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Chronic administration of cryptolepine nanoparticle formulation alleviates seizures in a neurocysticercosis model

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ABSTRACT

Worldwide, neurocysticercosis remains an important cause of acquired epilepsy. We therefore seek to investigate the effectiveness of the nanoparticle formulation of cryptolepine in alleviating seizures in a neurocysticercosis model.

A solid-lipid nanoparticle formulation of extracted cryptolepine was prepared. The parasites were maintained in T. crassiceps metacestode (ORF strain) - infected female BALB/c mice. Cryp (5 mg/kg), SLN-CRYP (5 mg/kg), ABZ (50 mg/kg) DXM (0.5 mg/kg), and PHE (30 mg/kg).

were assessed for in vitro cysticidal, in vivo cysticidal and/or antiseizure activity in 70 mice that had developed seizures from infection with T. crassiceps. General pathologic processes were studied in the host tissue and inflammatory mediators were quantified from isolated mice brains.

All treatments (CRYP, SLN-CRYP and ABZ) caused significantly reduced viability of T. crassiceps cysts. Treatment with SLN-CRYP significantly shrunk cysticerci and resolved ventricular expansion and deviation similar to albendazole on examination of encephala. SLN-CRYP inhibited hyperemia but was more effective against microgliosis, calcification, edema and meningitis. Mean seizure score was significantly reduced in models administered with SLN-CRYP ($p < 0.0001$); as were frequency ($p < 0.0001$) and duration ($p < 0.0001$) of seizures. SLN-CRYP significantly reduced brain homogenate levels of IL-10 ($p = 0.0016$) and IFN-γ ($p < 0.0001$).

Our study shows that the chronic administration of the nanoparticle formulation of cryptolepine is effective in alleviating seizures associated with neurocysticercosis in a mouse model.

1. Introduction

The larval form also known as metacestode of *Taenia solium* is the causative organism of Neurocysticercosis (NCC). The Central Nervous System (CNS) may be infected with cysts when *Taenia solium* eggs are accidently ingested (Alvarez et al., 2010; Mishra et al., 2009). This condition has become a major menace in countries in the tropics and remains the principal cause of epilepsy in these areas (De Bittencourt et al., 1996; Mewara et al., 2013; Montano et al., 2005).

Experimentally, the most commonly used parasite for cysticercosis models is *Taenia crassiceps*. This specie has a rapidly developing cycle, is easy to maintain with antigenicity similar to *T. solium* (Sciutto et al., 2007) The neurological syndrome in NCC is dependent on the location of the cysticerci. This may be cerebellar, intraventricular, medullar, meningeal or parenchymal (Matushita et al., 2011).

Mostly, in human cases of NCC, viable parasites cause only slight levels of inflammation in their cellular environment, resulting in asymptomatic NCC. However, majority of symptomatic NCC cases present with intense inflammatory reaction in the host tissue (Cardona et al., 2003). Intraventricular and meningeal cysticerci occur less frequently than the parenchymal but are more aggressive in their clinical manifestations (Khade et al., 2013). Intraventricular invasions of parasites may lead to focal compression and distension of the ventricle (Khade et al., 2013). Patients with parenchymal and calcified parenchymal cysticercosis usually present with recurrent seizures. These seizures are thought to be caused by breakdown of the granuloma which release parasite antigens (Webb and White, 2016). The antigens trigger seizures in the parenchymal region via a brain immune-inflammatory response,

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implicating cytokine production and cellular infiltration (de Lange et al., 2019). At 90 days after *Taenia crassiceps* infection, there is mononuclear cells are predominant in the inflammatory infiltration.

Clinical diagnosis as well as management of NCC is not clear-cut as a result of the wide variation in clinical symptoms and neuropathology. Randomized control trials have shown that antihelmintic therapy like Albendazole, resolves vesicular cysts in the brain parenchyma (Carpio et al., 2008). However, the management of NCC has become increasingly difficult due to factors such as failure of anthelmintic drugs to protect the patient from getting a reinfection and relapse as well as the overwhelming presence of anthelmintic resistance (Jung-Cook, 2012). Albendazole's effect on seizure outcome has also been explored. Research has found no significant difference in the risk of seizure recurrence after treatment in addition to a disparity in effect on seizure rate; this effect varies widely according to seizure type. Antiepileptic drugs (AEDs) such as carbamazepine, have an important function in NCC management. These drugs when prescribed for NCC induced seizures, are continued until live or degenerating cysts and/or brain inflammation surrounding the cysts completely resolves. Choice of AED in NCC is based on factors such as efficacy and affordability of drug - with affordability being particularly important in developing countries.

