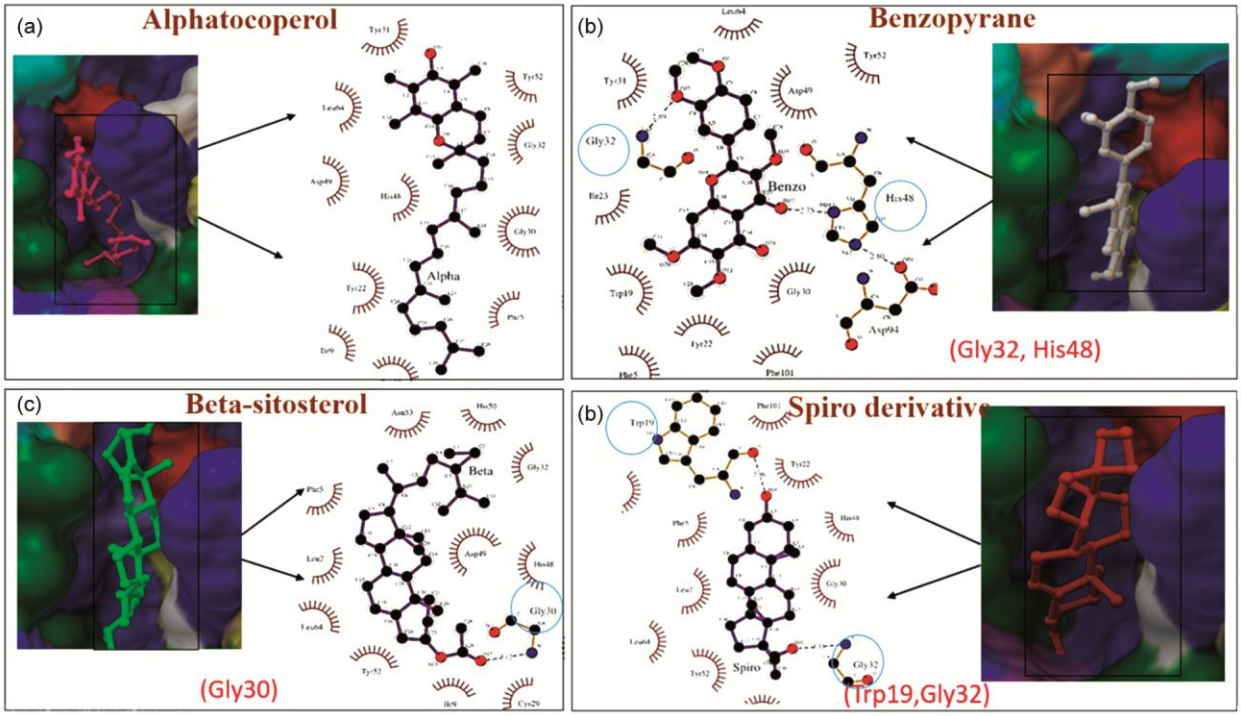

Fig. 3 — Representative structures of the protein-ligand complex depicting the intermolecular interactions between PLA_2 and (a) alpha-tocopherol, (b) benzopyran (c) beta-sitosterol (d) spiro derivative. LigPlot was used to represent the interactions.

Table 1 — Molecular docking analysis for selected compounds against snake venom PLA_2. The binding energy given is derived from the five best structures (mean ± sd) of each complex.

