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Original Article

In Silico Characterization and Epitope Mapping of *Echinococcus granulosus* Annexin Protein: Novel Insights for Vaccine Design

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Abstract

Background: A neglected zoonosis, cystic echinococcosis (CE) is the most common diseas in the developing nations worldwide. Vaccination is helpful in preventing the disease. Predicting main biochemical properties of the *E. granulosus* annexin (ANX) and its possible B-cell and human leukocyte antigen (HLA)-binding epitopes as a viable vaccine candidate was the goal of the current study.

Methods: This study was done in Neyshabur University of Medical Sciences, Neyshabur, Iran. Predictions about transmembrane domain, subcellular localization, post-translational modification (PTM) sites, physico-chemical, antigenic, and allergenic profiles, secondary and 3D structure, tertiary model refinement, and validations were done using online servers.

Results: The cytoplasmic 79.05 kDa protein was non-allergenic, hydrophilic (GRAVY: -0.490), stable (instability: 39.30), with improved thermotolerance (aliphatic: 80.07) and 122 post-translational modification sites. The secondary structure mostly included helices and extended strands. The 3D model was generated using Robetta server (confidence: 0.59) and was refined and validated subsequently. Shared B-cell epitopes were discovered using ElliPro, ABCpred and SVMTriP servers with antigenicity, allergenicity, and solubility screening. Moreover, multiple human and mouse MHC-binding epitopes were predicted and screened in *E. granulosus* ANX.

Conclusion: This work offers a foundation for further investigation regarding designing an effective vaccination against CE. Further empirical research using examined protein alone or in conjunction with other antigens/epitopes is needed in the future.

Introduction

The larval stages of *Echinococcus granulosus* are the causative agents of a significant zoonosis called hydatidosis or cystic echinococcosis (CE), rendering a public health concern (1). With canids as their primary hosts, adult tapeworms lay gravid proglottid in the gut lumen, leading to the environmental contamination via feces (2). Herbivores as intermediate hosts ingest ova during grazing on pastures, so that hydatid cysts containing protoscoleces (PSCs) would develop in liver and lungs (3). Human are only accidental hosts, getting infected in the same manner as herbivores (4) and affected people may experience impotency and disability (3, 5).

Control programs on CE have been principally relied on promotional health education in endemic settings along with slaughter hygiene and regular dosing of dogs (6). Also, side effect including drug resistance as well as drug residues in dairy products are inevitable following pharmacotherapy of CE (7). Vaccination strategies are likely more efficient in prevention strategies (8, 9). Different vaccine candidates have been utilized so far in immunization studies, showing variations in the elicited immune response profile. One of the successful examples is the oncospheral EG95 with prophylactic activity against *E. granulosus* infection in several counties (10). Located throughout eukaryotic cells, annexins (ANX) are a class of phospholipid-binding proteins that have the ability to bind calcium ions. They are involved in calcium signaling. In addition to their numerous roles in membrane repair, membrane transport, and cell anti-inflammatory action, annexins are also likely involved in cell differentiation, proliferation, and death. The host immune response has been observed to be regulated by the parasite-derived ANX, which have also been suggested as possible targets for the development of novel drugs and vaccines. Annexin proteins have been found in PSCs and hydatid fluid, and an ANX-based vaccine candidate would prevent the infection in intermediate hosts (11-13).

Precise identification of *E. granulosus* molecules as diagnostic and follow-up biomarkers and targets for vaccination has been made possible through unprecedented computer-aided techniques (14, 15) in a time- and cost-effective manner. The present study was aimed to investigate the immunoinformatics features and immunogenic epitopes *E. granulosus* ANX using comprehensive *in silico* methods.

Materials and methods

This study was done in Neyshabur University of Medical Sciences, Neyshabur, Iran.

Amino acid sequence retrieval

The amino acid sequence of *E. granulosus* ANX was obtained by UniprotKB, available at https://www.uniprot.org/, as FASTA format (Accession ID: U6JES0).