There is evidence that herbal medicines may be a remedy for worm infestation which dates far back to 3500 years BC. Recent data shows that herbal medicines are still used in the management of parasitic infections in countries such as India and China which are endowed with great resources of medicinal plants (Tandon et al., 2011).

Cryptolepis sanguinolenta of the family Apocynaceae is a plant with several species distributed across the tropical rainforest sectors of Africa. In Ghana, *C. sanguinolenta* is vastly distributed in the mountainous regions of Ghana particularly the Kwahu and Akuapem mountains (Addy, 2003; Iwu, 2014). Osafo et al. reported that *Cryptolepis sanguinolenta* from the West African region, for centuries, is largely used in managing various infectious and non-infectious diseases in African Traditional Medicine (Osafo et al., 2017).

C. sanguinolenta and its major alkaloid, cryptolepine, have been extensively studied for their effects in the management of malaria. The mode of action is linked to its rapid blood schizonticidal action as well as its potent anti-inflammatory activity (Bugyei et al., 2010; Olajide et al., 2009). Similarly, research has been conducted to establish the antimicrobial activity of cryptolepine and its analogues (Mazu et al., 2011; Zhu et al., 2007). However, its potential cysticidal action is yet to be studied. Cryptolepine (Cryp) is known to possess anti-inflammatory properties (Bugyei et al., 2010). The authors have previously shown that the SLN formulation of cryptolepine aids the molecule to cross the blood brain barrier and exert improved anti-seizure activity (Mante et al., 2021).

Hence, combining its blood brain barrier crossing capability and its cysticidal activity will make it beneficial monotherapy for the management of neurocysticercosis at controlled doses. This study therefore seeks to investigate a nanoparticle formulation of cryptolepine for potential cysticidal activity and usefulness against seizures induced by neurocysticercosis.

2. Materials and methods

2.1. Chemicals

Phenytoin (PHE), Substance P (SP), Dexamethasone (DXM), Albendazole sulfoxide (ABZ), Dimethylsulfoxide (DMSO) were obtained from Sigma-Aldrich, Saint Louis MO 63103 USA.

2.2. Formulation of solid-lipid nanoparticles of cryptolepine (SLN-CRYP)

Extraction of cryptolepine (Cryp) from *Cryptolepis sanguinolenta* was done as described by Mante et al. (2021).

The solid-lipid nanoparticles of cryptolepine (SLN-CRYP) were prepared using the solvent-evaporation method as reported by (Amasya et al., 2016). Poloxamer-188 (4 mg), acting as a surfactant, was dissolved in 10 mL of water. The co-solvent used was Propylene glycol (3 mg) which was added to the surfactant solution after which the lipid formulation was obtained by adding 50 mg of stearic acid. Cryptolepine (15 mg) was dissolved in ethanol (10 mL) which was subsequently mixed with the lipid formulation. This resultant mixture was stirred for 45 min using a magnetic stirrer after which sonication for 15 min and centrifugation at 12,298*g* force at room temperature (25 °C) for 15 min was done. Solid pellets obtained were freeze dried. SLN-CRYP prepared was kept at 4 °C.

Physicochemical properties of SLN-CRYP has been added as supplementary data.

2.3. Parasite maintenance

The parasites were maintained in *T. crassiceps* metacestode (ORF strain) - infected female BALB/c mice with weights between 20 and 25 g as reported by (Palomares-Alonso et al., 2015). The mice were sacrificed 2 months post infection via cervical dislocation. Larval forms in the peritoneal cavity were then removed and washed with sterile normal saline solution repeatedly. Intact non-budding cysts of approximately 3 mm with a complete vesicular membrane and translucent fluid confirmed with a stereoscopic microscope were used. Mice were housed in a facility under 12 h light/dark cycles with 40–70% relative humidity and temperature of 18–23 °C. Food and water were provided *ad libitum*. Ethical standards were observed in handling of animals based on standard laboratory animal care and use principles (National Research Council, 2010). Ethical clearance for the study was obtained from the Faculty Animal Ethics Committee. All animals were obtained from the Departmental Animal Facility.