S. No	Compound	Binding affinity (kcal/mol)	Donor: acceptor(H-bond)	ADME Value (Log P)
1	Spiro[androst-5-ene-17,1'-cyclobutan]-2'-one, 3-hydroxy-[3.beta.,17.b (Spiro)	-9.41±0.10	SpiroH56:PLA_2Trp19O PLA_2Gly32O:SpiroO22	4.12
2	Alpha-tocopherol (Alpha)	-8.15±1.01	AlphaH81: PLA_2Tyr31OH	8.57
3	Beta-sitosterol acetate (Beta)	-8.69±1.43	PLA_2Gly30HN:BetaO27	7.63
4	Squalene (Squa)	-7.17±1.35	No H bond	9.38
5	4h-1-Benzopyran-4-one, 2-(3,4-Dimethoxyphenyl)-5,6,7-Trimethoxy (Benzo)	-6.84±0.29	PLA_2Gly32HN:BenzoO27 PLA_2His48:BenzoO12 BenzoN:PLA_2Asp94	3.10

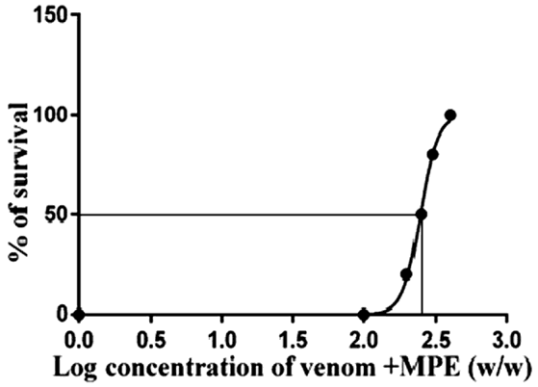

Fig. 4 — Neutralisation of Krait venom toxicity by the methanolic extract *C. reticulata.* ED_{50} of the extract was determined to be log concentration= 2.3979 (250 mg/kg).

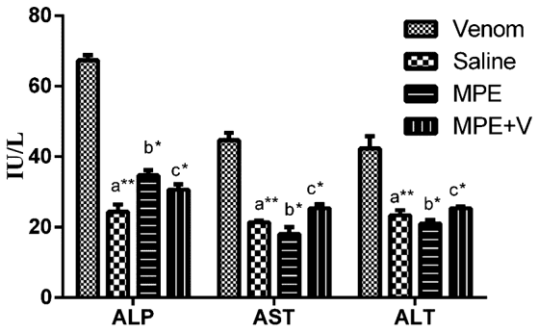

Fig. 5 — Histochemical analysis for liver function (ALP-Alkaline Phosphatase, AST-Aspartate aminotransferase, ALT- Alanine aminotransferase). The levels of all three enzymes were normal in animals treated with venom pre-incubated with the methanolic extract. Values were expressed as mean±SD, n=3 in each group (*p<.05, **p<.01), a**=comparison of venom with saline, b*=comparison of venom with MPE, c*=comparison of venom with MPE+V.

Biochemical analysis

The biochemical parameters were used to assess liver function. AST, ALT, and ALP levels were higher in animals treated with snake venom than the animals injected with only saline (Fig. 5). Increased enzyme activity in venom treated group might be due to the damage that occurred in the liver tissue/cell. Mice treated with venom pre-incubated with MPE samples showed complete neutralization of snake venom-induced toxicity. Further, the toxicity was not evident in the case of mice treated only with MPE.

Histopathological changes

Histopathological analysis of the organs harvested from mice treated with snake venom indicates several abnormalities. The heart section showed haemorrhage, congestion, and avascular tubular necrosis. In the kidney section, mononuclear and inflammatory cell

