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Leishmania tarentolae Cell-Free System for Filamentous Protein Expression

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ABSTRACT

Background: In earlier studies, *Leishmania tarentolae* is known as an expression system due to its post-translational modification. SAG1 and Cyc18 proteins of *T. gondii* was evaluated for protection against toxoplasmosis. A surface glycoprotein of *T. gondii*, is a promoter of the immune response and previously studied for vaccine development during the infection. Cyc18 induces IL-12, which can drive Th1 and CD8+ T cell development when bound to CCR5, it rolled to adjuvant the immune response of *T. gondii* surface protein.

Objectives: This study was performed to evaluate the efficiency of SAG1 (Surface Antigen 1) and TgCyc18 (Cyclophilin 18) considered a a CCR5 chemochine for *Toxoplasma gondii* vaccine when expressed in *L. tarentolae*.

Methods: SAG1 and TgCyc18 genes were cloned and transfected into *L. tarentolae*. Protein expression and secretion were effectively validated by PCR, immunoblotting, enzyme activity, ELISA, and immunofluor. And features of SAG1 were evaluated mass spectrometry.

Results: Successful transfection of SAG1 and TgCyc18 plasmids and transfected into *Leishmania tarentolae,* and was confirmed by two steps of PCR. Current study verified that the culture supernatant included positive expression and secretion of the *T. gondii* protein (SAG1) into the media, indicated by acid phosphatase assay. The characteristics of the SAG1 protein were established using immunofluorescence, immunoblot, ELISA, and mass spectrometry.

Conclusion: This study confirms that *L. tarentolae* effectively can produces and secretes recombinant *T. gondii* proteins (SAG1 and TgCyc18), offering a potential platform for vaccine development against toxoplasmosis.

Keywords: Expression system, *Leishmania tarentolae*, proteins of *Toxoplasma gondii,* SAG1, Cyclophilin 18.

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INTRODUCTION

In this study, we hypothesize that *Leishmania tarentolae* is a eukaryotic system for protein expression as its nonpathogenic protozoan parasite [1]. *L. tarentolae* can express the filamentous protein using its efficient gene expression system and in vitro translation reactions as molecular machinery was approached by applying its promastigotes [2]. Using *L. tarentolae* would enable precise eukaryotic post-translational modifications (PTMs) that the bacterial system failed to perform. Additionally, other mammalian cells are known to be costly and complex. Therefore, *L. tarentolae* offers an alternative for expressing glycoprotein proteins in its applications in antibody production. [2].Even so, using the amastigotes stage of this parasite requires various culture environments for cultivation [3]. Previously, *L. tarentolae* promastigotes had the potential to express the filamentous protein for immunological and therapeutic studies. [4, 5]. *L. mexicana* is a pathogenic *Leishmania*, which is responsible for cutaneous or visceral disease worldwide [6]. This parasite can be an efficient gene expression system in vitro translation reactions[7]. The live vector of *L. mexicana* promastigote secretes a phosphomonoesterase protein in filamentous form, previously used to carry a (SAG1) [8]. Therefore, one copy gene *Imsap1* encoded for a short filamentous protein of acid phosphatases with the characteristic traits of serine/threonine-rich regions of 32 amino acids long[9], which is activated by enzymes which compiled to form the secreted acid phosphatase filaments (SAP1) [10–12]. *Toxoplasma gondii*'s surface proteins, SAG1 and Cyc18, are crucial for adherence and host cell invasion [13, 14]. Due to SAG1 being critical for the immune system's reaction during the earlier phases of infection, affecting together the cellular and humoral immune responses, previous research has concentrated on it concerning vaccination [15, 16]. The chemokine mimic *T. gondii* Cyclophilin 18 (Cyc18) interacts with the cysteine-cysteine chemokine receptor 5 that is found on the dendritic and macrophage surfaces. [17]. Subsequently, these cells induce IL-12 production, creating an immunological response. [18]. Thus, the objective of this work is to create constructs of the filamentous protein-derived *L. mexicana* for the expression of *T. gondii* (SAG1 and Cyc18) in the system of *L. tarentolae* for vaccination purposes. [19]. Subsequently, the SAG1 protein was purified using varying ammonium sulfate concentrations to engage the filamentous complex.

MATERIALS AND METHODS

Preparation of Plasmid Constructs for transfection into *Leishmania tarentolae* promastigotes:

In the present study, the expression pLEXSY-ble2.1plasmid (Jena Bioscience) was used. The cloning site of this plasmid was constructed to express the secreted acid phosphatase 1 (SAP1) protein, resulting in a fusion of the concerned proteins, which includes *T. gondii* (SAG1) and (Cyc18). The entire DNA construct is meant to be integrated into *L. tarentolae's* (SSU) RNA locus. The plasmids pLPhSAP1sCyc18 and pLPhSAP1sSAG1 were produced by two cloning processes. Hence, the plasmids were separated, and the sample was cleaved using *NcoI, SwaI*, and *HindIII* + *BamHI*, respectively. Electroporation was utilized to transfect 100 µl of DNA into *L. tarentolae* cell using NucleofectorII (Amaxa Biosystems, Lonza, Germany). After harvesting the cells at 3x107 cells/ml density, the samples were centrifuged at 5,600 × g/ 2 min. After being electroporated, The diluted cells at 1:2 and 1:40 and added to 96-well plates along with 40 µM Puromycin. Later, for a period of 10-14 days, the plates were incubated at 27°C or till cloudy wells showed signs of the recombinant parasite growth. After that, 40 µl of positive clones were inoculated into 10 ml of SDM79 media supplemented by phleomycin (40 µg/ml). These clones were

then cultured at 27°C for 96 hours till they reached the late log. Phase. The protein production was implied using a modified method designated by [20]. One liter of culture was incubated at 27ºC using a shaking incubator to increase the protein amount. When parasites achieved the log phase 1×10^7 cells/ml, it centrifugated at 2,500 ×g, 4ºC, 15 mins, and then the supernatant was harvested for further procedures.

