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REPURPOSING CNS DRUGS ZONISAMIDE AND PERAMPANEL AS A POTENTIAL THERAPEUTIC AGAINST ACANTHAMOEBA-INDUCED GAE

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ABSTRACT:
A free-living, soil- and water-borne, biphasic, opportunistic FLA, **Acanthamoeba castellanii**, causes severe CNS infections especially in human being, such as GAE and pariculry eye infection like AK. Treatment challenges stem from the absence of safe and effective drugs, barrier-impaired entry into the central nervous system (i.e., the BBB), as well as the amoeba's cyst-forming ability. Most of the drug is currently under development, and there is limited information regarding appropriate dose or concentrations, administration schedule, timing parameters, therapeutic purpose as well as delivery pathway. Therefore, repurposing drugs is an ideal strategy for promptly addressing A. castellanii infection. This reseach sought to determine the effectiveness of two approved anti-epileptic drugs, Zonisamide (A) and perampanel (B) against T4-genotype A. castellanii in combating amoebic pathogens. Both drugs (A and B) exhibited potent antiamoebic activity with IC_{50}/24 hr values of 58.02 ± 1.22 and 57.155 ± 0.00 µg/mL. Tthey reduced cyst viability by 70% and 62%, respectively. Zonisamide inhibited phenotypic transformation significantly at 50 µM, whereas perampanel required higher concentrations. Both drugs effectively reduced host cell cytopathogenicity and demonstrated moderate toxicity towards HaCaT cells at lower concentrations. Amoebic morphological changes induced by drug treatment were observed using light microscopy during the assessment. These results showed that both drugs exhibit promising potential as repurposed drugs against AGE caused by A. castellanii, representing a significant advancement in drug development targeting all stages of the pathogen.

KEYWORDS: Acanthamoeba, Granulomatous Amoebic Encephalitis (GAE), Drug repurposing, in vitro, mode of action.

INTRODUCTION:

A widespread, microscopic, free living amoeba, A. castellanii found in environmental sources, is well known for infecting the CNS and the retina of animals, particularly human beings, leading to lethal and blinding conditions such as granulomatous amoebic encephalitis and Acanthamoeba keratitis, respectively. It is an opportunistic

protozoa widely distributed in various environments like sea water, swimming pools, tap water, and soil, with pathogenic species causing serious infections like blindness and brain/spinal cord infections [1], [2]. The biological characteristics, classification, disease, and pathogenesis of A.castellianii are key areas of study in understanding this organism. The life cycle of A.castellianii involves trophozoite and cyst stages, with trophozoites feeding on various particles and forming cysts in response to adverse conditions [3] [4].

GAE is an uncommon but extremely deadly infection of the CNS, often resulting in severe neurological damage and finally having a high mortality rate. A. castellianii-induced infections are typically marked by clinical signs such as cognitive disturbances, convulsions, and special focal neurological abnormalities. A. castellianii can enter the central nervous system through various routes, including the BBB and potentially the olfactory epithelium, leading to infecting especially immunocompromised individuals [5], including those with HIV/AIDS [6], organ transplant recipients, and patients with conditions like diabetes and lupus, but can also occur in immunocompetent individuals

GAE cases are often underdiagnosed due to the nonspecific nature of the symptoms and the rarity of the infection, requiring strong clinical suspicion and specialized laboratory techniques for early diagnosis and intervention. The current treatment regimen for GAE includes a combination of five or six drugs: pentamidine, sulfadiazine, flucytosine, fluconazole, and miltefosine, with the addition of a macrolide (azithromycin or clarithromycin) [1], [7]. However, these drugs pose several limitations, such as cysts being resistant to absorbing them, which increases the risk of recurrence, and the potential for host-cell toxicity from prolonged treatment. These drugs are delivered intravenously and needs to cross the blood-brain barrier in order to reach the target parasite in the brain [8],[9]. Along this process, the respective drugs administered at a therapeutic concentration, would pass through various tissues and potentially impacting their functions before reaching it's target. Therefore, repurposing already-approved drugs for disease targets offers a valuable strategy with the challenges faced in new drug discovery and obtaining clinical approval. Drug repurposing is a highly effective approach to drug development, as it significantly reduces research time, cuts costs, and minimizes the resources required to advance lead candidate drugs into clinical trials.

In this study, we present the outcomes associated with approved pharmacological therapies such as perampanel and zonisamide on A.castellianii. Zonisamide is an anticonvulsant primarily used in the treatment of epilepsy. It is been authorized as an add-on therapy, it targets partial-onset seizures in both children and adults populations. Zonisamide also has off-label uses, such as for migraine prevention, bipolar disorder, and Parkinson's disease [10]. The drug works by blocking sodium and calcium channels, thereby stabilizing neuronal membranes and reducing abnormal electrical activity in the brain. Patients with epilepsy, Parkinson's disease are recommended with initial dosage of 25 mg/day, up to 400 mg/day depending on the disease severity [10], [11], [12]. Perampanel is a medication used alongside other treatments to help control partial-onset seizures and generalized tonic-clonic seizures in people with epilepsy. It works by blocking AMPA receptors, which are important for fast signaling in the brain, to reduce excessive neuron activity [13]. Patients with epilepsy disease are recommended with initial dosage of 2 mg/day, up to 12 mg/day depending on the disease severity [14], [15]. Both of the drugs is legally sanctioned by the FDA and certified by the WHO for use as an adjunctive therapy under antiepileptic drugs list [16].

METHODS AND METHODOLOGY:

Chemicals.

Commercially sourced, analytically graded substances and regents were used through out this investigation. Well known consumable, Chlorhexidine, was purchased from Sigma-adrich, based in San Francisco, USA. Further chemicals used, like media basic components; tablets of phosphate buffered saline, yeast extract (Y), D-glucose (G), proteose peptones (P), dimethyl sulfoxide (DMSO) solvent and cryoprotectant, sodium dodecyl sulfate (SDS), and a trypan blue dye were supplied by Thermo Fisher Scientific, based in Massachusetts, USA. However, Elabscience, based in Texas, USA, provided Roswell Park Memorial Institute 1640 medium (RPMI-1640).

Acantamoeba castellanii Culture and Maintenence.

In present analysis a single A. castellanii was used. However, an important clinical isolate of T4-genotype that was provided by American type culture collection (50429), was used through out assessment in order to obtain a consistant results. The cuture of the said strain was maintained in a sterile freshly prepred PYG medium and maintained in 75 cm^2 clear bottom plastic culture flasks and placed in incubator environmented at 30 °C [17]. The cells were monitored regularly under microscope. When the flask were confent 90%, the cells were harvested either for subculture or biological assessments.