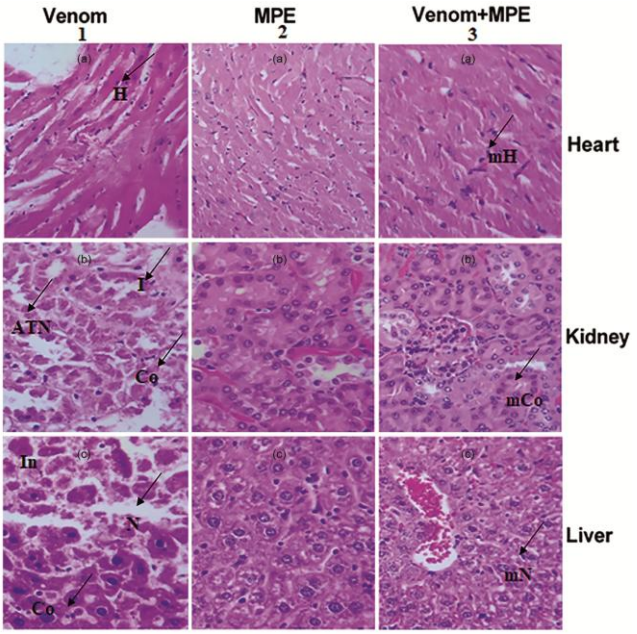

Fig. 6 — Histopathological observations of the heart (a), liver (b), and kidney (c); 1(a-c) organs of mice injected with only venom, showing pathological changes (H- Hemorrohage, Co-congestion, I-infiltration), 2(a-c) organs of mice injected with only MPE ofC. reticulata, showing no major abnormalities, 3(a-c) organs of mice injected with a pre-incubated sample of venom and MPE (1:300 w/w), (mCo- mild congestion, mI- mild infiltration, In-Inflammation) showing strong neutralization in the venom toxicity.

infiltration,and the liver section showed inflammation in a hepatic vein (Fig. 6 (a-c). However, there was a significant reduction in venom toxicity in the case of animals injected with venom pre-incubated with MPE, which showed haemorrhage in the case of heart tissue, mild necrosis in the liver, and mild congestion in kidney tissue (Fig. 6 (a-c). *C. reticulata* methanolic peel extract injected animal tissue (control) did not show any abnormality (Fig. 6.2 (a-c).

Discussion

Bungarus caeruleus envenomation cannot be detected easily as the visual local effects are not very predominant. In many instances, krait envenomation is misinterpreted as dry bite[22] since the local effect produced is minimum and, therefore, barely observable. This probably is the reason why traditional healers administer herbal decoction orally, as first aid treatment, and do not apply them topically. Indian medicinal plants are being recognized, practiced, and recommended as a remedy for snakebites[11].

Administration of anti-venom restores haemostasis and cardio dysfunctions, whereas prevention of tissue damage and other neuro-related effects remains

controversial[23]. Administration of anti-venom, in case of krait bite, fails to reverse paralysis. The prophylactic effects are still contradictory[24]. Hence, there is a requirement to delay the spreading factor of venom enzymes from the site of the bite to enter the circulation. Plant metabolites can be used as additives if they can delay/inhibit the toxic effect of the snake venom proteins. In addition, such a strategy will also reduce the amount of anti-venom used for treatment and therefore lower the post-treatment regional toxic impact of the anti-venom.

About 68.0% of *B. caeruleus* venom contains PLA_2 and bungarotoxins[25]. Relatively higher amount of PLA_2 (only) present in *B. caeruleus* (Sri Lanka) venom. These toxic proteins have been isolated, purified, and characterized earlier[26,27].

Phospholipase A_2 and proteases are key enzymes in Elapidae venom and are responsible for mortality among the human population[16]. Plant extracts of *Mimosa pudica* (0.14 mg and 0.16 mg) were shown to possess significant anti-venom effects against *Naja naja*, and *Bungarus caeruleus* venom, and the mechanism(s) of their action was suggested[10]. However, there is no primary evidence available on the venom neutralization studies of non-edible parts of plants[14]. Research on the effect of *Citrus limon* (L.) Burm. f. against snake venom showed 100% inhibition,which is a positive way forward in this direction[28].

Secondary metabolites present in peel extracts of *C. aurantium* and *C. reticulata* Blanco were qualitatively analysed. They showed the presence of molecules with a wide range of polarity[21,29]. The identified secondary metabolites are effective against stress tolerance in the plant system[30]. It has been proven that plant metabolites can interfere with the activities of various enzymes, either by inhibiting or by stimulating or altering their activity. Studies report that polyphenolic compounds are capable of eliciting anti-inflammatory effects[31].