PCR technique for genomic DNA separation of *L. tarentolae* :

PCR was carried out in two stages following genomic DNA extraction from recombinant cells using the "isolate II genomic DNA kit from Bioline, UK." of forwards LeishSSU for (5'-GATCTGGTTGATTCTGCCAGTAG-3') and SAP2mod2C.for2 (5'- AGCGACGTCCCTTCCTTCAA-3') employed. Reverse primer sequences were pLexyup1.rev (5'-CCTACGTCAATCGCAGACCT-3); SAG1-2 reverse (5'-CCACTACTGCAGCGGCACGA-3') and Cyc18.rev (5'- CTGGTGGTTCTCGAAGTCGC-3'). Using a PE thermocycler, the reaction mixture was performed using the 25 µl PCR tube.

Acid Phosphatase Secretion Test :

Protein expression in *Leishmania* cloned supernatants was detected using an acid phosphatase test. The supernatants were made from 25µl of culture at the late log. phase by centerfugation at 5600 × g, 4°C, for 2 minutes. A 96-well plate held 100 µl of total volume per well, of which 20 µl was the media sample and 70 µl contained 50mM p-nitrophenyl phosphate in 100mM sodium acetate pH 5. For thirty minutes, the experiment was raised at 37°C. This response was stopped by adding 10µl of 2M sodium hydroxide 69 solutions, and a noticeable color shift (pink, no activity; yellow, positive reaction) occurred. A spectrophotometer (Spectramax M5, Molecular Devices, USA) was used at 405nm to quantify absorbance readings.

Precipitation of Ammonium Sulfate (AS):

The purification of proteins was achieved in line with the methods reported by [21] By adding the ammonium sulfate. Supernatants were extracted from parasite cultures. Centrifugation was used to extract *Leishmania* cultures, with a 2,500 ×g setting,20 minutes, (at 4°C). The supernatant then was mixed with solid ammonium sulfate to achieve 20%, 30%, 40%, 50%, and 60% saturation. Precipitation was permitted to occur for sixty minutes. Standard buffers could be used to solubilize the pelleted protein easily.

Immunoblot Analysis:

A Western blot analysis evaluated the amount of SAG1 and Cyc18 produced by *L. tarentolae* clones. The Biometra (DAKO, Hamburg, Germany) device was used to transfer recombinant proteins from a 10% SDS-gel to a PVDF membrane at a rate of 4 mA/cm2 for a duration of 90 minutes. The PVDF membrane was stimulated by 100% methanol before transferring, and it was then immersed in the transfer buffer for five minutes. Then, the membrane was incubated in (5%) milk in PBST (1 hour at 37°C). Afterward, the PVDF was incubated with monoclonal antibody LT8.2 (1:2,000) or anti-His antibody (1:5,000) in a blocking buffer at 37°C for an hour while gently stirring. The membrane was washed four times for five minutes at room temperature using 1 × PBST. The blocking solution was added with IgG HRP-conjugated (goat anti-mouse) of 1:2,000–1:5,000 dose (DAKO, Hamburg, Germany), and it was incubated at 37°C for an hour. As before, three washing steps were completed. The Super Signal West Pico Chemiluminescent Kit (Fischer Scientific, UK) exposed the immunoblot to X-ray films containing solutions for five minutes.

Imaging using Fluorescence Microscopy.

After fixing recombinant cells on a 10-well slide, each well was washed with 50µl of 70% ethanol and 20µl of 0.1mg/ml polylysine in 1× PBS and allowed for settling for a period of 15 minutes. Next, 50µl of 1×PBS solution

was used to wash each well twice. After fixing 20 µl of culture supernatant with 20µl of 4% p-formaldehyde, and was kept for 15 minutes at room temp. Every well underwent a washing phase using 50 µl of washing buffer (1× PBS solution, 50 mM NH4Cl, 0.1% Saponin). Each well received 50µl of blocking buffer, and kept at room temp. For 15 minutes. After using 20 µl of blocking buffer containing primary Ab mAb LT8.2 (diluted 1:40), the slide was incubated for one hour at room temperature. This was followed by two washing procedures using the same method. After adding 20 µl of secondary antibody (1:500 of goat anti-mouse IgG-conjugated fluorescent) to the blocking buffer, it was incubated for an hour at room temperature and washed thrice. After applying 10µl of Mowiol/DABCO solution, it was covered with a coverslip and until dry at room temp. The fluorescence was detected by magnification of 60× of epifluorescence microscope.

Enzyme-Linked Immunosorbent Assay (ELISA):

The humoral response was reviewed with [23], while ELISA measured IgG1 and IgG2a antibody titers. The TLA protein was coated with a concentration of 5µg/ml of 50 mM sodium carbonate buffer, pH 9, all night in each well of an ELISA plate. Three rounds of PBS washings with 0.05% Tween 20 were performed on the plates. Following 200 µl/well blocking with 5% BSA in PBS for a period of 1 hour, the plates were incubated at 37 °C. 100 µl of serially diluted serum was added to each well in blocking buffer. After one hour of incubation at 37 °C, the plates were rinsed thrice using 0.05% Tween-20 in PBS 0.01M. Using dilution at 1:5,000 in blocking buffer, goat anti-mouse IgG^{1} and IgG^{2a} (polyclonal) conjugated with (HRP) (Invitrogen, USA) applied at 100µl to detect antibodies after incubation for 1 hour. 100µl of substrate of sodium acetate pH 5.5, 100 µl of (TMB), and 5µl H2O2 were implied. To stop the reaction, 50µl of H2SO4 was added to each well while the plate was read using a Spectramax spectrophotometer (BioTek, E-800, USA) at 450 nm.