For subculturing, the trophozoites were harvested by placing flsk for 8 minutes on ice and tapped mechanically. The cells were centrifuged shortly and resuspended in sterile freshly prepared medium and distributed in two clear bottom flasks. However, for assessment, the trophozoites were detached by mechanical means as described earlier

and resuspended in RPMI in order to maintain the number of trophozoites. As the trophozoites can grow and divide in RPMI. Aspecific number of cel were used for 24 and 96-well clear bottom microtiter plates.

Culturing and maintenance of HaCaT cells.

In the present assessment, a single human normal cellline was use. The American type culture collection provided these cells. The HaCaT cells were freezed at negative196 °C. Before assessment, the cells were thawed quickly in water bath already environmted at 37 °C. The cryoprotectant was removed by mixing with pre-warmed freshly prepared sup-RPMI medium. Centrifuged the cells at 1000 xg and the pellet was mixed with fresh sup-RPMI.

The suspended cells were seeded in a cleas bottom plastic cell flask having an area of 75 cm^2. The cells were maintained in CO_2 gas incubator environmented at humidified 37 °C required for normal growth of human cells. The media was regularly changed after 2 days, after taking note of a medium colour. The cells were not disturbed until a monolayer of 90% confluent flask was obtained. For subculture and assessment of cytotoxicity assays, the cells were harved by enzymatic means. As mechanical means will destroyed the cells. Foe which cells were treated with trypsin at 37 °C for 4-5 minutes and transferred to falcon tubes. The cells were gently centrifuged and immediately transferred to 20 mL of sup–RPMI. The cells were distributed in 2 flasks and maintained at 37 °C. However, for assessment, the cells were resuspended in 23 mL medium, while 20 mL was usedfor seeding in 96-well microtiter plate and the remaining for flask. The flasks and microtiter plates were maintained in a humidified, CO_2 gas incubator [2].

Amoebicidal assay.

This assay was performed against A. castellanii using 24- and 96-wellmicrotiter clear bottom plates. The assay was performed to assess the efficacy of tested CNS drugs, zonisamide and perampanel against trophozoites as described earlier [18]. In short, a specific number of cells were enumerated and tranfered to microtiter plates. Whole assessment was performed in RPMI to maintain specific number through out experiment. This step act as preliminary screening of zonisamide and perampanel. The trophozoites were exposed to 25, 50, 75, 100, 150, and 200 µg/mL concentration of tested drugs. As for positive control, Chlorhexidine was used. However, DMSO was employed as solvent control, while keeping its concentration less than 2% in each well. Both control are necessary for accurate assessment of the efficacy of tested drugs. The challenged trophozoites were transferred to incubator for 24 hours. Trypan blue dye was used for counting the number of live cells appeared white under microscope, while compromised cells were appeared as dark purple. Haemocytometer was used fortis purpose.

The cells were also counted by microplate reader for which vibilty formulae was used given below.

$$\%\text{VIABILITY} = \frac{\text{ABSORBANCE OF CHALLANGED TROPHOZOITES (TC)}}{\text{ABSORBANCE OF CONTROL TROPHOZOITES (CC)}} \times 100$$

A. castellanii is bi-phasic amoeba. Depending on environmental condition one phase is alternative to anther phase. If condition are supportive for life, A. castellanii exist in an active and reproductive trophozoites stage. However, lack of food and harsh ecological conditions compel the trophozoites to transform in to cysts stage. This transformation process is known as encystation. After finding an appropriate contition, the cysts again physiologically transform in to trophozoites through excystation process, consequently leading to the full progression of its deelopmental cycle.

Trophozoites-to-Cysts assessment

As discussed in earlier section, the bi-phasic A. castellanii have a dormant form emerge from trophozoites. The conversion of trophozoites-to-Cysts is known as encystation. In short, assay was performed by conting trophozoites and seeded in 24- and 96-well plates. The wells contained PBS, $MgCl_2$, and high conent of glucose. These components compel the trophozoites to convert to cysts. The trophozoites were challenged with both zonisamide and perampanel at 25, 50, 75, 100, 150, and 200 µg/mL concentrations, while keeping the volume of DMSO less than 2%. The trophozoites were challenged for 72-hours in sterile incubator. Following treatment with SDS. Only fully matured cysts were computed for 1-2 minutes under microscope.

Cysts-to-trophozoites assessment

As discussed earlier, A. castellanii have two phenotypic form. The conversion of Cysts-to-trophozoites is known as excystation. For this assay the cysts were prepared by transferring the trophozoites from culture flask to agar plates. Due to harsh condition trophozoites were transformed to cysts in 15 days. The cysts were scraped and specific number of cysts were enumerated under microscope. Mature cysts were trasfrered to 24- and 96-well microtiter clear bottom plates. PYG medium was used to provide favourable environment to cysts to transform to trophozoites. The cysts then challenged with both zonisamide and perampanel at 25, 50, 75, 100, 150, and 200 µg/mL concentrations. As usual, DMSO and chlorhexidine were employed as solvent and positive controls, respectively. Cysts were placed in incubator environmented at 30 °C. Newly emerged trophozoites were stained with trypan blue dye and only trophozoites were counted under microscope.

Toxicological examinations.

LDH assay kit and one human cell line was used for this purpose. The normal cell line (HaCaT) was provided by American type culture collection. The HaCaT cells were seeded in 96-well microtiter clear bottom plastic plates and placed in CO_2 gas, properly humidified incubator, environmented at 37 °C. The monolayer was properly

monitored under microscope. A complete monolayer was challenged with zonisamide and perampanel at concentrations described above. The assay was performed in RPMI to maintain the effect of challenged drugs. After 24-hours incubation with tested drugs, the specified wells of HaCaT were challenged with Triton X-100 (1% octyl phenol ethoxylate) serverd as positive control. The remaining un-challenged wells were specified as untreated or negative control. Following the incubation, cell-free supernatants were collected from each well and the LDH kit was employed to determine the extent of LDH released by damaged or dead or compromised HaCaT cells. The reading was measured at 490 nm. The following formulae was used to determine viability or toxicity.

$$\% \text{ toxicity} = \frac{(A0 - NC0)}{(PC0 - NC0)} \times 100$$

(A0 = absorbance values of a treated cells), (NC0 = absorbance values of a Negative control), (PC0 = absorbance values of a positive control) The data are represented as the means and standard errors of various independent experiments performed in duplicate.

Determination of proposed Mode of action

Programmed cell death determination

The mode of action in A. castellanii was also detrmined for the tested drugs. A double stain method was used for this purpose. Hochest/PI 33342 was employed to stain DNA for florescence microscope. The trophozoites were preliminary challenged with zonisamide and perampanel at pre-determined IC_{50} values. The treated cells were washed with PBS and stained with Hochest/PI at 2-5 µg/mL as acclaimed by producer. The stain was diluted, fixed and washed with PBS. After specified time, the cells were visualized with correct filter set to visualize chromatin or nucleus under florescence microscope.