Prediction of physico-chemical, antigenic, allergenic and solubility profiles

Physico-chemical parameters of the examined protein were evaluated *in silico* using ProtParam server. The grand average of hydropathicity (GRAVY), instability and aliphatic indices, half-life, charged residues, isoelectric point, the molecular weight (MW) and total length were predicted accordingly (16). The VaxiJen v2.0 (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) was used for the prediction of antigenicity, while AllergenFP v1.0 (https://ddg-pharmfac.net/AllergenFP/) and AllerTOP v2.0 (https://www.ddg-pharmfac.net/AllerTOP/) were used to evaluate allergenicity of the whole protein sequence with 88.9% and 85.3%, respectively. The protein solubility was forecasted by the Protein-

Sol server, available at https://protein-sol.manchester.ac.uk/.

Post-translational modification (PTM) sites

One of the important PTM sites are phosphorylation regions (serine, threonine, and tyrosine), which were predicted by the NetPhos 3.1. server (https://services.healthtech.dtu.dk/). Moreover, acetylation, N-glycosylation and O-glycosylation sites were predicted using the GPS-PAIL (https://www.biocuckoo.org/), NetNGlyc 1.0, and NetOGlyc 4.0 (https://services.healthtech.dtu.dk/) web tools, respectively.

Transmembrane domains and subcellular localization

The DeepLoc 2.0 server at https://services.healthtech.dtu.dk/services/DeepLoc-2.0/ as a multi-label predictor forecasts the subcellular localization of the eukaryotic proteins such as *E. granulosus* ANX. The likely presence of the signal peptides and transmembrane helices in the protein sequence was evaluated using SignalP-6.0 (https://services.healthtech.dtu.dk/services/SignalP-6.0/) and TMHMM 2.0 (https://services.healthtech.dtu.dk/services/TMHMM-2.0/) servers, respectively.

Prediction of secondary and tertiary structures

The NetSurfP-3.0 was used to evaluate disordered regions, secondary structure, surface accessibility, and phi/psi dihedral angles in the protein sequence (https://services.healthtech.dtu.dk/services/NetSurfP-3.0/). Next, a completely automated three-dimensional (3D) model prediction server, Robetta of the Baker lab, was used for tertiary structure predictions based on a confidence score (C-score), ranging between 0 (zero confidence) to 1 (100% confidence) (https://robetta.bakerlab.org/).

3D model refinement and validation

The best 3D model provided by the Robetta server was subsequently refined using the GalaxyRefine server (http://galaxy.seoklab.org/cgi-bin/subunit.cgi?type=REFINE), with side-chain establish and repack, causing regional and total structural improvements (17). The crude and refined models were compared in terms of a number of parameters, including GDT-HA, RMSD, MolProbity, Clash Score, Poor rotamers and Rama favored, and the best refined model was selected to validate the refinement process using Ramachandran plot analysis by PROCHCK online tool (https://saves.mbi.ucla.edu/) (18). Also, the Z-score of both crude and refined models were estimated using the Prosa-web server (https://prosa.services.came.sbg.ac.at/prosa.php).

Prediction of continuous and conformational B-cell epitopes

To predict the linear B-cell epitopes, a multi-method approach was applied using SVMTriP (http://sysbio.unl.edu/SVMTriP/prediction.php), ABCpred (http://crdd.osdd.net/raghava/abcpred/ABC_submission.html), and linear B-cell epitope prediction tool of the ElliPro server (http://tools.iedb.org/ellipro/). Next, shared linear B-cell epitopes among at least two servers were highlighted and further assessed in terms of antigenicity, allergenicity and solubility, using VaxiJen v2.0, AllergenFP v1.0 and PepCalc (https://pepcalc.com/) online servers, respectively. Different properties of the antigen sequence, such as beta-turn, accessibility, antigenicity, hydrophilicity, etc. were used to predict B-cell epitopes, based on the antigen sequence properties tool of the IEDB web server (http://tools.iedb.org/bcell/). Moreover, ElliPro tool of the IEDB server was used to predict conformational B-cell epitopes of ANX of E. *granulosus* using default settings of 0.5-min score and 6 Å max-distance. This server performs a three-step prediction, by neighbor residue clustering, res-

idue protrusion index (PI) and protein shape estimation (http://tools.iedb.org/ellipro/) (19).