RESULTS

PCR Results for Detection and Confirmation of the correct DNA constructs integration into *L. tarentolae* SSU Locus:

To verify that the matching constructs were appropriately inserted into the 18S rRNA gene locus, PCR was performed (Figure 1). The integration of accurate size of SAG1 and Cyc18 constructions and predicted fragment size were represented in the schematic figure in (appendix 1). The PCR results displayed no band for *L. tarentolae* wild type and an 862 bp amplicon was indicated of SAG1 and Cyc18 integration to *L. tarentolae* locus ofribosomal. The PCR reaction's 628 bp and 333 bp fragments demonstrated that the filamentous SAP1 phosphatase () had correctly fused to SAG1 and Cyc18 (Figure 1).

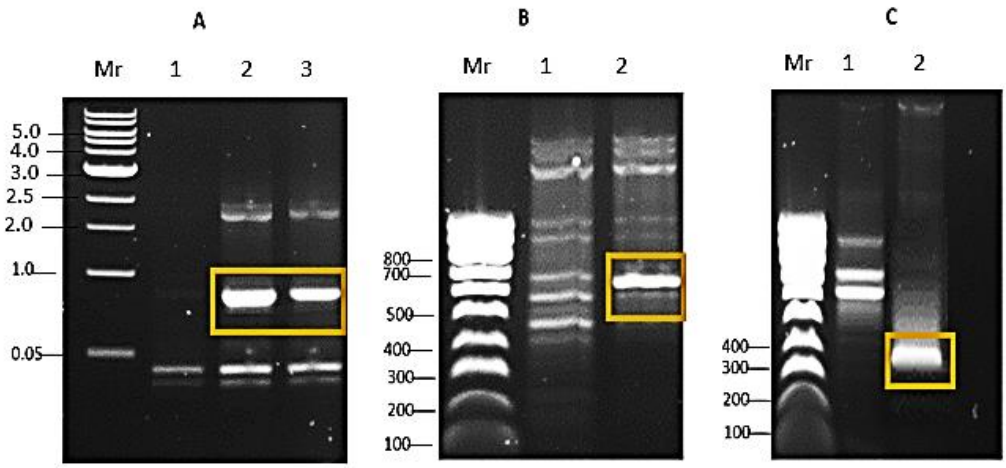

Fig-1: Confirmation using PCR fragments was correctly integrated into the rRNA gene locus. *(A) SAG1 and Cyc18 constructs are integrated into L. tarentolae. Lane 1, L. tarentolae WT; lanes 2 and 3, L. tarentolae clones (size 862 bp). (B) The SAP1SAG1 fusion. Lane 1, L. tarentolae WT; lane 2, L. tarentolae with integrated SAG1 (size 628 bp). (C) The SAP1 Cyc18 fusion. Lane 1, L. tarentolae WT; lane 2, L. tarentolae with integrated Cyc18 (size 333 bp). Mr is 1kb and 100 bp ladder.*

Evaluation of Phosphatase Enzyme in Supernatants of *L. tarentolae* culture:

The enzyme of Secreted acid phosphatase (SAP1) function as part of recombinant proteins expressed in the culture was determined in dilutions of the supernatants. By measuring the absorbance at 405nm, p-nitrophenyl phosphate was utilized to assess the enzyme activity as a substrate in the testers. The culture of wild *L. mexicana* supernatant was a positive sample., whereas the wild type of *L. tarentolae* was negative. (D9, F7, D11, D5) clones of *L. tarentolae* electroporated with the SAP1SAG1, and two clones (A11, D5) of SAP1Cyc18constructed are shown a notable enzyme activity in the 10% of iFCS supplemented SDM79 media. (Figure 2 A). After reducing the supplied iFCS to 2%, only SAP1SAG1 (D9) and SAP1Cyc18 (A11) showed enzyme function. These activities were higher than those of *L. mexicana* wild type (Figure 2 B).