Reactive oxgen species determination

Intracellular ROS was determined inside treated cells. The ROS was calculated by the non-florescent and non polar 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) compound provided by Sigma Aldrich. For this, the stock solution of DCFH-DA was prepared using DMSO. A final concentration of 5 mM was used. The stok was store at negatve 20 °C.

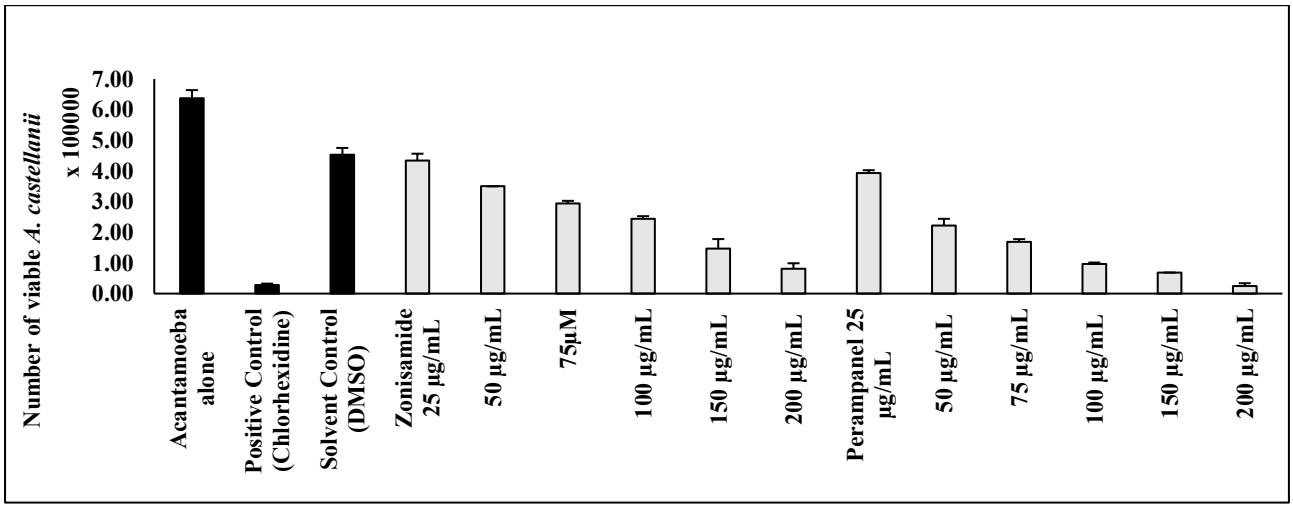
In short. The trophozoites were challenged with zonisamide and perampanel as described in amoebicidal section in 96-well microtiter clear bottom plates. After specified incubation, the DCFH-DA was diluted in appropriate culture medium to 10 µM. The trophozoites were incubated for 30 minutes in complete dark. The dye was gently aspirated and washed twice with PBS. A florescent micro plate reader was employed for recording of absorbance at excitation at ~485 nm and emission at ~535 nm.

For recoding of micrograph, florescent microscope was employed using a specified correct filter set to visualize ROS generated inside cell after challenging with drugs.

RESULTS:

3.1 CNS-targeting medication exhibited notable anti-amoebic effect.

Preliminary screening of both Zonisamide and Perampanel at different concentrations ranging from 25 – 200 µg/mL. **Figure 1A** depicted that the treatment with both drugs (Zonisamide and Perampanel) led to amarked reduction in the viability of A. castellanii, demonstrating significant amoebicidal effect.



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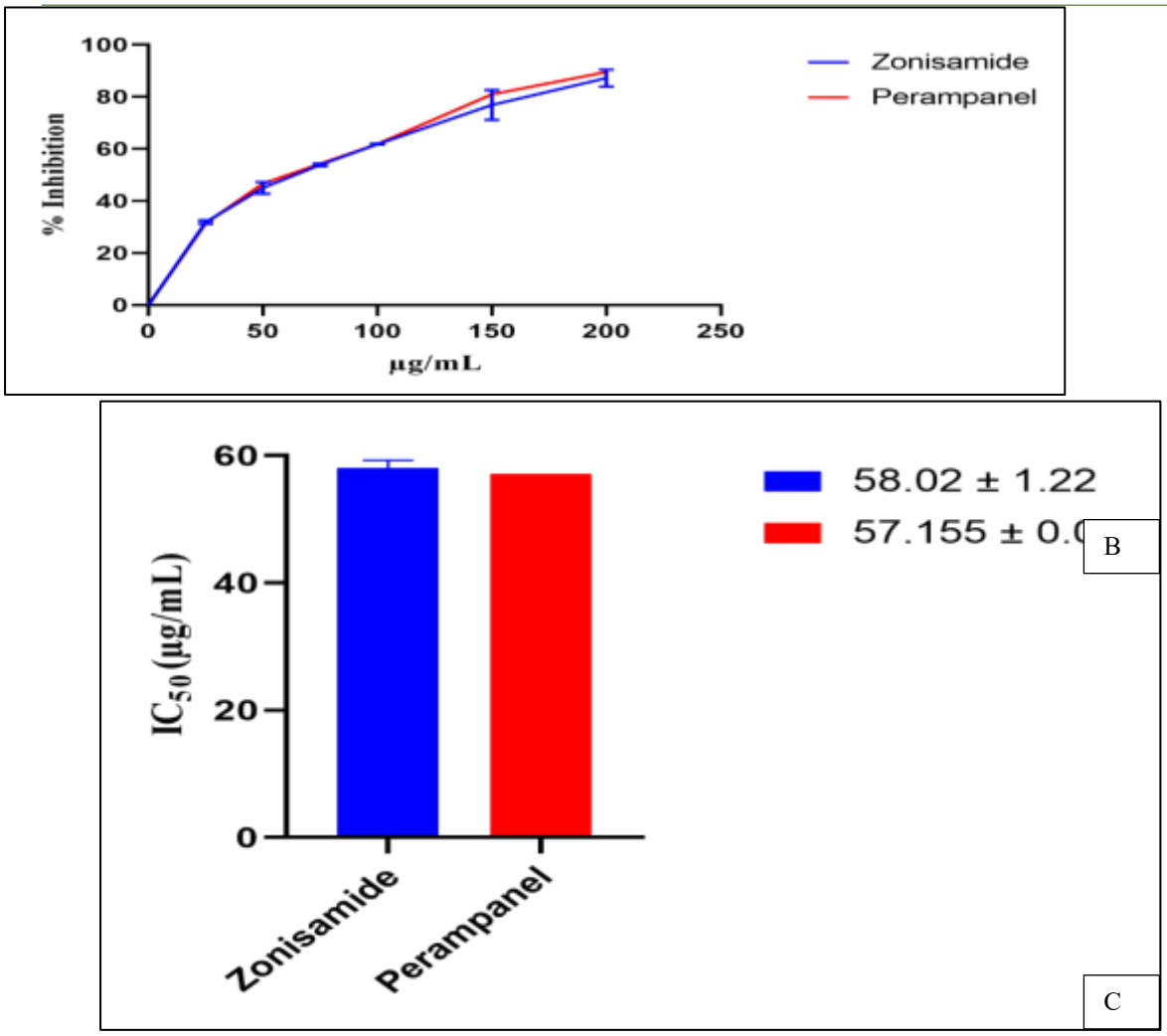