2.4. Assessment of cysticidal activity

2.4.1. In vitro cysticidal assay

Cryp and SLN-CRYP concentrations of 3 μM were used. This corresponds to the effective concentration at which 50% of the cysts are killed (EC_{50}) obtained from unpublished toxicity data. A stock solution of Cryp (5 mg/mL) was prepared in DMSO. Serial dilution was performed to obtain 3 μM solution in DMEM. SLN-CRYP, dispersed in distilled water was diluted with DMEM to obtain required concentration. Dilution of ABZ (5 mg/mL) in DMEM was done to obtain a resultant concentration of 0.5 μM. The negative control used was 0.2% DMSO in culture medium. A 24-well cell culture flat-bottom microplate (Thermo Scientific, UK) was filled with 2 mL of culture medium either containing each drug alone or DMSO 0.2%. Each well contained ten cysts which were then incubated at 98% of relative humidity, 37 °C and 5% CO_2 atmosphere and for 11 days. The culture medium was changed on alternate days. Triplicate of each experiment was conducted. A daily check of the parasites for motility, integrity and morphological changes under an inverted light microscope ICM 405 (Leica DMi8, Germany) was done. The parasites that showed loss of motility and vesicular fluid as well as damage to their tegument were tagged non-viable. Trypan Blue exclusion test was used for confirmation on day 11 as described by (Palomares-Alonso et al., 2017).

2.4.2. In vivo cysticidal assay

About 40 *T. crassiceps* non-budding cysts were suspended in 60 mL of sterile Hank's Balanced Salt Solution (HBSS) and were injected into 3–5 weeks old female BALB/c mice (25–30 g) intracranially using a 25-gauge needle and 1 mL syringes post anesthesia (0.1 mL/10 g of Xylazine 2% and Ketamine 10% solution); Injected at the junction of the transverse sutures and superior sagittal to a 2 mm depth. The control mice were administered with 60 mL sterile HBSS. On day 91 post-infection (DAI), mice which developed seizures (40% of total population (n = 175)) were divided randomly into experimental groups of ten animals each. Animals received a) Cryp 5 mg/kg b) SLN-CRYP 5 mg/kg; c) ABZ 50 mg/kg; d) DXM 0.5 mg/kg; e) control (distilled water 0.5 mL). Each mouse was

given a maximum of 0.5 mL of the drug preparations intragastrically for 28 consecutive days (Matos-Silva et al., 2012).

2.5. Substance P-induced seizures

Due to unpredictability of seizures, Substance P (SP) was used to induce seizures on day 28, 1 h after treatment. Substance P was injected subcutaneously into the soft neck fold of the mice and seizure activity examined using video recording. SP (10 nM) was injected with resultant severe behavioral seizures determined using the Racine score. The recording was scored for the highest seizure score, determined as the highest observed seizure score for the whole 30 min observation period using the racine score:

Stage 0 (no response); Stage 1 (hyperactivity, restlessness and vibrissae twitching); Stage 2 (head nodding, head clonus and myoclonic jerks); Stage 3 (unilateral or bilateral limb clonus); Stage 4 (forelimb clonic seizures); Stage 5 (generalized clonic seizures with loss of postural control). Mice were also scored for frequency and duration of seizures.

Afterwards animals were sacrificed by cervical dislocation and their encephala removed for analysis. Animals (n = 10) received a) Cryp 5 mg/kg b) SLN-CRYP 5 mg/kg; c) ABZ 50 mg/kg; d) PHE 30 mg/kg; e) Distilled water 0.5 mL.

2.6. Assessment of general pathologic processes

General pathologic processes of the encephala were examined in the host tissue for manifestation of inflammation i.e., meningitis, edema, hyperemia, calcification, fibrosis, and microgliosis. They were described using a semi-quantitative method in accordance with the following measures: absent; + discrete with up to 25% of the affected area; ++ moderate from 25 to 50% of the affected area and +++ accentuated above 50% of the affected area.