Prediction and selection of epitopes associated with the human leukocyte antigen (HLA)

The multifunctional IEDB server was utilized to predicted HLA-binding epitopes of E. granulosus ANX. For this aim, the helper T-lymphocyte (HTL; 15-mer) and cytotoxic T-lymphocyte (CTL; 12-mer) epitopes were predicted using the mhci and mhcii tools, respectively. All predictions were performed using NetMHCIIpan 4.1 EL (recommended epitope predictor-2023.09) and the IEDB HLA reference set options (20). Subsequently, top discovered HTL epitopes were further screened regarding antigenicity, IFN-γ, and IL-4 induction using VaxiJen v2.0, IFNepitope (https://webs.iiitd.edu.in/raghava/ifnepitope/predict.php) and IL4pred (https://webs.iiitd.edu.in/raghava/il4pred/index.php) web servers, respectively. Moreover, top CTL epitopes were screened in terms of immunogenicity and IFN-γ induction.

Results

General characteristics

This 713-residue protein showed a MW of 79059.61 dalton, a speculated pI of 5.91 and charged residues, either positively (n = 93) and negatively (n = 103) ones. The estimated half-life *in vitro* in mammalian reticulocytes was 30 hours. The protein was stable in the laboratory milieu with an instability index of 39.30. In addition, the GRAVY score of -0.490 and aliphatic index of 80.07 rendered the protein to be highly hydrophilic and thermotolerant in a wide range of temperatures, respectively. Antigenicity prediction using VaxiJen server demonstrated that this protein was antigenic in nature with a VaxiJen score of 0.5844 (threshold: 0.4), and it was totally non-allergenic in nature as evidenced by AllergenFP v1.0 and AllerTOP v2.0 servers. Also, no IgE epitopes and MEME/MAST motifs were present within the protein sequence, as evidenced by the AlgPred web server. The solubility score of the protein was found to be 0.507 by Protein-Sol server, showing a relatively high solubility (Fig. 1).

Prediction of PTM sites of E. granulosus annexin

E. granulosus ANX protein possessed 92 Phosphorylation sites (serine: 38, tyrosine: 33, threonine: 11) (Fig. 1), 4 N-linked glycosylation sites (positions: 54, 156, 182, and 287), and 26 O-linked glycosylation sites (positions: 11, 24, 56, 113, 124, 132, 134, 135, 139, 147, 148, 155, 158, 167, 177, 184, 185, 199, 200, 214, 218, 238, 329, 333, 336, and 341).

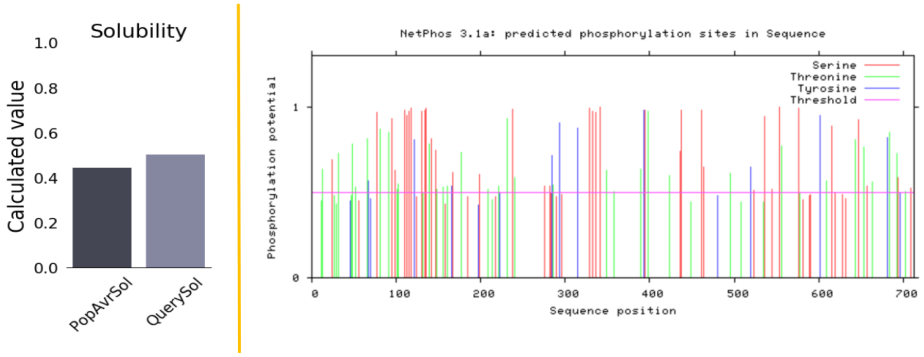

Fig. 1: Prediction of solubility and phosphorylation sites of the *E. granulosus* ANX protein using Protein-Sol and NetPhos 3.1 web servers, respectively. The solubility showed a score above average (score: 0.507) solubility in *Escherichia coli*. Also, there existed 92 phosphorylation sites in this protein

Transmembrane domains, signal peptide, and subcellular localization

Neither a signal peptide nor transmembrane domain was found in the ANX protein of *E. granulosus*, as shown by the SignalP-6.0 and TMHMM 2.0 server output. Also, this protein was estimated to be a cytoplasmic protein by DeepLoc server with a prediction score of 0.7764.