Figure 1. Exploring the amoebicidal properties of Zonisamide and perampanel on A. castellanii in its trophozoite stage. **(A)** Bar graph showing an anti-amoebic effect. Briefly, A. castellanii ($2.5X10^4$) trophozoites were treated with different concentrations of CNS drugs for 24 hr. **(B)** Illustrate the percent inhibition of Zonisamide and perampanel against A. castellanii trophozoites. The error bars showed the standard deviation. **(C)** Showed an IC_{50} values calculated after 24 hr treatment of both tested compounds at different tested concentrations.

Both Zonisamide and Perampanel demonstrated a significant dose-dependent reduction in cell viability. A. castellanii trophozites was initially counted at $5 X 10^5$ cells and after 24 hr it increased to 6.4×10^5 cells as shown in **Figure 1A**. In comparison to both CNS drugs, Perampanel at 200 µg/mL shows a significant reduction of viability to $2.5 X 10^4$ (96.04% inhibition of viability), as compared to the negative control and IC_{50} of 57.177 ± 0.000 µg/mL were recorded. As for Zonisamide, at 200 µg/mL, significantly reduced the viability to $8.1 X 10^4$ (87.18% inhibition of viability) and showed an IC_{50} of 58.02 ± 1.22 µg/mL was recorded (**Figure 1B and C**). Perampanel at 200 µg/mL shows a lower viability of A. castellanii in comparison to the positive control with a reduction in viability at $2.8 X 10^4$. The viability images of A. castellanii trophozoites against lowest and highest concentration of Parempanel and Zonisamide in a 24-well plate recorded at 200 X is illustrated in **Figure 1D**.

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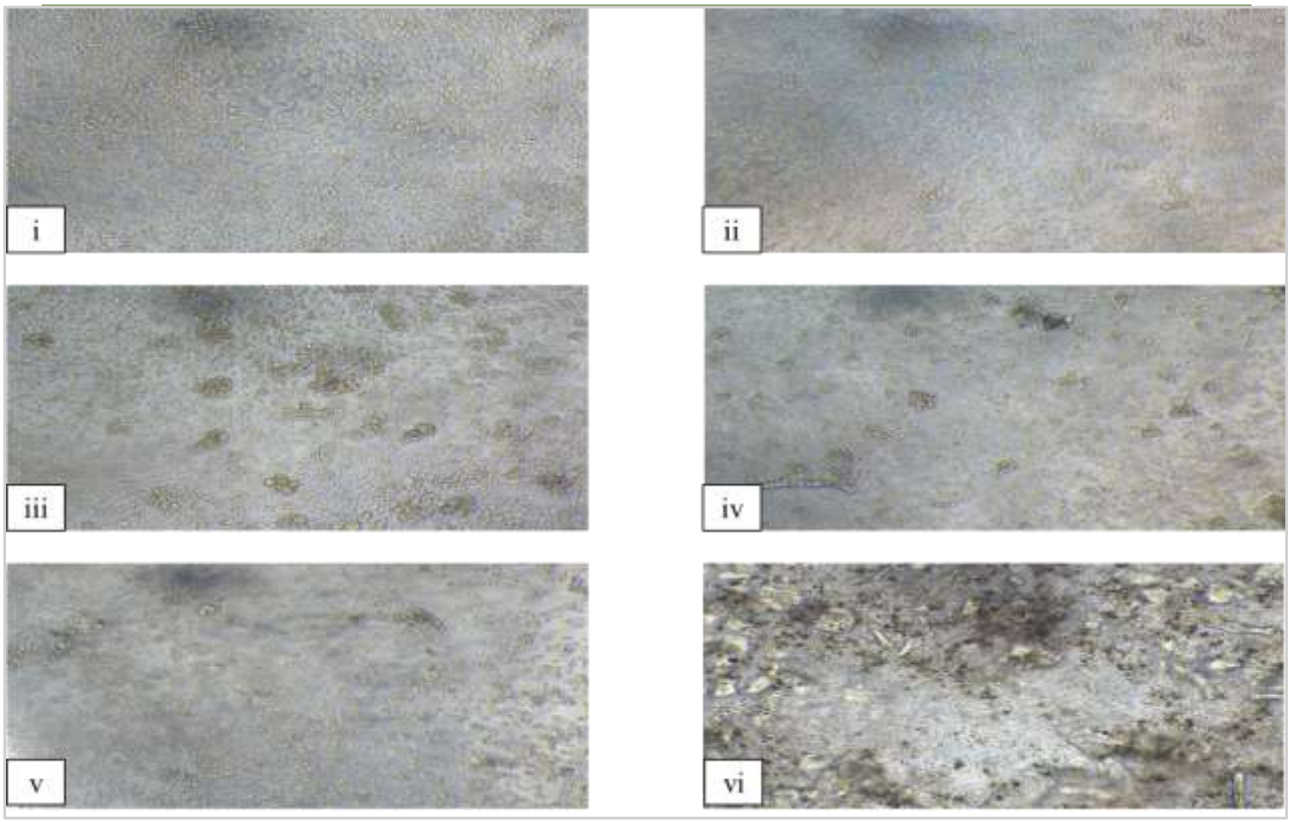


D

Figure 1D Illustrative images of individual wells from a 24-well plate recorded at 200 X, showing the effects of Zonisamide and Perampanel on the viability of A. castellanii. Negative control (i), solvent control (ii), trophozoites treated with 25µM of Zonisamide (iii), trophozoites treated with 25µM of Perampanel (iv), trophozoites treated with 200µM of Zonisamide (v), and trophozoites treated with 200µM of Perampanel (vi).

3.2 CNS drugs showed significant effects on the encystation of A. castellanii

The encystation assay was conducted to evaluate the inhibition of A. castellanii trophozoites from transforming into cysts. Zonisamide and Perampanel compounds showed a significant reduction of encystation at all tested concentrations as shown in Figure 2. As indicated by the findings, both CNS drugs significantly inhibited the encystation of A.castellanii with a minimum concentration of 25 µg/mL by more than 50% compared to the negative control. The viability at 25 µM for Zonisamide is 2.6 X 10^5 whereas for 25 µM of Parempanel, the viability is reduced to 2.9 X 10^5 in comparison to 7.2 X 10^5 trophozoites in negative control.