2.7. Cytokines ELISA assay

The secreted IFN-γ, IL-6 and IL-10 levels in the homogenized brain tissue of the mice were measured using Mouse, IL-6, IL-10 and IFN-γ ELISA following the manufacturer's manual (Abcam Plc, Cambridge, CB2 0AX). The detection limit was 62.5 pg/mL for IFN-γ and 15.6 pg/mL for IL-6 and IL- 10. Treatment groups assessed were a) Cryp 5 mg/kg b) SLN-CRYP 5 mg/kg; c) ABZ 50 mg/kg; d) DXM 0.5 mg/kg; e) control (distilled water).

2.8. Data analysis

Data represent Mean ± S.E.M. Significant differences among means were analyzed with one-way analysis of variance (ANOVA) with Tukey *post hoc* test. $P < 0.05$ was considered significant in all instances. Statistical analyses were done with Graph Pad Prism® Version 8.0 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. In vitro cysticidal assay

3.1.1. Effect of drug treatments on T. crassiceps viability

All treatments (Cryp, SLN-CRYP and ABZ) significantly reduced viability of *T. crassiceps* cysts. Effects were not significantly different among treatment groups but significantly different from control (see Table 1).

3.1.2. Effect of drug treatments on T. crassiceps morphology

All treatments (Cryp, SLN-CRYP and ABZ) significantly reduced viability of *T. crassiceps* cysts. Effects were not significantly different among treatment groups but significantly different from control.

Cysts incubated in 0.2% DMSO exhibited normal shape, size and movements (Fig. 1A). Cysts incubated with Cryp (Fig. 1B), SLN-CRYP (Fig. 1C) and ABZ (Fig. 1D) showed a decrease in size with loss of robustness and altered movement. In some cysts, there was complete loss of cystic fluid.

Table 1
In vitro cysticidal activity of Cryp, SLN-CRYP, ABZ on *T. crassiceps* cysts.

Treatment	Viability (%)
Control (0.2% DMSO)	100
Cryp (3 μM)	41.1 ± 6.3*
SLN-CRYP(3 μM)	40.2 ± 7.3*
ABZ (0.5 μM)	38.5 ± 4.5*

Mean ± S.E.M. Each well contained n = 10 cysts. Each treatment measured in triplicate. *P < 0.05.

3.2. In vivo cysticidal assay

All *T. crassiceps*-infected mice had cysticerci in brain ventricles with resultant inflammation, expansion to ventricles and median line deviation. Treatment with SLN-CRYP (Fig. 2D) completely eliminated cysticerci and resolved ventricular expansion and deviation similar to albendazole (Fig. 2B). Control and Cryp treated mice had viable cysticerci. Treatment with DXM resulted in shrunken cysticerci (Fig. 2E). Fig. 2F shows presence of viable cysticerci with inflammatory process in ventricle at 91 days post-infection.

3.3. General pathologic processes analysis of host tissue

SLN-CRYP inhibited hyperemia but more effective against microgliosis, calcification and edema. Dexamethasone was more effective than albendazole completely inhibiting meningitis, edema, calcification and hyperemia. Microgliosis was inhibited to a lesser extent. None of the treatments were effective against fibrosis. Albendazole partly inhibited microgliosis, meningitis and edema but completely prevented hyperemia (see Table 2).

3.4. Effect of drug treatments on SP-induced seizures

SLN-CRYP caused a significant reduction in mean seizure score ($p < 0.0001$, $F(5, 54) = 40.71$; Fig. 3A), frequency ($p < 0.0001$, $F(5, 54) = 130.6$; Fig. 3B) and duration ($p < 0.0001$, $F(5, 54) = 732.0$; Fig. 3C) of seizures. Cryptolepine alone showed activity in reducing duration of seizures. Phenytoin was effective against all parameters while albendazole showed no activity.

3.5. Cytokines ELISA assay

SLN-CRYP significantly reduced brain homogenate levels of IL-10 ($p = 0.0016$, $F(4, 45) = 57.29$; Fig. 4B) and IFN-γ ($p < 0.0001$, $F(4, 45) = 88.98$; Fig. 4C). Dexamethasone significantly reduced all cytokine levels $p < 0.0001$, $F(4, 45) = 88.98$). Albendazole was effective against all cytokines with the exception of IL-6.