The present study indicates that *C. reticulata* is more effective than *C. aurantium* against snake venom PLA_2. PLA_2 is the most crucial enzyme in the pharmaceutical sector as it plays a critical role in pro/anti effects on toxicity (myo-, neuro- and cyto-) levels[32]. *Bungarus caeruleus* venom has a cocktail of PLA_2, 3FTxs, LAAOs, SMVPs, CRISPS, and AchEs, of which the percentage of PLA_2 is higher than the other venom components[33]. Hence, inhibition or suppression of PLA_2 activity caused by snake venom isthe primary objective of anti-venom developers. Research revealed many effective secondary metabolites against snake venom enzymes[16,27].

The snake venom does not possess any observable proteolytic activity on the substrate (azo-casein) used in the study, thus indicating no major effect of venom metalloprotease. According to Rusmili *et al.*[4] serine protease is not substantial in *Bungarus* species as it is mainly detected at the transcript level. Similarly, krait venom does not affect the pro-coagulant activity *in vitro*. However, Utkin *et al.*[34]. Have reported that serine proteases of krait venom interfere with the coagulation process and have anti-coagulant properties. Literature indicates that krait venom contains 5% of Kunitz-type serine protease inhibitor (KSPI) that interferes with blood coagulation and fibrinolysis[35].

Extracts of *C. aurantium* and *C. reticulata* are very effective in inhibiting the hemolytic activity of the venom. Methanolic extract of *C. reticulata* shows comparatively significant inhibition of hemolytic (*82%)* activity compared to other extracts. Previously, anti-hemolytic activity was reported by extracts of *C. hystrix* (92.69%) and *C. maxima* (93.60%) during the stress conditions by preventing RBC rupture[36]. A study carried out on *Citrus reticulata* revealed the possibility of oxidative stress inhibition[37]. The mechanism of venom enzyme neutralization can be directly related tothe competitive binding of metabolites present in the extract at the substrate binding site of the enzyme[38].

PLA_2 of krait venom has 118 amino acid residues compared to 119 residues in the cobra PLA_2. The binding sites of cobra venom PLA_2 comprises of 15 amino acid residues (PHE5, ILE9, TRP18, PHE21, ALA22, TYR27, CYS28, GLY29, ARG30, CYS44, HIS47, ASP48, TRY51, TYR63, and PHE63). Sequence alignment indicates 70 conserved amino acids (Supplementary Fig. S1). Both these proteins belong to D-type PLA_2 (containing aspartic acid at the 49^{th} position) which catalyses the hydrolysis of sn-2 bonds in phospholipids. Despite the differences in the amino acid sequence, both these molecules contain identical catalytic site residues. These amino acid residues along with others that are involved in the formation of homo-oligomers dictate the activity state of these toxins. Krait PLA_2 trimer has been shown to have aligned ordered active sites on the enzyme, whereas the cobra PLA_2 trimeric form renders the enzyme inactive though the stability is moderately enhanced[39]. Therefore, the conformation of these proteins is crucial in their activity.

Interactions were seen in all the targeted ligands, and the major residues *(*TRP19, GLY30, TYR31, GLY32, and HIS48) are involved in hydrogen bond formation with the ligand. Other additional interactions, such as hydrophobic alkyl, and pi-alkylwith the residues LEU2, TRP19, CYS29, LEU64, HIS47, and PHE101, are also identified between PLA_2 and target metabolites. These non-covalent interactions contribute substantially to the binding free energy, in addition to the conventional H-bonded interactions. Moreover, the complementarity in the binding site between the protein and the ligand, and the involvement of residues in the cavities, especially the hydrophobic amino acid residues contribute significantly to the enthalpy of binding[40]. Compared with the interaction with cobra venom, PLA2 reported earlier[21], the three amino acid residues (CYS29, GLY30, and HIS48) seem to play a crucial role in substrate binding with the target ligands. Further, ligand-induced oligomerization of PLA_2 has been reported[27,41] and it is believed to lower toxicity of the snake venom PLA_2.