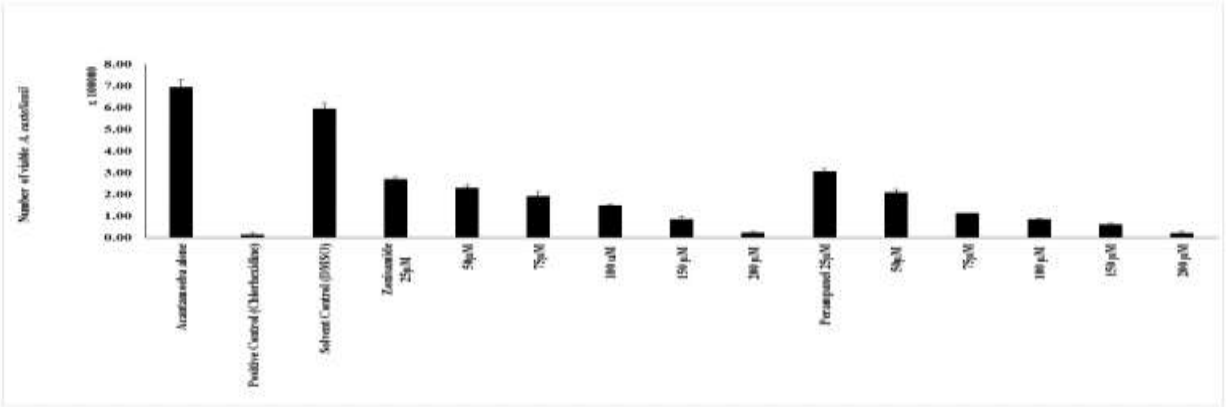


Figure 2 CNS drugs inhibited A. castellanii encystation. This bar chart depict how CNS drugs, Zonisamide as well as Perampanel, influence the phenotypic shift of FLA, i.e., A. castellanii. Take note that shift is from trophozoites-to-cysts. In summary, A. castellanii (1.0X10^5) was inoculated in encystation media and treated with

various concentrations of both CNS drugs to assess their influence on this morphological transition.

3.3 CNS drugs showed significant effects on the excystation of A. castellanii

The excystation assay was conducted to determine whether both Zonisamide and Perampanel compounds exhibited inhibitory effects on the conversion of A. castellanii cysts into trophozoites. According to the results, as illustrated on figure 3(a), both CNS drugs shows a strong inhibitory effect on excystation. Perampanel effectively reduced the emerging trophozoites from 2.3 X 10^5 cysts at 25 µg/mL to 9.0 X 10^3 cysts at 200 µg/mL in comparison to 5.2 X 10^5 cysts in negative control. As for Zonisamide, the emerging trophozoites were reduced from 3.5 X 10^5 at 25 µg/mL to 5.6 X 10^4 at 200 µg/mL in comparison to the negative control. The Perampanel compound effectively inhibited the conversion of cysts to trophozoites at a concentration of 200 µg/mL, which was significantly lower compared to the positive control, which resulted in 1.5 x 10^4 cysts. Figure 3(b) illustrate the images on phenotypic change of cysts to trophozites of A. castellanii treated with both Parempanel and

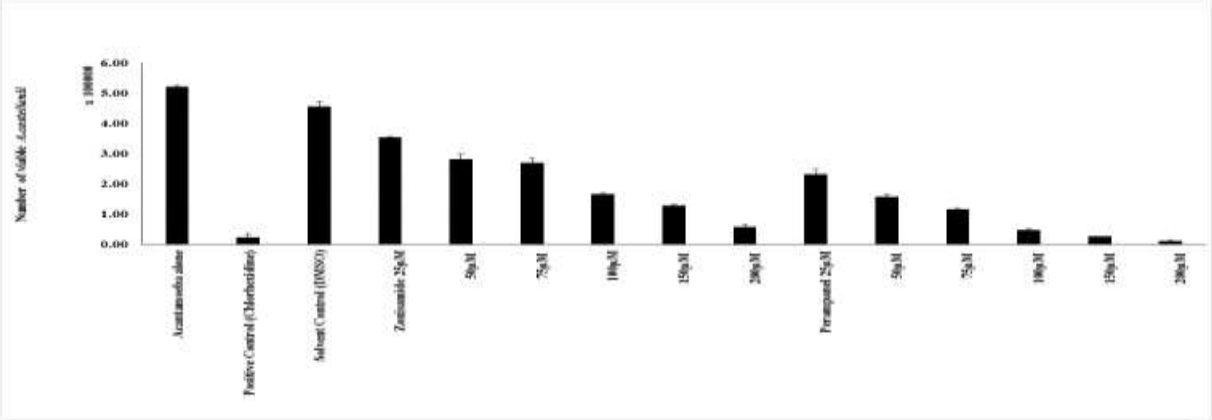


Figure 3(a) Excystation assay. The bar graph highlights the impact of CNS drugs, Zonisamide as well as Perampanel on morphological shift. In summary, the cuture was subjected to diffent concentrations (described is earlier sections) of each CNS drugs (25µM, 50µM, 75µM, 100µM, 150µM, and 200µM). Take note that the shift is from the cysts stage-to-trophozoite stage. The sign (*) was employed for indication of significane, which shows comparison between the treated cells with sovent-treated cells.