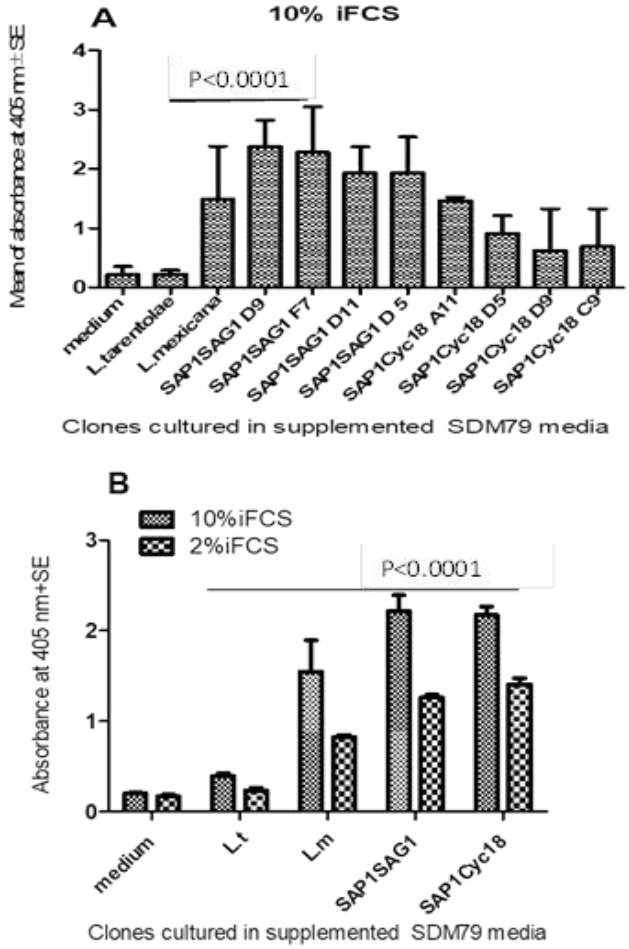

Fig-2: Activity of (SAP) in *L.tarentolae* promastigotes'. *(A) SAP1SAG1 and SAP1Cyc18 in 10% of iFCS supplement. (B) SAP1SAG1 and SAP1Cyc18 in 2% iFCS.*

Protein purification ofSAP1SAG1 by Ammonium Sulfate Precipitation (AS):

Ammonium sulfate (AS) salt was precipitated to purify the fusion protein t. Enzymatic activity was performed from serial ammonium sulfate precipitation with AS from culture supernatants. Partially refined SAP1SAG1 showed high enzyme function at 60% saturation (Figure 3 A). The average of SAP1SAG1 protein was presented in Figure 3 B at 60% AS precipitation. A 64 kDa band confirmed the presence of BSA protein obtained from 40-60% AS enrichment from the culture of *L. tarentolae* wild type. Meanwhile, the band around 82 kDa at 60% of AS enrichment from recombinant cells confirms the presence of SAP1SAG1 protein.

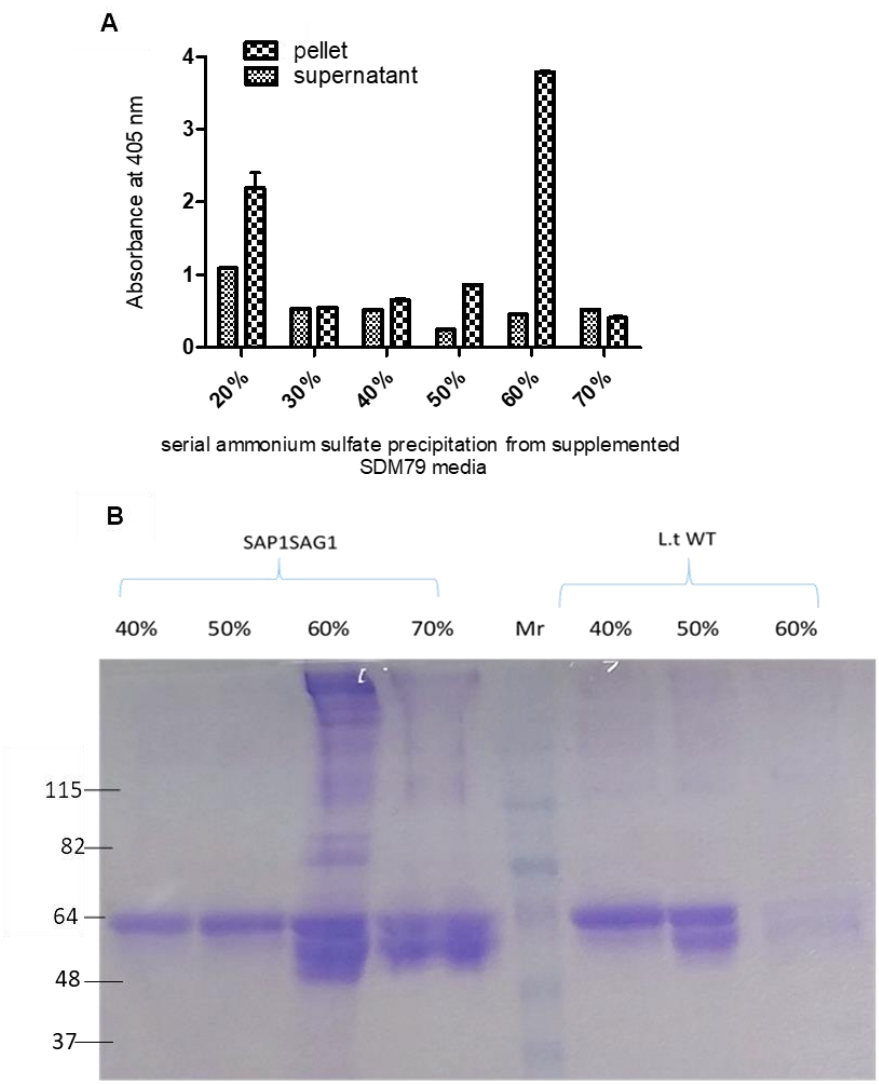

Fig-3: Ammonium sulfate enrichment of SAP1SAG1. (A) SAP activity in ammonium sulfate precipitation. *(B) Coomassie-stained 10% SDS-PAGE of L. tarentolae. An 82 kDa band represents the SAP1SAG1; the 64 kDa band is BSA.*

Confirmation of SAP1SAG1 Fusion Protein by Using Immunoblot Analysis:

A strong band around 82 kDa presented on SDS-PAGE equivalent to the molecular size of SAP1SAG1 purified with 60% AS precipitation. Similarly, the same band showed 20%,30%, and 40% of AS saturations. It disappeared at 50% of AS enrichment (Figure 4 A). Anti-hexahistidine-tag antibody was used in Immunoblot analysis

and found that the clones of precipitated cells of AS (20-70%) enrichments presented bands sized 82 kDa which conformed to the SAP1SAG1 (Figure 4 B). The full-length of SAP1SAG1 protein was identified by hexahistidine situated at the C-terminal and that identification was repeated to detect the fluorescence signal of the same size by infrared immunoblot (LI-COR) It detected a (Figure 4 C). Since the wild type of *L. tarentolae* showed no signal against an anti-His antibody for comparison.