Liver dysfunction and pathology are life-threatening conditions. Biochemical assay of the liver revealed an increased ALT level, AST and ALP cause a high rate of liver damage[42]. Histopathological studies showed major destruction in the structural integrity of the organs. Pathological impressions revealed heavy infiltration and congestion in the kidney. The main route of elapid venom elimination from the living system is through excretion; hence, kidney dysfunction could be possible[43]. Inflammation, along with tissue necrosis of liver cells, is also reflected in liver function testsas inferred by elevation in the level of marker enzymes. Haemorrhage is also observed in heart muscles. Mice treated with pre-incubated venom and MPE showa significant reduction in toxicity in the animal system, possibly due to the binding of venom enzymes with the extract's peel metabolites. The animals injected only with MPE, and saline does not show any toxic activity.

Further in-depth studies are required to explore the lead molecule against snake venom enzymes isolated and studied for their potential inhibitory activity. Combinatorial approaches, with commercial anti-venom, can also be further investigated in detail to reduce the dosage of anti-venom used for the therapy. Hence, metabolites from the natural source can act as complementary drugs for their unique properties or can use as a first-aid procedure, wherein delayed medical procedures with anti-venom are expected[44].

Conclusion

For the first time, Citrus species were examined for their inhibition of krait venom toxicity. The current study reveals that Citrus reticulata Blanco's methanolic extractinhibits PLA_2 (93%) and haemolytic (82%) activities. In vivo, animal studies reveal 100% neutralization potential with 400 mg/kg. Thus, secondary metabolites present in the methanolic extract indicate possible binding with PLA_2, making way for the development of molecules to neutralize venom enzymes. Hence, further synthesis and formulation of phytoconstituents against the target enzyme can reduce the effect of toxicity. As the World Health Organisation has given a clear road map to mitigate snake envenomation, these metabolites can serve as supplements to the existing anti-venom therapy.

Supplementary Data

Supplementary data associated with this article is available in the electronic form at https://nopr.niscpr.res.in/jinfo/ijtk/IJTK_23(01)(2024)67-76_SupplData.pdf

Acknowledgement

The authors like to thank Vellore Institute of Technology, Vellore for providing the necessary support for carrying out this research work.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Author's Contributions

GJ and SR conceptualised and formatted the study design. SR performed, analysed, interpreted, and wrote the first draft. GJ supervised, revised, and reviewed the manuscript. All authors read and approved the final manuscript.

References

1 Naik B S, "Dry bite" in venomous snakes: A review, Toxicon, 113 (2017) 63 7, https://doi.org/10.1016/j.toxicon.2017.04.015.

2 Brunda G, Sashidhar R & Sarin R, Use of egg yolk antibody (IgY) as an immunoanalytical tool in the detection of Indian cobra (*Naja naja naja*) venom in biological samples of forensic origin, *Toxicon,* 48 (2) (2006) 183-94. https://doi.org/10.1016/j.toxicon.2006.04.011.

3 Sarkhel S, Chakravarty A, Das R, Gomes A & Gomes A, Snake venom neutralizing factor from the root extract of *Emblica officinalis* Linn, *Orient Pharm Exp Med,* 11 (1) (2011) 25-33. https://doi.org/10.1007/s13596-011-0008-4 .

4 Rusmili M R A, Yee T T, Mustafa M R, Hodgson W C & Othman I, Proteomic characterization and comparison of

Malaysian *Bungarus candidus* and *Bungarus fasciatus* venoms, *J Proteom*, 110 (2014) 129-44. https://doi.org/10.1016/j.jprot.2014.08.001.