Extrapolation of secondary and tertiary structures

The secondary structures in the ANX sequence were predicted using the NetSurfP-3.0 online tool, revealing that helices were mostly frequent secondary structure C-terminally, while extended strands were more prevalent at the N-terminal of the protein. Most of the residues were exposed and disordered regions were present in the first half of the sequence (105-210 and 325-385 residues). Graphical illustration of this prediction is provided in Fig. 2. The powerful Robetta server generated the 3D model of *E. granulosus* ANX protein using a finely-tuned homology modeling approach called RoseTTAFold. A high confidence score (0.59) was obtained for this prediction, indicating the higher quality of the generated model. In this study, model number 1 was chosen based on lower error estimates (Fig. 3).

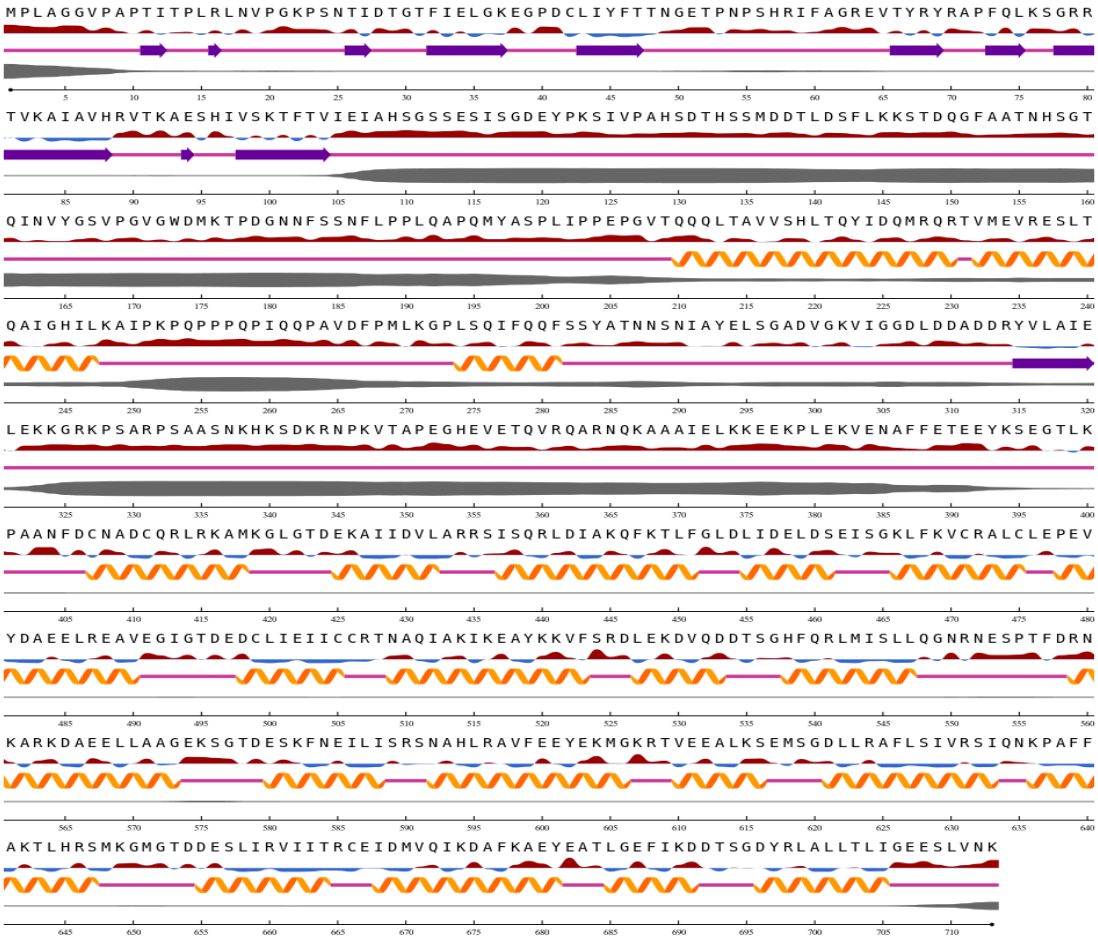

Fig. 2: Secondary structure prediction for *E. granulosus* ANX protein using NetSurfP-3.0 server (**Yellow spiral:** helices; **Blue arrows:** strands; **Violet straight lines:** coils). Extended strands and helices were the most abundant secondary structures in this protein. Also, there observed some disordered areas in first half of the protein sequence, which influence the protein function

3D model refinement and quality assessment

The 3D model of *E. granulosus* ANX predicted by Robetta was subsequently rehashed to improve structurally. Among top 5 refinements provided by the GalaxyRefine server, model 3 was selected as the best refined model with the following parameters: GDT-HA (0.9779), RMSD (0.337), MolProbity (1.742), Clash score (10.1), Poor rotamers (0.7) and Rama favored (96.6). Next, the Ramachandran plot analysis of the initial 3D structure demonstrated 90% of residues in the most favored region, 8.8% in additionally allowed region, 0.3% in the generously allowed regions, and 1% as outliers. After refinement, these were improved as follows: 93.3%, 5.4%, 0.1%, and 1.1% of the residues in the respective regions by order (Fig. 3 & supplementary Fig. 1). Moreover, Prosa-web analysis showed that the Z-score of the crude and refined models were -9.3 and -10.56, respectively (Fig. 3 & supplementary Fig. 1).