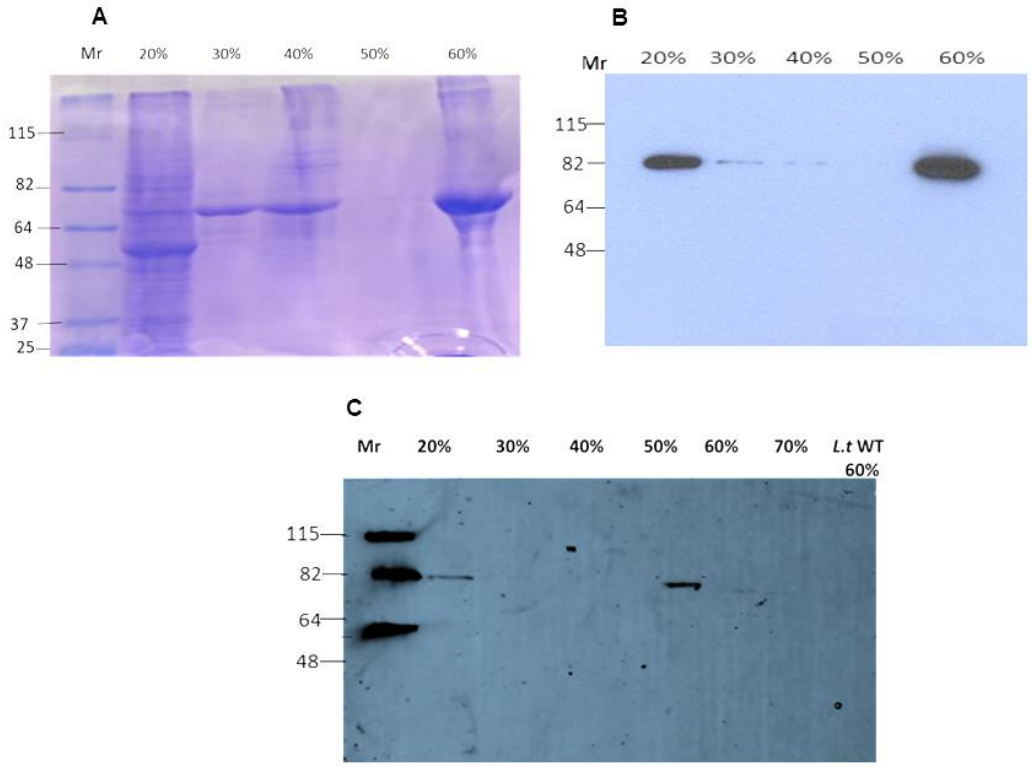

Fig-4: Anti-His-tag immunoblot expressing SAP1SAG1 obtained from supernatants afterward disparity AS precipitation. *(A) Coomassie-stained 10% SDS-PAGE SAP1SAG: A band refers to SAP1SAG1 after 60% AS precipitation. (B) Immunoblot analysis: Strong bands are visible for 20% and 60% AS of SAP1SAG1. (C) Infrared immunoblot: The fluorescence signal confirms the SAP1SAG1 of 60% AS saturation; lane 7, no reaction of L. tarentolae WT.*

ELISA Analysis:

An ELISA test was performed to corroborate further the refinement of the SAP1SAG1(recombinant protein) with 60% ammonium sulfate precipitation. Following the SAP1SAG1 protein coating of the ELISA plate it was subjected to both LT8.2 and anti-SAG1 antibodies. When the serial dilutions were reduced, the results for LT.8.2 revealed robust reactivity for the full protein at 60% AS enrichment at high dilution (Figure 5 A). The same result was shown with a particular anti-SAG1 antibody for the SAP1SAG1 protein (Figure 5 B).