5 Tzeng M C, Interaction of presynaptically toxic phospholipases A2 with membrane receptors and other binding sites, *J Toxicol*, 12 (1) (1993) 1-62. https://doi.org/10.3109/15569549309084185.

6 Daltry J C, Wüster W & Thorpe R S, Diet and snake venom evolution, *Nature*, 379 (6565) (1996) 537-40. https://doi.org/10.1038/379537a0.

7 Gupta Y K & Peshin S S, Snakebite in India: current scenario of an old problem, *J Clin Toxicol*, 4 (1) (2014) 182.

8 Momoh S, Friday E T, Raphael E, Stephen A & Umar S, Anti-venom activity of ethanolic extract of *Bridelia ferruginea* leaves against *Naja nigricollis* venom, *J Med Res*, 1 (5) (2012) 69-73.

9 Fox J W & Serrano S M, Approaching the golden age of natural product pharmaceuticals from venom libraries: an overview of toxins and toxin-derivatives currently involved in therapeutic or diagnostic applications, *Curr Pharm Des*, 13 (28) (2007) 2927-34. https://doi.org/10.2174/138161207782023739.

10 Meenatchisundaram S, Priyagrace S, Vijayaraghavan R, Velmurugan A, Parameswari G, *et al.*, Antitoxin activity of Mimosa pudica root extracts against *Naja naja* and *Bangarus caerulus* venoms, *Bangladesh J Pharmacol*, 4 (2) (2009) 105-9. https://doi.org/10.3329/bjp.v4i2.2276.

11 Upasani S V, Beldar V G, Tatiya A U, Upasani M S, *et al.*, Ethnomedicinal plants used for snakebite in India: a brief overview, *Integr Med Res*, 6 (2) (2017) 114-30. https://doi.org/10.1016/j.imr.2017.03.001.

12 Lakhmale S, Acharya R N, Chavan S S, Ashok B K & Ravishankar B, A protective effect of *Symphorema polyandrum* Wight seeds against *Naja naja* venom, *Pharmacol Eval*, 7 (4) (2016) 328-333.

13 Mabberley D, Citrus (Rutaceae): A review of recent advances in etymology, systematics and medical applications, *Blumea-Biodivers Evol Biogeogr Plants*, 49 (2-3) (2004) 481-98. https://doi.org/10.3767/000651904X484432.

14 Feitosa C M, Cardoso M F K, Oliveira L S G & Melo C H S, Antioxidant and anticholinergic properties of *Citrus sinensis* (L.) Osbeck (Rutaceae) essential oil in mice hippocampus, *Indian J Nat Prod Resour*, 11 (3) (2020) 199-205.

15 Gamage D G, Dharmadasa R M, Abeysinghe D C, *et al.*, Emerging herbal cosmetic production in Sri Lanka: Identifying possible interventions for the development of the herbal cosmetic industry, *Scientifica*, (2021). https://doi.org/10.1155/2021/6662404.

16 17 Angulo Y, Estrada R, Gutiérrez J, Clinical and laboratory alterations in horses during immunization with snake venoms for the production of polyvalent (Crotalinae) anti-venom, *Toxicon*, 35 (1) (1997) 81-90. https://doi.org/10.1016/S0041-0101(96)00077-3.

17 Gopi K, Anbarasu K, Renu K, Jayanthi S, Vishwanath B, *et al*., Quercetin-3-O-rhamnoside from *Euphorbia hirta* protects against snake venom induced toxicity, *Biochim Biophys Acta*, 1860 (7) (2016) 1528-40. https://doi.org/10.1016/j.bbagen.2016.03.031.

18 Daina A, Michielin O, Zoete V, Swiss ADME: a free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules, *Sci Rep*, 7 (1) (2017) 42717. https://doi.org/10.1038/srep42717.

19 Laskowski R A & Swindells M B, LigPlot+: multiple ligand–protein interaction diagrams for drug discovery, *ACS Publications*, (2011) 2778-2786. https://doi.org/10.1021/ci200227u.