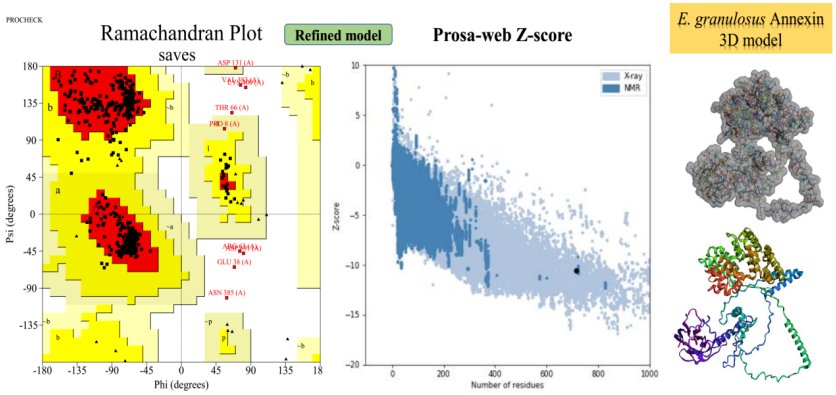

Fig. 3: Tertiary (3D) structure of the *E. granulosus* ANX protein, provided by the Robetta server, and subsequent validations of the refined model. The protein 3D model has been illustrated as surface (**up**) and ribbon (**down**). The Ramachandran plot analysis of the refined 3D structure demonstrated that 93.3%, 5.4%, 0.1%, and 1.1% of the residues in the most favored, additionally allowed, generously allowed, and outliers regions, respectively. Also, the Z-score of the refined model was estimated as -10.56

Linear and conformational B-cell epitope prediction

Using ABCpred web tool and a threshold of 0.8, various linear B-cell epitopes were predicted in the ANX protein of *E. granulosus*. Moreover, prediction of continuous B-cell epitopes was done by the SVMTriP and ElliPro online tools for cross-checking and increase the prediction confidence. Finally, 14 linear epitopes shared among at least 2 web servers' output were extracted and screened in terms of antigenicity (VaxiJen), allergenicity (AllergenFP), and solubility (PepCalc). Out of 14 shared epitopes, only 3 were ultimately shown to possess adequate (above 0.45) antigenicity, good water solubility, and without allergenicity (Table 1). Moreover, epitopes predicted using antigen sequence properties were illustrated using the appropriate tool in the IEDB web server (Fig. 4). Conformational B-cell epitopes were predicted using ElliPro tool of the IEDB server, showing 11 potential epitopes with length and scores as follows: I) 6 residues (0.942); II) 23 residues (0.939); III) 15 residues (0.83); IV) 46 residues (0.81); V) 112 residues (0.737); VI) 14 residues (0.727); and VII), 44 residues (0.665), VIII) 85 residues (0.657); IX) 4 residues (0.626); X) 16 residues (0.53); and XI) 7 residues (0.51) (Fig. 5).