4. Discussion

A good pathological model provides a precise characterization of the disease condition and facilitates proper investigation and the attainment of credible results. The observance of expansion to ventricles, inflammation and median line deviation is characteristic of a successful NCC model consistent with other studies conducted (Matos-Silva et al., 2012; Moura et al., 2020).

In contrast to the negative control, the similar percentage viability of *T. crassiceps* observed in the culture medium with SLN-CRYP and albendazole indicates significant in-vitro cysticidal activity.

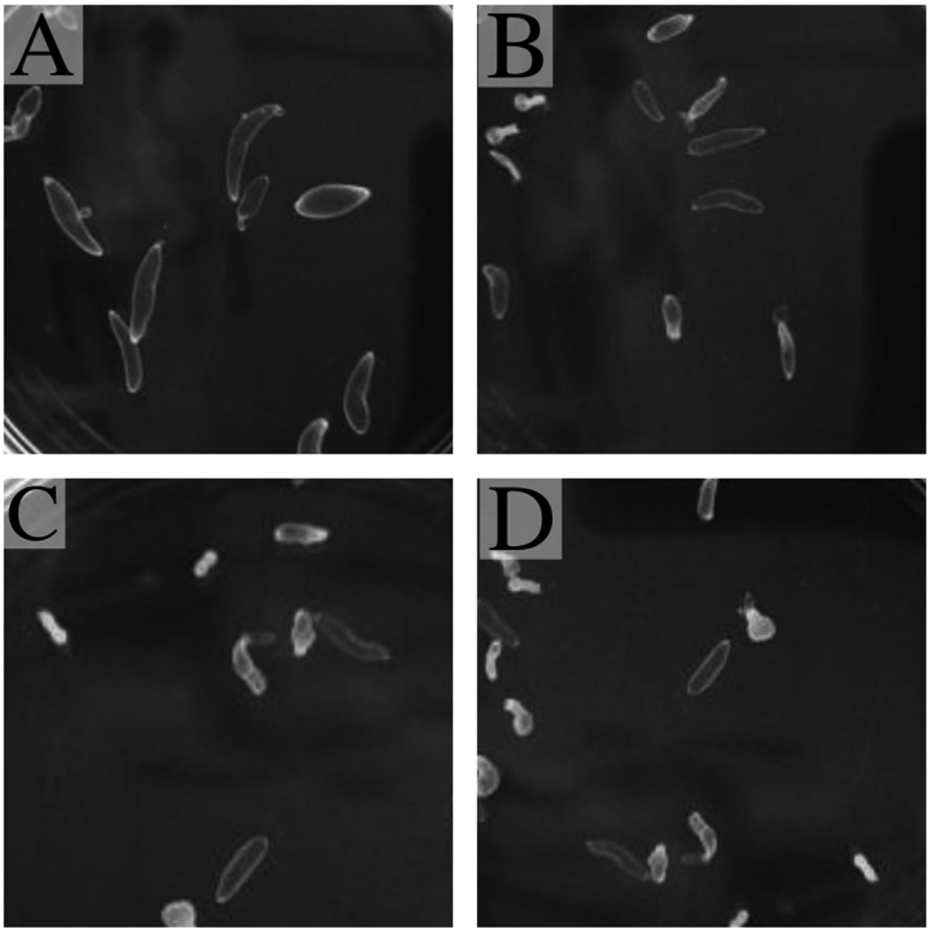

Fig. 1. Morphological appearance of cysts of *T. crassiceps* post treatment in vitro. A) control 0.2% DMSO; B) Cryp 3 μM; C) SLN-CRYP 3 μM; D) ABZ 0.5 uM.