20 Meier J & Theakston R, Approximate LD50 determinations of snake venoms using eight to ten experimental animals, *Toxicon*, 24 (4) (1986) 395-401. https://doi.org/10.1016/0041-0101(86)90199-6.

21 Srimathi R & Jayaraman G, Metabolites from the citrus extracts inhibit the activity of selected proteins in Indian Cobra (Naja naja) venom, *J Ethnopharmacol*, 252 (2020) 112575. https://doi.org/10.1016/j.jep.2020.112575.

22 Warrell D A, Looareesuwan S, White N J, Theakston R, Warrell M, *et al.*, Severe neurotoxic envenoming by the Malayan krait *Bungarus candidus* (Linnaeus): response to anti-venom and anticholinesterase, *Br Med J*, 286 (6366) (1983) 678-80. https://doi.org/10.1136/bmj.286.6366.678.

23 Kularatne S, Common krait (*Bungarus caeruleus*) bite in Anuradhapura, Sri Lanka: A prospective clinical study, 1996-98, *Postgrad Med J*, 78 (919) (2002) 276-80. http://dx.doi.org/10.1136/pmj.78.919.276.

24 Gawarammana I B, Kularatne S A M, Kumarasiri R P, Senanayake N, *et al.*, Parallel infusion of hydrocortisone±chlorpheniramine bolus injection to prevent acute adverse reactions to anti-venom for snakebites, *Med J Aust*, 180 (1) (2004) 20-3, https://www.mja.com.au/system/files/issues/180_01_050104/gaw10181_fm.pdf.

25 Oh A M F, Tan C H, Ariaranee G C, Quraishi N & Tan N H, Venomics of *Bungarus caeruleus* (Indian krait): Comparable venom profiles, variable immunoreactivities among specimens from Sri Lanka, India and Pakistan, *J Proteom*, 164 (2017) 1-18. https://doi.org/10.1016/j.jprot.2017.04.018.

26 More S S, Kiran K, Veena S & Gadag J, Purification of an L-amino acid oxidase from *Bungarus caeruleus* (Indian krait) venom, *J Venom Anim Toxins incl Trop Dis*, 16 (1) 2010, 60-76. https://doi.org/10.1590/S1678-91992010005000002.

27 Singh G, Gourinath S, Saravanan K, Sharma S, Bhanumathi S, *et al*., Sequence-induced trimerization of phospholipase A2: Structure of a trimeric isoform of PLA2 from common krait (*Bungarus caeruleus*) at 2.5 Å resolution, *Acta Cryst F: Struct Biol Cryst Commun*, 61 (1) (2005) 8-13. https://doi.org/10.1107/S1744309104025503.

28 Otero R, Núñez V, Jiménez S, Fonnegra R, Osorio R, *et al*., Snakebites and ethnobotany in the northwest region of Colombia: Part II: Neutralization of lethal and enzymatic effects of Bothrops atrox venom, *J Ethnopharmacol*, 71 (3) (2000) 505-11. https://doi:10.1016/s0378-8741(99)00197-x.

29 M'hiri N, Ioannou I, Ghoul M & Mihoubi Boudhrioua N, Phytochemical characteristics of citrus peel and effect of conventional and nonconventional processing on phenolic compounds: A review, *Food Rev Int*, 33 (6) (2017) 587-619. https://doi.org/10.1080/87559129.2016.1196489.

30 Justin J S, Milton A & Natesan G, Phytochemical evaluation of peel of Citrus reticulata Blanco using various solvent extracts, *Int J Pharm Sci Bus Manag*, 2 (9) (2014) 26-35.

31 Ahmed T, Potentiation of the anti-inflammatory effects of NSAIDs, steroids and statins using antioxidants, (2018). http://hdl.handle.net/10361/11067.

32 Kini R M, Venom phospholipase a2 enzymes, John Wiley, (1997). https://agris.fao.org/agris search/search.do?record ID=US201300307716.