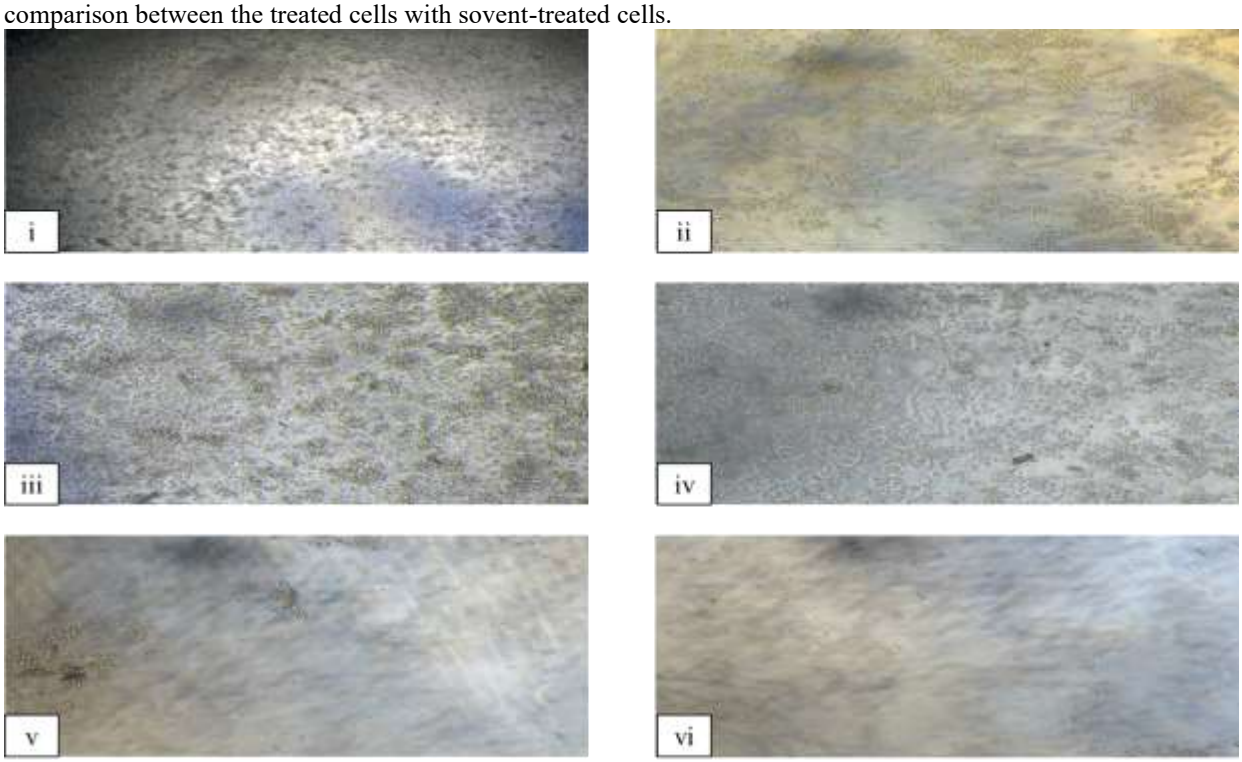


Figure 3B. These are visual microscopic representation of A.castellanii trophozoites captured in microtiter clear bottom plastic plates highlights the impact of treated drugs. The transition is from cysts_to_trophozoites. The experimental outfit include: (i) Negative control, (ii) solvent control, (iii) 25 µg/mL Zonisamide-treated cells, (iv)

25 µg/mL Perampanel-treated cells, (v) 200 µg/mL Zonisamide-treated cells, and finally (vi) 200 µg/mL Perampanel-treated cells.

3.4 CNS drugs showed moderate toxicity against HaCat cell lines

To determine an imminent cytotoxicity of both CNS drugs, Zonisamide and Perampanel, on human normal cels, (i.e., HaCaT cells) an LDH test was employed to demonstrate varying degrees of cell death at different concentrations. **Figure 4** revealed that Zonisamide at all the tested concentrations recorded minimal toxicity, i.e., ≥ 40 %, compared to the Perampanel compound illustrating a minimal toxicity, 30% and 39% at lower tested concentrations, 25 µg/mL and 50 µg/mL, respectively.

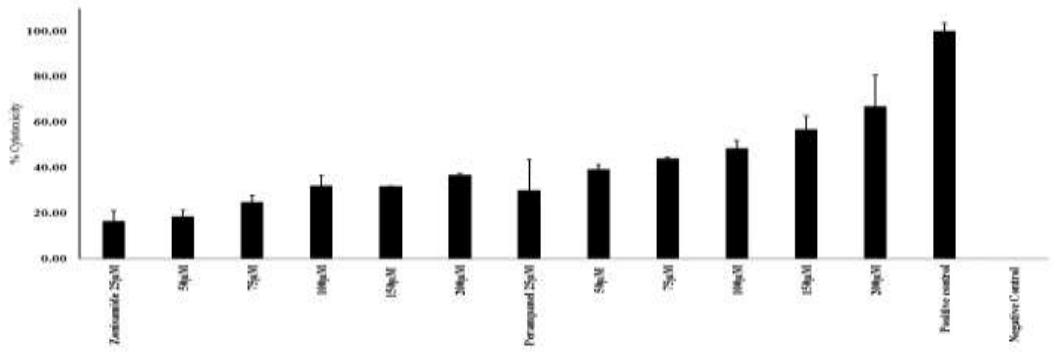


Figure 4: In vitro cytotoxicity induced by CNS drugs against HaCaT cell line. The bar graph represents potential toxic impact of Zonisamide and Perampanel on human normal cell line (HaCaT cells). The human cell line was subjected to different concentrations of each CNS drug. The impact of drugs were increasing dose-dependently, as depicted by bars. However, no impact was documented for negative-control. Note that imact was recorded after 24 hours.

Mode of action

Both Zonisamide and Perampanel treatment induced programmed cell death (PCD)

The mode of action of cell death induced by Zonisamide and Perampanel in Acanthamoeba trophozoites was observed using a Hoechst 33342/Propidium Iodide (PI) double staining kit. The florescence micrograph illustrated in **Figure 5** proved that treatment of both compounds trigger brogrammed cell death (PCD) in A. castellanii trophozoites. After treatment of trophozoites with highest concentration (200 µg/mL) of compounds for 6 hr, the micrograph showed that dark blue florescence was observed in treatment group. While a low or fainted blue florescence was obsrvered in neative control, indicating healthy cells. The only dark blue and red color in merged section showed programmed cell death (apoptosis), while the only red color indicate the dead cells.