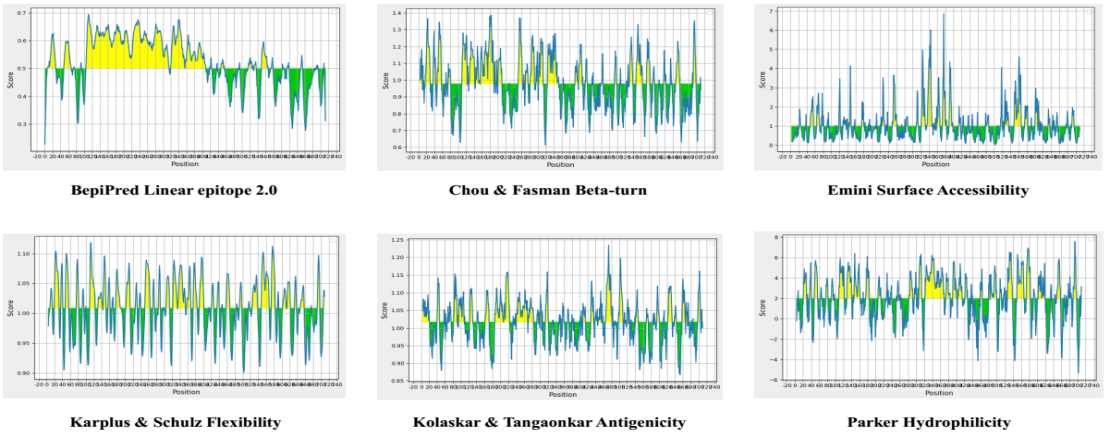

Fig. 4: B-cell epitopes predicted based on different antigen sequence properties of *E. granulosus* annexin via the IEDB server

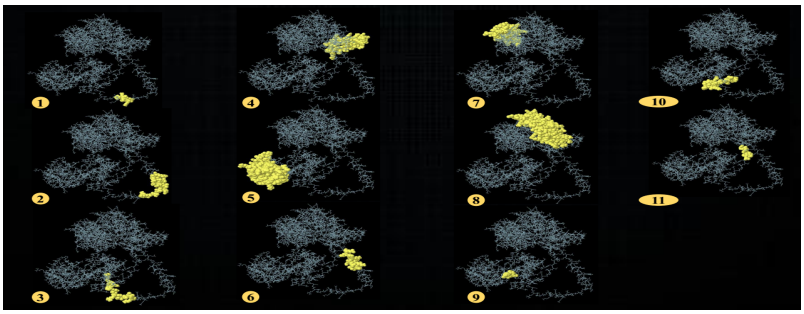

Fig. 5: Conformational B-cell epitopes of the *E. granulosus* ANX protein predicted by ElliPro web tool of the IEDB multi-functional server

Table 1: Shared continuous B-cell epitopes for *E. granulosus* ANX predicted using three different web servers (ABCpred, SVMTriP, and ElliPro) and screened in terms of antigenicity, allergenicity and solubility