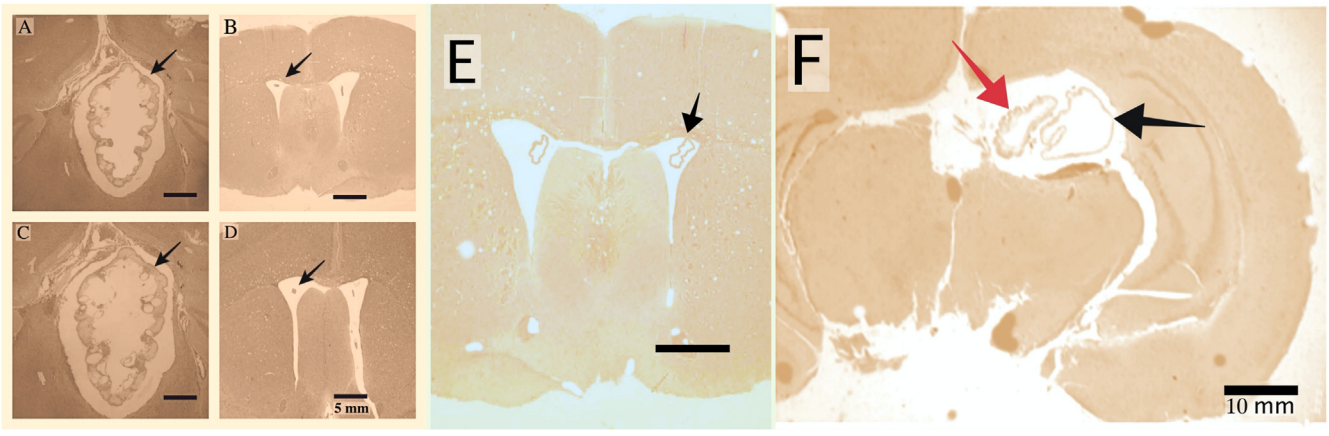

Fig. 2. Photomicrographs of encephala of BALB/c mice infected with *T. crassiceps* cysticerci, stained with H/E. A) Control 0.2% DMSO B) ABZ 0.5 μM C) Cryp 3 μM D) SLN-CRYP 3 μM E) DXM 0.5 mg/kg day at 29 after oral treatment. F) Untreated control at day 91 post-infection. Arrows in panels A and C show the presence of cysticerci and resultant disruption of midline. Arrows in panels B, D, E show shrunken cysticerci and restoration of midline. Black arrow in F shows presence of cysticerci and red arrow in F shows presence of inflammatory process in ventricle.

Several studies have identified the occurrence of inflammatory processes such as meningitis, edema, hyperemia, microgliosis and calcification and fibrosis in neurocysticercosis (Nash and Garcia, 2011; Simão et al., 2018). Edema as well as inflammation around lesions and meningitis have been observed in extra-parenchymal neurocysticercosis of which intraventricular is the most common (Anadure et al., 2020). The resolution of these classical features therefore provides a good measure of drug efficacy since they mirror microscopical tissue changes that occur in brain cells in the pathology of NCC. In addition, inflammation is cardinal in the pathophysiology of NCC and its control is key to the limitation of disease morbidity and mortality (Nash and Garcia, 2011).

The presence of calcification in the tissue samples may indicate that the disease process progressed to the destructive stage of neurocysticercosis (also described as the calcified nodular stage) which is characterized by calcification (Coyle and Tanowitz, 2009; Moskowitz and Mendelsohn, 2010; Mullins et al., 2017).

Table 2
Pathological processes observed in infected tissue of BALB/c mice.

	DMSO 0.5%	CRYP (5 mg/kg)	SLN-CRYP (5 mg/kg)	ABZ (50 mg/kg)	DXM (0.5 mg/kg)
Calcification	++	–	+	+	
Fibrosis	+	+	+	+	+
Microgliosis	++	++	+	+	+
Meningitis	+++	+++	–	+	–
Edema	+++	+++	+	+	–
Hyperemia	+++	+++	++	–	–

- absent; + discrete with up to 25% of the affected area; ++ moderate from 25 to 50% of the affected area and +++ accentuated above 50% of the affected area.

An absence of meningitis upon treatment with SLN-CRYP, coupled with a reduction of microgliosis and edema in the compromised area from moderate to discrete and change from accentuated to moderate in hyperemia indicates some anti-inflammatory effect of SLN-CRYP. Similarly, treatment with albendazole showed some anti-inflammatory effect- an accentuated/moderate to discrete level change in the occurrence of microgliosis, meningitis and edema, absence of hyperemia. However, Dexamethasone remained superior to SLN-CRYP in its anti-inflammatory effect. Compared to cryptolepine, the higher anti-inflammatory effect of SLN-CRYP in brain tissues may be due to increased BBB penetration of the nanoparticle formulation of cryptolepine as was previously shown by the authors (Mante et al., 2021).