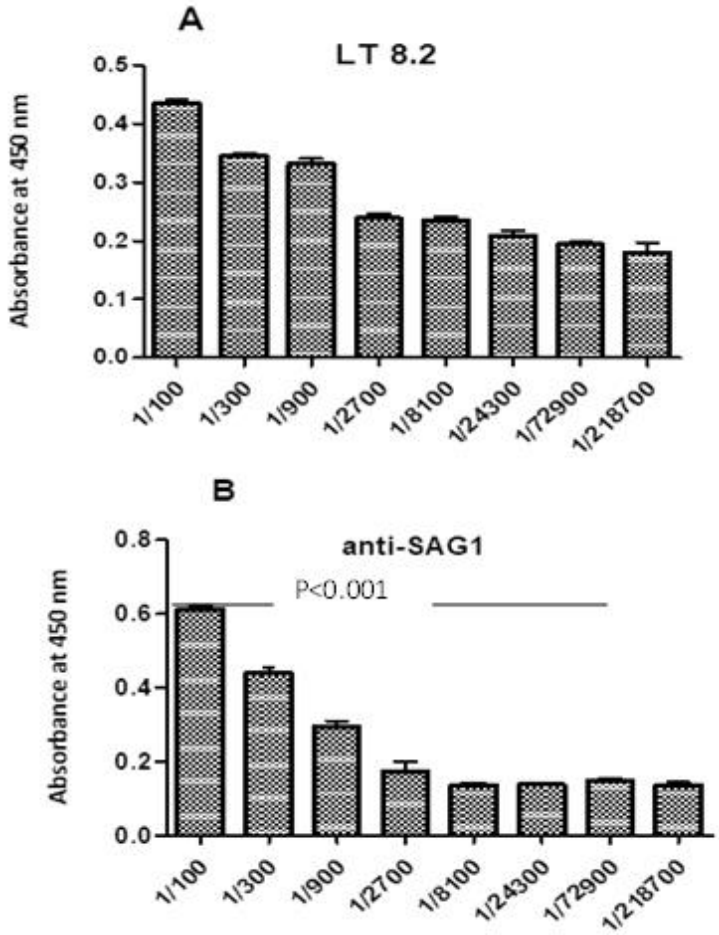

Fig-5: ELIZA of LT8.2 and anti-SAG1 antibodies are shown to recognize the recombinant SAP1SAG1 protein produced by 60% ammonium sulfate enrichment. *The SAP1SAG1 protein was applied to plates, and the LT8.2 and anti-SAG reactivity was evaluated. (A) It shows that SAP1SAG1 is recognized by mAb LT8.2 at serial dilutions. (B) It shows that SAP1SAG1 is recognized by anti-SAG1 at serial dilutions.*

Immunofluorescence Analysis:

Recombinant SAP1SAG1 protein expression and secretion in culture supernatants by *L. tarentolae* clones were directly observed using immunofluorescence analysis at 1-2 × 10^7 cells/ml density. Since the monoclonal Antibody of LT8.2 epitope is present in the recombinant protein and phosphatase enzyme (SAP1) of *L. mexicana*, it can be utilized the protein in the supernatant of the medium. Bright-field images visualized *Leishmania*. The wild type of *L. tarentolae* exhibits no fluorescence staining (negative control). The presence of secreted acid phosphatase filaments was shown by brilliant green structures in the supernatant of the wild-type *L. mexicana* (positive control). Strong green structures, even more, brilliant than those observed in the wild type of *L. mexicana*, were observed in the supernatant of *L. tarentolae* SAP1SAG1 recombinant cells (Figure 6 E and F). The filamentous protein released from a promastigote cell's flagellar compartment is seen in Figure 6 G. A concentrated solution of SAP1SAG1 molecules was observed in the SAP1SAG1 recombinant protein purified using 60% AS precipitation, as indicated by increasing fluorescent filaments (Figure 6H).