Epitope No.	Epitope Sequence	Antigenicity (VaxiJen server)	Allergenicity (AllergenFP server)	Solubility (PepCalc server)
1	HSDTHSS	1.9113	Yes	Good
2	SSMDDTLDSFLKKS	0.2562	No	Good
3	GEFIKDDTS*	0.9982	No	Good
4	DTSGDYRLAL	0.4208	No	Good
5	PDCLIYFTTNGETPNP	0.7140	No	Poor
6	IRVIITRCEID	0.2285	No	Good
7	EKSGTDESKFNEI*	0.4503	No	Good
8	PGVGWDMKTP	0.9329	Yes	Good
9	PSAASNKHKSDKRNPK	1.1287	Yes	Good
10	KAIPKPQPPPQPIQQP	-0.0276	Yes	Good
11	TQAIGHILKAIPK	0.2851	Yes	Poor
12	KTLFGLDLIDELDSEI	-0.2282	No	Good
13	HSSMDDTLDS*	0.7988	No	Good

*** Indicates potentially qualified epitopes.**

Prediction and selection of HLA-binding epitopes

In the present study, 4 IFN-γ inducing and 4 IL-4 inducing human HTL epitopes were forecasted, among which only a single potent good HLA-binding, highly antigenic, IFN-γ inducing, and non-IL4-inducing HTL epitope was predicted for the *E. granulosus* ANX: GTQINVYGSVPGVGW$_{39-53}$ (antigenicity: 0.7509) (Supplementary Table 1). Also, regarding those HTL epitopes with binding affinity to mouse MHC-II molecules, 2 and 3 mouse HTL epitopes possessed IFN-γ and IL-4-inducing activity, while no potent epitopes were found exerting both functions simultaneously (Supplementary Table 2). In terms of human CTL epitopes, 3 epitopes were found to be immunogenic and capable to induce IFN-γ as the representative cytokine of Th1 response, respectively. Accordingly, the predicted human epitopes were as follows: TVKAIAVHR$_{12-29}$ (immunogenicity: 0.18954), KPAFFAKTL$_{36-44}$ (immunogenicity: 0.12201), and RYRAPFQLK$_{9-16}$ (immunogenicity: 0.05133) (Supplementary Table 3). Moreover, two mouse CTL epitope were EVTYRYRAPFQL$_{4-15}$ (immunogenicity: 0.16486) and VTYRYRAPFQLK$_{5-16}$ (immunogenicity: 0.13094). Full characteristics of the T-cell epitope predictions and screening are provided in Tables (for HTL) and (for CTL), respectively (Supplementary Table 4).

Discussion

Annexins are widely distributed in all eukaryotes and are members of a multigene family. Certain annexins in parasites have been thought to guard against structural disintegration during parasitism and control the host's immune system, rendering parasite survival in a host (21). The latter study may demonstrate that *E. granulosus* ANX may also demonstrate protection against CE infection, hence deserves to be investigated in details using *in silico* methods.

Using the ProtParam ExPASy service, the theoretical physico-chemical characteristics of *E. granulosus* ANX were first discovered. The MW of 79.05 KDa for this 713 amino acid protein indicates appropriate immunogenicity (antigens with MWs greater than 5–10 KDa are considered powerful immunogens) (22). The protein was estimated to be stable and somewhat acidic, as shown by the calculated pI (pH at which net charge becomes zero) and instability score of 5.91 and 33.88, respectively. The estimated aliphatic index resulted in a score of 80.07. The higher the aliphatic index, the more stable the protein is throughout a broad temperature range. Moreover, *E. granulosus*-associated ANX's negative GRAVY score (-0.490) indicated that it was a hydrophilic molecule with better interactions in water-based milieu. Of note, higher positivity in GRAVY score would indicate the lower hydrophilicity (or solubility) in a given protein (23). This protein was shown to be soluble in nature, as substantiated by a score of 0.507 using Protein-Sol web server. All things considered; these initial biochemical characteristics might be advantageous for further extraction/purification procedures in subsequent experimental studies. The VaxiJen v2.0 server was used to assess the protein's possible antigenicity; the results showed antigenic scores of 0.5844 that is above the threshold (0.45). As we discovered with the *E. granulosus* ANX using different web servers (AllerTOP v2.0, AllergenFP v1.0, and AlgPred), a vaccine candidate should not exhibit allergenicity.