33 Choudhury M, McCleary R J, Kesherwani M, Kini R M & Velmurugan D, Comparison of proteomic profiles of the venoms of two of the 'Big Four'snakes of India, the Indian cobra (*Naja naja*) and the common krait (*Bungarus caeruleus*), and analyses of their toxins, *Toxicon*, 135 (2017) 33-42. https://doi.org/10.1016/j.toxicon.2017.06.005.

34 Utkin Y N, Gantsova E, Andreeva T, Starkov V, Ziganshin R, *et al.*, Venoms of kraits *Bungarus multicinctus* and *Bungarus fasciatus* contain anti-coagulant proteins, *Dokl Biochem Biophys*, 53 (2015). https://doi.org/10.1134/S1607672915010159.

35 Mukherjee A K, Mackessy S P & Dutta S, Characterization of a Kunitz-type protease inhibitor peptide (Rusvikunin) purified from *Daboia russelii russelii* venom, *Int J Biol Macromol*, 67 (2014) 154-62. https://doi.org/10.1016/j.ijbiomac.2014.02.058.

36 Abirami A, Nagarani G & Siddhuraju P, In vitro antioxidant, anti-diabetic, cholinesterase and tyrosinase inhibitory potential of fresh juice from *Citrus hystrix* and *C. maxima* fruits, *Food Sci Hum Well*, 3 (1) (2014) 16-25. https://doi.org/10.1016/j.fshw.2014.02.001.

37 Fomani M, Happi E N, Bisoue A N, Ndom J C, Waffo A F K, *et al.*, Oxidative burst inhibition, cytotoxicity and antibacterial acriquinoline alkaloids from *Citrus reticulata* (Blanco), *Bioorg Med Chem Lett*, 26 (2016) 306-9. https://doi.org/10.1016/j.bmcl.2015.12.028.

38 Alam M I, Gomes A, Snake venom neutralization by Indian medicinal plants (*Vitex negundo* and *Emblica officinalis*) root extracts, *J Ethnopharmacol*, 86 (1) (2003) 75-80. https://doi.org/10.1016/S0378-8741(03)00049-7.

39 Fremont D H, Anderson D H, Wilson I A, Dennis E A & Xuong N H, Crystal structure of phospholipase A2 from Indian cobra reveals a trimeric association, *Proc Natl Acad Sci*, 90 (1) (1993) 342-6. https://doi.org/10.1073/pnas.90.1.342.

40 Fischer A, Smiesko M, Sellner M, Lill M A, Decision making in structure-based drug discovery: Visual inspection of docking results, *J Med Chem*, 64 (2021) 2489-500. https://dx.doi.org/10/1021/acs.jmedchem.0c02227.

41 Soares A M, Mancin A C, Cecchini A L, Arantes E C, *et al*., Effects of chemical modifications of crotoxin B, the phospholipase A2 subunit of crotoxin from *Crotalus durissus terrificus* snake venom, on its enzymatic and pharmacological activities, *Int J Biochem Cell Biol*, 33 (9) (2001) 877-88. https://doi.org/10.1016/S1357-2725(01)00065-6.

42 Chatterjea M N & Shinde R, Text Book of Medical Biochemistry, 8th Edition, JP Medical Ltd, 2011.

43 Al-Mamun M, Rahman M, Hasan R, Rahmann Z & Haque K, Histopathological alterations induced by common krait *Bungarus caeruleus* venom on hepatic, renal and cardiac tissues of albino mice, *Digestion*, 6 (2015) 10.

44 Adriao A A X, dos Santos A O, de Lima E J S P, Maciel A B, Paz W H P, *et al*., Plant-derived toxin inhibitors as potential candidates to complement antivenom treatment in snakebite envenomations, *Front Immunol*, 13 (2022) 842576. https://doi.org/10.3389/fimmu.2022.842576