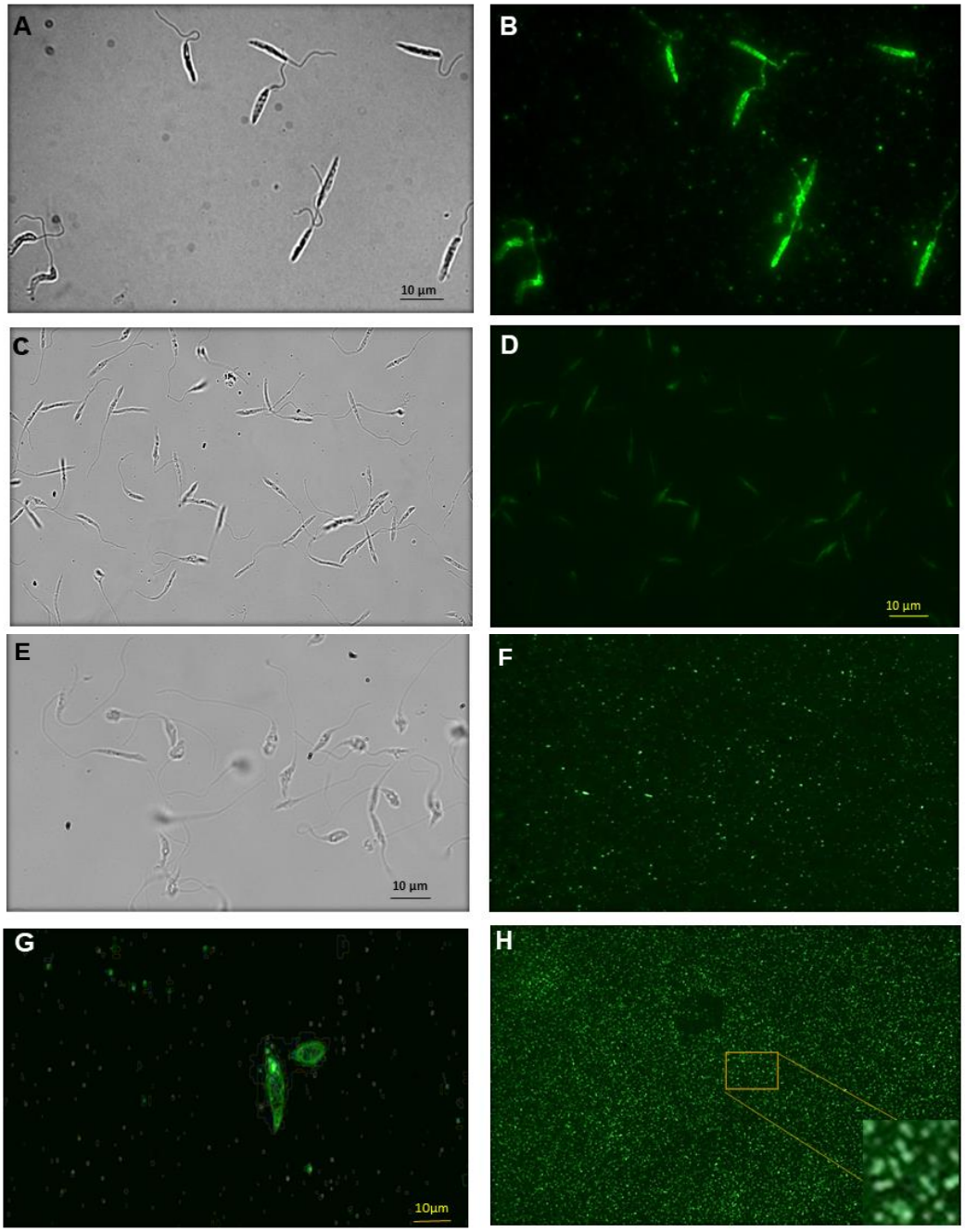

Fig-6: Immunofluorescence of *Leishmania* with mAb LT8.2. (A) *L. mexicana* WT bright field; *(B) L. mexicana WT FITC (positive control); (C) L. tarentolae WT bright field; (D) L. tarentolae WT FITC (negative control); (E) L. tarentolae expressing SAP1SAG1 bright field; (F) L. tarentolae expressing SAP1SAG1 FITC; (G) L. tarentolae expressing SAP1SAG1 showing the filamentous protein secreted from the flagellar pocket; (H) SAP1SAG1 of 60% AS precipitation. Bar size, 10 µm.*

Mass Spectrometry (MS) Analysis:

Mass spectrometry was used to verify the identity of a dyed with coomassie blue band that was sliced from SDS-PAGE of 60% AS enrichment lane of cell clones that expressed SAP1SAG1 recombinant protein (Glasgow Polyomics Facility, University of Glasgow). User protein sequences were compared to data using Batch-TagWeb (http://msviewer.ucsf.edu/prospector/mshome.htm) blasted with protein sequences. Mass spectrometry-detected aminoacid sequences verified that the isolated protein is SAP1SAG1. Nine peptides corresponding to the sequential amino acids of the acid-phosphatase portion and one peptide corresponding to SAG1(Figure 7) were among the sequences highlighted in red to show the similarity between the sequences in the table.

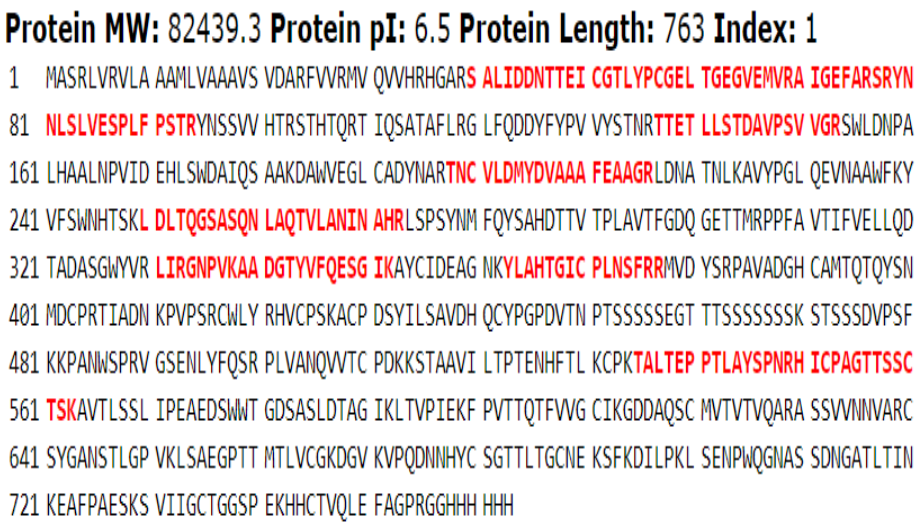

Fig- 7: Mass spectrometry analysis of SAP1SAG1 fusion protein.

DISCUSSION

This work aimed to create two plasmids that had the coding sequence of the *L. mexicana* protein of secreted acid phosphate that fused to *T. gondii* SAG1 or Cyc18 as a vaccine candidate for toxoplasmosis. This secretory protein of SAP1 is produced from the flagella of *L. mexicana* promastigotes [24]. With its N-terminal phosphatase domain [25], SAP1 served as a transporter of both *T. gondii* proteins(SAG1 and Cyc18), which were generated at C-terminal constructs. As a result, *L. tarentolae* is employed in synthesizing recombinant proteins and heterologous gene expression [26, 27]. In this work, the recombinant proteins SAP1SAG1 and SAP1Cyc18 were secreted to medium supernatants using the *L. tarentolae* system [28]. were Successful cloned cells of *L. tarentolae* promastigotes with pLPhSAP1sSAG1 and pLPhSAP1sCyc18 by electroporation [29]. Phleomycin protein was utilized to produce stable plus long-lasting transfections, making these secretory proteins easily produced [30, 31]. High and steady growth rates can be achieved when cultivating *L. tarentolae* cell lines [32]. Cloned cells were cultured to a density of 1×10^8 parasites/ml afterward incubation of 4-5 days [20]. The integration of the delivery gene cassette of *L. tarentolae* by homologous recombination was diagnosed by two PCR steps [33]. The proper transformation of the expression constructs in the locus of the short 18S subunit (SSU) rRNA of *L. tarentolae is* shown in the first phase of the PCR. To demonstrate the fusion of SAP1 to SAG1 or Cyc18, a second PCR was necessary. For the clones that were