About 30% people with epilepsy or seizures in most endemic countries suffer from neurocysticercosis (Ndimubanzi et al., 2010). Recurrent seizures are commonly the major manifestation of parenchymal brain cysticercosis (Nash et al., 2004). Although mechanisms of epileptogenesis in neurocysticercosis are debatable, they are likely to be partly due to the formation of reactive gliotic scars local and inflammation. In a study that demonstrated the presence of SP- producing cells localized in inflammatory areas in the brain of NCC patients, researchers concluded that seizures in neurocysticercosis are substance P – mediated (Robinson et al., 2012). The anti-seizure effect of SLN-CRYP as demonstrated by a significant decrease in the mean seizure score, frequency as well as duration therefore demonstrate the potential use of SLN-CRYP in the amelioration of seizures in NCC, especially in calcified neurocysticercosis. Studies have demonstrated that calcified neurocysticercosis is an important factor for development of drug resistant epilepsy (Rathore et al., 2013). The efficacy of SLN-CRYP may therefore be an indication of potential use in resistant epilepsy.

Although inflammation may be important in the destruction of cysticerci, poor regulation may exacerbate the condition. The implication of biological modulators produced by the host on inflammation is therefore key and the management of the levels of such may be essential in the resolution of Neurocysticercosis. In NCC, immune responses at the cellular level are characterized by increased levels of interferon-gamma,

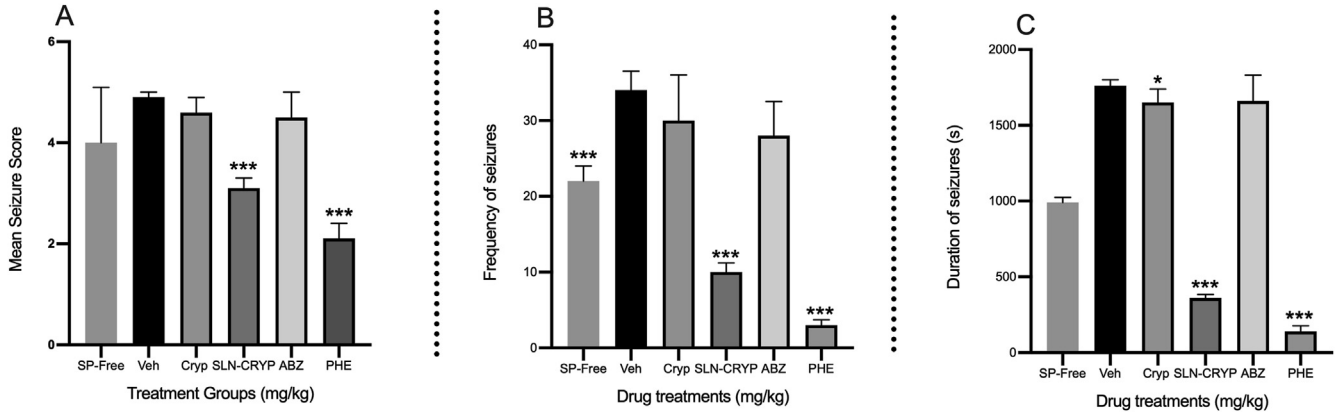

Fig. 3. A) Mean Seizure Score B) Frequency of seizures C) Duration of seizures in mice with *T. crassiceps* infection and treated with Cryp (5 mg/kg; p. o.); SLN-CRYP (5 mg/kg; p. o.); ABZ (50 mg/kg; p. o.); PHE (30 mg/kg; p. o.); and seizure-induced with SP. Data are presented as Mean ± S.E.M; ***p < 0.001 one-way analysis of variance followed by Tukey post-hoc test.