The crudely produced proteins undergo a number of enzymatic changes, including as acylation, phosphorylation, and glycosylation—all of which are referred to as PTMs (24). These changes are essential for phosphorylation (signal transmission), acylation (membrane anchoring), glycosylation (changing the half-life of proteins), and other cellular regulatory processes (25). Furthermore, identifying PTM sites in eukaryotic proteins—like those found in parasites—is a crucial step in choosing the right expression hosts for the

synthesis of recombinant proteins (26). Using NetPhos 3.1, which was employed in this investigation to identify putative phosphorylation sites, 92 sites including 38 serine, 11 threonine, and 33 tyrosine were found in the sequence. In addition, there were 4 N-linked and 26 O-linked glycosylation sites in the protein sequence. The presence of various PTMs may implicate the utilization of expression methods based on yeast and mammals for recombinant production. This protein was cytoplasmic as demonstrated by the DeepLoc server. Generally, secondary structure in polypeptide chains is represented by hydrogen bonds between amino hydrogen and carboxyl oxygen, often including α-helices and β-structure (27). Additionally, the tertiary structure of a protein is defined by its current bonds and interactions. The inclusion of a beta-turn and an alpha helix inside a protein structure with a high hydrogen bond energy may help to preserve the protein's shape and improve its interaction with antibodies (28). Based on the NetSurfP-3.0 server, major secondary structures in the ANX sequence were helices (at the C-terminal) and extended strands (at the N-terminal), and the first half of the protein sequence was internally disordered. In the following the Baker lab's Robetta server provided the 3D modelled structure of the *E. granulosus* ANX with the best confident (score: 0.59), which was further refined and validated using GalaxyRefine and PROCHECK online tools, respectively. Ramachandran plot analysis showed relative improvement in the protein sequence, comparable to the crude model.

To better manage the CE infection, it is essential to induce acquired immune compartments, i.e. cellular and humoral responses. *In silico* strategies for vaccine design offer a foundation for directly triggering these immune responses to enclose the parasite (29). A cross-validating approach was performed for continuous B-cell epitope prediction through ElliPro linear epitope prediction tool, ABCpred and SVMTriP servers. Ultimately, 13 commonly shared epitopes were found in the *E. granulosus* ANX, among which 3 immunodominant fragments adequately antigenic, water-soluble and without allergenicity, including "GEFIKDDTS", "EKSGTDESKFNEI", and "HSSMDDTLDS". Also, 11 conformational B-cell epitopes found in the protein, which are important in terms of antigen-antibody interactions. With respect to T-cell-based immunity, HLA reference set- and mouse MHC-associated predictions were performed and high-binding epitopes with lower percentile ranks were forecasted. Based on the screening steps performed for HTL (antigenicity, IFN-γ, and IL-4) epitopes, "GTQINVYGSVPGVGW" (antigenicity: 0.7509) demonstrated positive IFN-γ and negative IL-4 induction, nominating as a good vaccine candidate. In the following, three human CTL epitopes, $TVKAIAVHR_{12-29}$ (immunogenicity: 0.18954), $KPAFFAKTL_{36-44}$ (immunogenicity: 0.12201), and $RYRAPFQLK_{9-16}$ (immunogenicity: 0.05133) and two mouse-associated CTL epitope, $EVTYRYRAPFQL_{4-15}$ immunogenicity: 0.16486) and $VTYRYRAPFQLK_{5-16}$ (immunogenicity: 13094) were predicted in the *E. granulosus* ANX protein, showing good immunogenicity and IFN-γ induction. As a final word, a strong multi-epitope vaccine construct might be created using these qualified epitopes alone or in combination with those from additional prospective antigens, and it could then be tested against CE in further research.

The current study met some limitations: i) multiple web servers with different cut-offs and trained algorithms are present which could impact the findings and their utilization was not possible, due to time and space limitations; ii) the *in silico* findings represented here are only computer-based predictions and should be validated in future wet experiments.

Conclusion

Multiple immunodominant regions were predicted for *E. granulosus* ANX, which should

be further evaluated in terms of cross-reaction, effective dosage, lymphocyte proliferation, total antibody titers, and cytokine production tests along with potential toxicity in the context of appropriate animal model challenges.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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