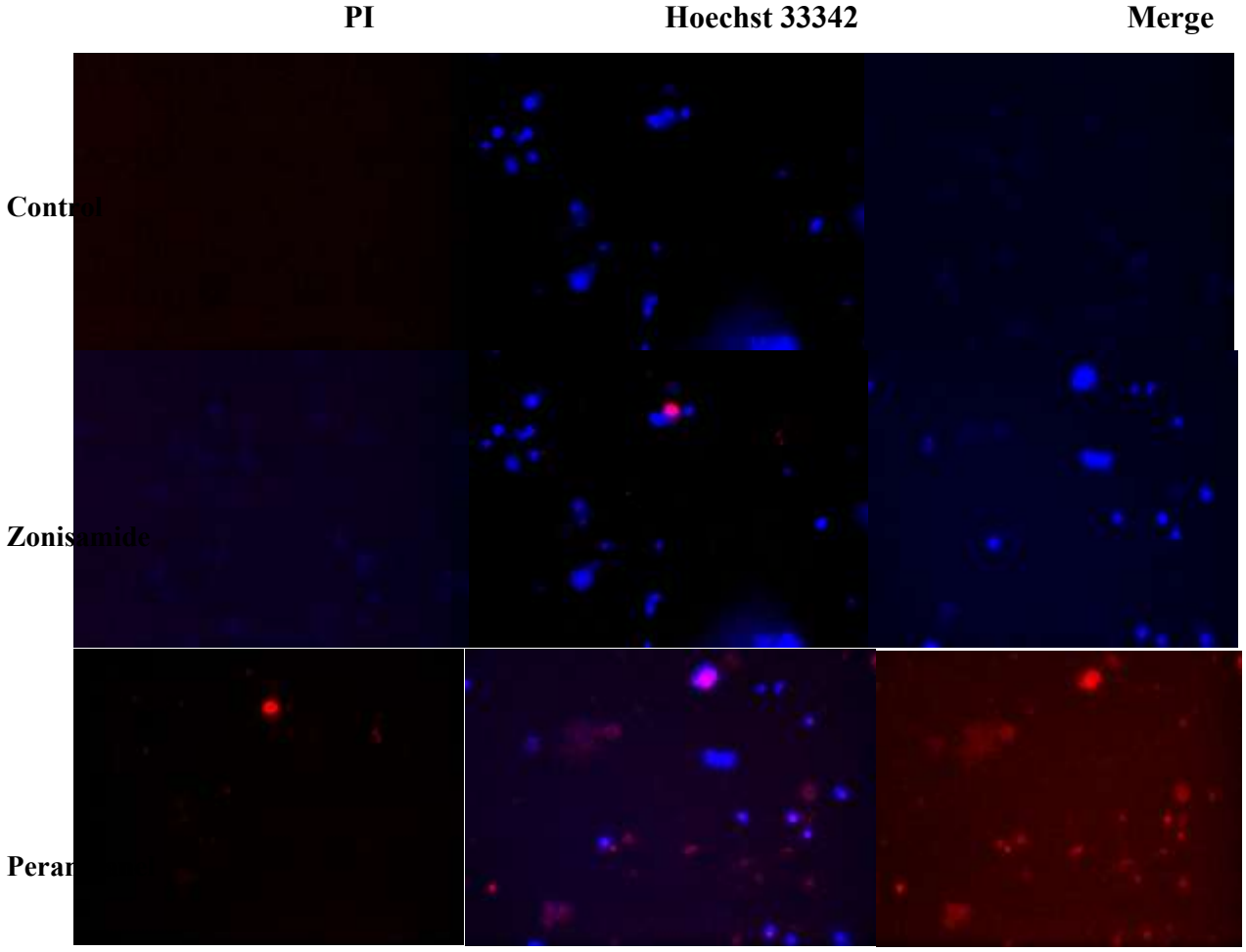
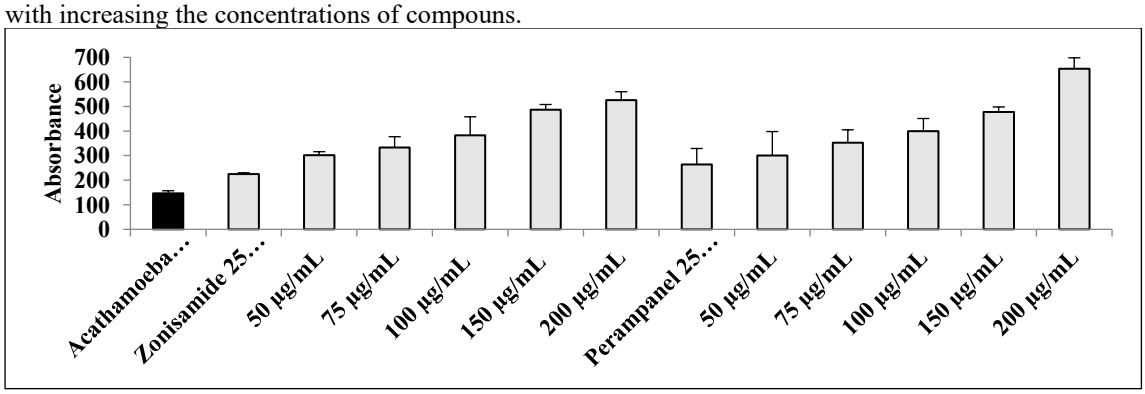
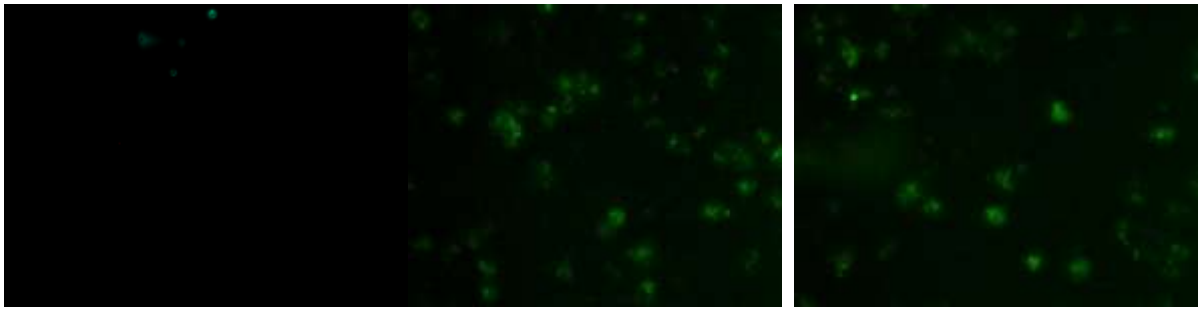


Figure 5: Shows florescence microscopy images of Acanthamoeba trophozoites after treatment with highest tested concentration (200 µg/mL) of Zonisamide and Perampanel. The light blue indicate healthy cell, the blue and red showed apoptotic cell and red indicate dead cells.

Treatment of both compounds significantly enhanced the level of ROS

In order to investigate the mode of action by which both the tested compounds (Zonisamide and Perampanel) induced PCD (apoptosis) in A. castellanii trophozoites, intracellular level of ROS was measured by convertion non florescent DCF-DA in to florescent product inside cells. The florescent was measured using microplate reader. **Figure 6A** illustrate the intracellular level of ROS generated inside trophozoites after treatment with both compounds at different concentration. The bar grap showed that both Zonisamide and Perampanel significantly enhanced the level of ROS dose dependently after 24 hrs incubation. Florescence microscopy was performed to visualize the intrcellular ROS generated after treatment with compounds. The trophozoites were treated with highest concentration (200 µg/mL) of compounds for 24 hrs and visualize under florescence microscopy. **Figure 6B** further confirmed the elevated level of ROS generated inside the cells after treatment as compared to negative control, where low or no florescence was observed. In treated group, elevated dark green florescence was observed with increasing the concentrations of compouns.





Control **Zonisamide** **Perampanel**

Figure 6: Illustrate the effect of Zonisamide and Perampanel on the intracellular level of ROS after 24 hr treatment. **(A)** The histogram showed that the level of ROS elevated with increasing concentration of compounds concentrations. **(B)** The florescence micrograph visualize the level of ROS generated inside cell after treatment with highest concentration (200 µg/mL) of both compounds. The dark green color illustrate the elevated level of ROS as compare to negative control. A low level of ROS was generated in negative control.