examined, both PCR results were positive, confirming that the entire expression construct was present in the correct chromosomal region [34]. It is significant to remember that RNA polymerase I regulates transcription, allowing for high-level transcription [35, 36]. Using phosphatase activity in culture supernatants to detect the gene transcription in *L. tarentolae* cloned cells of recombinant SAP1SAG1 and SAP1Cyc18 proteins was ascertained [37, 38]. Santignific enzyme level was obtained from clone cells of SAP1SAG1 expressed protein and a lower signal was obtained from cloned *L. tarentolae* electroporated s with SAP1Cyc18 that cultured with 10% iFCS of supplemented media. Following reducing the outcome of bovine albumin on protein refinement, the iFCS in the media was diminished to 2% (v/v). Every electroporoted *L. tarentolae* cell expressed SAP1SAG1 or SAP1Cyc18 was designated. Then enzyme function was measured in this culture supernatant, high enzyme level was found in cell lines expressing both SAP1SAG1 and SAP1Cyc18, more significant than of wild *L. mexicana* parasites. The highest levels of SAP1SAG1 expressed cells were elected to refine the recombinant protein for more analysis of immunofluorescence and immunoblotting methods.The fundamental principle of ammonium sulfate usage is that the dissolvability of the proteins rises by consequential addition of this salt more than <15% meaning salting-in trem. With high concentrations, the protein dissolvability usually declines, resulting in more solubilization, which is salting out trem. Since SAP1SAG1 is a filamentous protein consisting of subunits that form filaments that might be purified by using changed proportions of ammonium sulfate. The macromolecular complexe***s*** fundament by salting out with more than 20% of AS saturation [21]. Previous studies used AS concentrations between 50-77% to precipitate Interleukin-1β [39]. Similarly, with 40-45% of AS, IgG has been precipitated from blood [22]. SAP1SAG1 was purified from culture supernatants using a modified approach of gradual additive of AS salt ranging 20-70% (v/v) precipitation in this work. Cloned *L. tarentolae* cells were cultured in SDM79 enriched with 2% iFCS phosphatase activity and then were assessed in precipitants and supernatants of SAP1SAG1 protein after stepwise accumulation of AS. Phosphatase level in crystallization of 60% AS saturation of recombinant proteins was observed higher compared to the positive control of *L. mexicana*. The process of protein precipitation was also carried out using culture supernatants of the WT *L. tarentolae* cultured under the same circumstances as *L. tarentolae* that expressed SAP1SAG1. The strong protein lane of 82 kDa of *L. tarentolae* expressed cells to SAP1SAG1 supplemented at 60% AS concentration. The filament of protein was identified by epifluorescence microscopy in cloned cells that were cultured with 60% AS precipitate except in *L. tarentolae* wild type, according to immunofluorescence using mAbLT8.2. The expression of SAP1SAG1 was noticed with mAb LT 8.2 antibody. This finding is consistent with previous research on SAP filament secretion in *L. mexicana* [40]. High acid phosphatase activity, an appropriately sized band in western blot investigation, and filament visualization in immunofluorescence microscopy are present in the 60% AS precipitate. By amplifying anti-hexahistidine antibodies that detect fusion proteins in the C-terminun end, immunoblot was approved for the fusion protein. With the western blot, SAP1SAG1 was identified in the 60% AS precipitations based on prominent bands at 82 kDa. According to the anti-hexahistidine antibody reaction, the whole SAP1SAG1 protein was performed and secreted from the cloned cells of *L. tarentolae.* Whereas monomeric protein precipitated with 60% AS, the multisubunit filamentous form of SAP1SAG1 was precipitated with 20% AS. An ELISA was carried out to verify that SAG1 was a component of the fused protein concentrated in 60% of ammonium sulfate precipitation since an anti-SAG1 antibody

was available. The mAbLT.8.2 was used in the ELISA as a control. 60% AS precipitate's SAP1SAG1 immunodominant antigens bind to anti-SAG1 antibodies with the particular mAb LT.8.2. The ensuing response resulted in a measurable signal from the recombinant protein. The immunofluorescence results were validated besides immunoblot and mass spectrometry analysis. Using mass spectrometry (MS), which is a dominant method with various submissions in biochemistry sciences, the sequences of SAP1SAG1 peptides were identified. The analysis revealed nine SAP1 peptides and one SAG1 peptide [14] (accession code 1KZQ). By separating molecules, MS determines the molecular weight of compounds based on their mass and charge [41]. Since *L.tarentolae* can be an encouraging system, further optimization is required to ensure a large yield of protein productions without degradation is one of the limitations of the recombinant proteins. Then preclinical can undergo to assess the effectiveness and potential immunity stimulation for human use.

CONCLUSION

L. tarentolae was electroporated with linear DNA fragments produced from pLPhSAP1sSAG1 and pLPhSAP1sCyc18, which had been effectively constructed. PCRs confirmed that the complete expression constructs had correctly integrated into the 18S ribosomal small RNA alleles of *L.tarentolae*. The effective expression of SAP1SAG1 secretion by *L. tarentolae* promastigotes was validated by enzyme activity, immunoblot, and immunofluorescence studies. Additionally, the protein was precipitated at 60% ammonium sulfate of the culture media. Mass spectrometry and ELISA analysis verified the recombinant fusion protein's presence of SAG1.

Ethical Considerations:

This study adhered to international and institutional biosafety standards for working with recombinant *Leishmania tarentolae*. Experiments were conducted under strict compliance to ensure the safe handling, storage, and disposal of genetically modified organisms. The data presented are accurate and free from manipulation, ensuring research integrity. Contributions from collaborators were appropriately acknowledged, reflecting the collaborative nature of the work. Any future preclinical applications will strictly adhere to ethical guidelines, including obtaining necessary approvals for animal studies. The authors are committed to transparency, reproducibility, and responsible scientific conduct.

Conflict of Interest:

The authors declare that there are no competing interests.

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Not received

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Authors contribution:

All authors contributed equally special thanks to Dalia Ahmed Khalef.

Consent for publication:

The author would like to do afterward acceptance.

Data Availability Statement:

The data supporting the findings of this study are available within the current article.

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