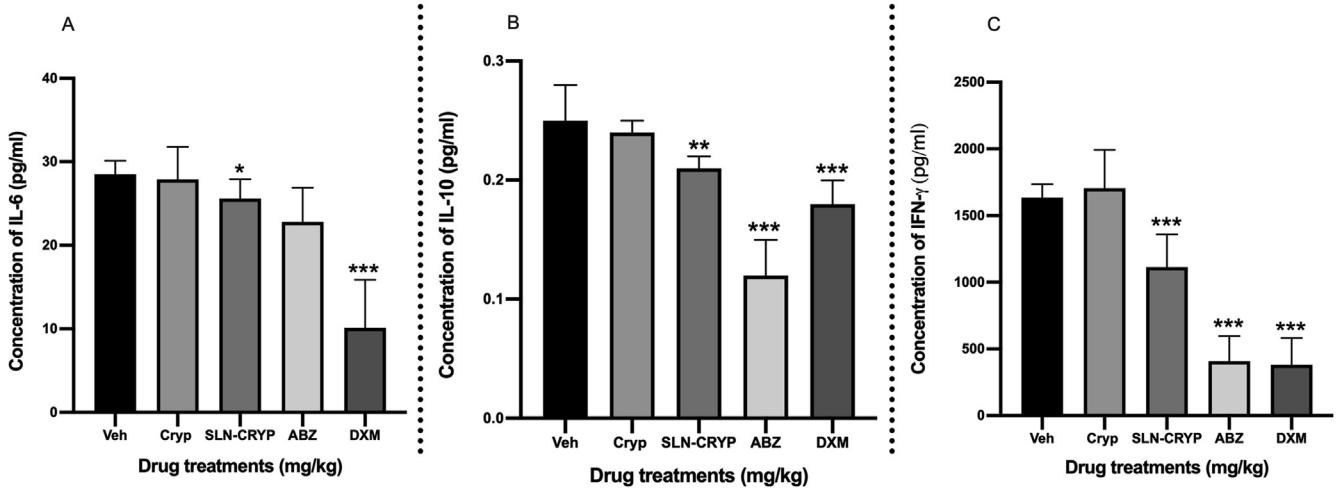

Fig. 4. Serum cytokine levels in mice brain homogenate infected with *T. crassiceps*. Mice were treated with Cryp 5 mg/kg; SLN-CRYP 5 mg/kg; ABZ 50 mg/kg; DXM 0.5 mg/kg. Data represent as Mean ± S.E.M; *p < 0.05, **p < 0.01, ***p < 0.001 one-way analysis of variance followed by Tukey post-hoc test.

IL-6 and IL-10 (Prodjinotho et al., 2020). An elevation in pro-inflammatory cytokines, IFN-γ and IL-6, is observed in several stages in the degeneration of the parasite (Fleury et al., 2016). According to (Arce-Sillas et al., 2016), production of high levels of interleukin 10 induced by regulatory T cell-mediated suppression of immune response creates a favorable immunomodulatory environment for *T. solium* development in the CNS. A decrease in the levels of these cytokines will therefore be critical in the management of NCC since the inflammatory process is also implicated in the pathogenesis of the disease. Our study demonstrated a significant reduction in the levels of IFN-γ, IL-6 and IL-10 and in the treatment of *T. crassiceps* - infected mice with SLN-CRYP. Cytokine reduction was however low in SLN-CRYP- treated mice relative to mice treated with dexamethasone and albendazole.

The general efficacy of SLN-CRYP in NCC, particularly in seizure alleviation, illustrated in this study has revealed its use as a potential therapeutic agent in the management of neurocysticercosis. SLN-CRYP may also be considered as an adjunct to conventional NCC therapy but further research on combination therapy is warranted to ascertain this.

4.1. Limitations

Given that infected mice exhibited spontaneous seizures after 90 days of *T. crassiceps* infection, observation of mice over the 28 days of treatment rather than an acute induction would have provided valuable data on seizure severity and seizure recurrence. This data is important with regards to epileptogenicity of *T. crassiceps* infection as well as pharmacological action of the various treatments in regards to epileptogenicity. Further investigation into the mechanisms by which SLN-CRYP acts in NCC is also of the essence.

5. Conclusion

Results of this study provide evidence for the usefulness of solid-lipid nanoparticle formulation of cryptolepine in the management of neurocysticercosis. Neurocysticercosis is of particular importance due to the neglected nature of the condition. Cryptolepine may present an opportunity for newer therapeutic options.

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CRediT authorship contribution statement

Priscilla Kolibea Mante: Conceptualization, Investigation, Methodology, Project administration, Formal analysis, Writing – original draft, Writing – review & editing. **Nana Ofori Adomako:** Methodology, Validation, Visualization, Writing – review & editing. **Paulina Antwi:** Methodology, Investigation, Writing – original draft. **Nana Kofi Kusi-Boadum:** Methodology, Formal analysis, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crphar.2021.100040.

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