4. DISCUSSION:

Members of the Acanthamoeba genus are considered facultative pathogens because they can infect vulnerable hosts and cause disease. GAE is a life-threatening infection of the brain and spinal cord, primarily affecting individuals with weakened immune systems [19]. It also impacts those with pre-existing health conditions such as diabetes, HIV, pneumonitis, and lupus erythematosus [20]. Finding antiamoebic drugs that are both safe and effective remains challenging. Although research over the past few decades has led to the development of numerous compounds with medicinal potential, their translation into practical treatments has not been fully explored [21].

To current date, many independent laboratories have identified several molecular targets, promising drugs, and lead compounds for synthesizing derivatives. However, these efforts have not captured the interest of major pharmaceutical companies, whose involvement is essential to fund expensive in vivo studies and clinical trials. This is consistent with the pharmaceutical industry's general lack of interest in developing treatments for parasitic diseases [22]. Current treatments for Acanthamoeba infections involve a combination of various medicinal compounds, including amidines, azoles, biguanides, and others [18]. However, most of these substances have shown significant efficacy only against the trophozoite stage of Acanthamoeba [23] and tend to be toxic to human cells at therapeutic doses.

The purpose of our research is to identify compounds that have not previously been reported to show activity against pathogenic amoebae and to potentially repurpose these approved drugs for the treatment of various amoebic diseases. Both Parempanel and Zonisamide are clinically used to treat central nervous system (CNS) related diseases such as epilepsy, Alzheimer's and Parkinson's [11], [15]. Parempanel showed potent antiemetic activity against A.castellanii trophozoites compared to Zonisamide drug in comparison to similar concentrations. The IC_{50} value is 57.155 µg/mL at 24 hr for Parempanel and 58.02 µg/mL at 24 hr for Zonisamide. In another study, both the CNS drug shows potential antitumoral effects [24], [25], [26] tested with glioma cells, which significantly decreases its viability.

Zonisamide functions as a blocker of the voltage-gated calcium and sodium channel. This causes stabilisation of cell membrane and prevents overexcitability [27]. They also change the pH, resulting in the inhibition of the carbonic anhydrase pathway [27]. Other amide-grouped drugs have reported this mode of action and inhibit the same carbonic anhydrase pathway when used on A.castellanii, resulting in the inhibition of trophozoite viability [28]. Here, it was hypothesised that zonisamide inhibition may have changed the pH in Acanthamoeba, resulting in affecting of trophozoite metabolism [28]. As Acanthamoeba rely on optimal pH for normal function, its alteration may affect the growth and viability of trophozoites [29]. So, it is also supposed that zonisamide may have blocked this pathway in A. castellanii trophozoites. This is reflective of the empirical data shown as the specificity of the drug lies in its ability to affect the Acanthamoeba carbonic anhydrase pathway, effectively suffocating the cell while leaving the anaerobic HaCat cells unbothered [29]. The anti-microbial potential of the zonisamide may be suggested due to the sulfonamide-related structure, which inhibits the folate synthesis in bacteria [30]. Acanthamoeba also has this pathway, and it is supposed that the compounds have also used the folate pathway as a potential avenue for action against Acanthamoeba castellanii.

Similarly, the primary mode of action of parampanel is the non-competitive AMPA receptor antagonism. They bind to the allosteric site on the AMPA receptor and inhibit its function without blocking glutamate binding [31]. The structure of perampanel showed that it contains N and O in its structure structure [32]. This may affect its

activities. The large number of N ions in perampanel and some empirical data suggested that a high concentration of perampanel may cause cytotoxicity and low cell viability in certain types of cells such as HaCat and A.castellanii as it is hypothesised that this cytotoxicity may cause oxidative stress mechanism [33], [34]. In the present study, we also showed that this compound increased the oxidative stress of A. castellanii. However, further investigation is necessary to correlate perampanel, redox imbalance, and apoptosis in Acanthamoeba castellanii.
Numerous studies have reported that both natural and synthetic compounds have been evaluated against the viability of A. castellani. However, most of them are unable to inhibit the cyst stage, resulting in the reoccurrence of infection. Most of the previously tested compounds and drugs are unable to cross BBB and also showed side effects. The majority of the compounds have no proposed mode of action [35]. Therefore, both Zonisamide and Perampanel were also evaluated for their possible mode of action. Various types of cell death, such as programmed cell death (PCD), autophagy, necrosis, etc., are proposed in different pathogens, including Acanthamoeba [36]. The PCD involves chromatin condensation, loss of mitochondrial membrane potential, decrease of ATP, and morphological changes [36], [37]. Apoptosis is the preferred mode of action in treated Acathamoeba as compared to necrosis [38]. The previous studies showed that natural compounds induced apoptosis in Acanthamoeba [36], [37]. Therefore, the present study used a double-stain Hoechst 33342/PI apoptosis kit to evaluate cell death. The analysis of the micrography showed that both tested compounds induced apoptosis in trophozoites. The apoptotic cell appeared to be dark blue with a light red color. Meanwhile, the healthy cells appear to be a light blue colour. The only dark red colour indicates dead cells. The Hoechst 33342 has an ability to penetrate the intact membranes of Acanthamoeba, while PI entered the compromised cell membranes of A. castellanii and bind to the nucleic acids [39].
The treatment often leads to oxidative stress, which is often characterized by increased levels of reactive oxygen species (ROS) [40]. In this study, the treatment of Zonisamide and Parempanel increased the level of ROS dose-dependently compared to the negative control. These elevated levels of ROS affect other proteins, DNA, mitochondrial dysfunction, and other cell components, resulting in cell apoptosis. In the present study, our findings clarify and elucidate programmed cell death (PCD) events in Acanthamoeba caused by two CNS drugs (Zonisamide and Parempanel) and offer a comprehensive framework for understanding the molecular mechanisms by which therapeutic agents may act against Acanthamoeba and other pathogenic free-living amoebae. These compounds can be further assessed in vivo and in vitro as they can cross the BBB. They have shown potential against both stages of Acanthamoeba and inhibit phenotypic transformation. This will help inhibit the reoccurrence of infection. Therefore, these compounds are highly recommended to be used as a potential compound for the treatment of GAE and AK infections casused by Acanthamoeba castellanii.

5. CONCLUSION

In this study, two CNS compounds, Zonisamide and Perampanel were evaluated against both stages of Acanthamoeba castellanii. Remarkebly, these compounds also demonstrated the potential to inhibit the phenotypic transformation of A. castellanii. Since both compounds have an ability to cross the BBB and exhibit potent anti-amoebic potential against trophozoites and cysts, they are highly recommended to be use for the treatment of GAE infection. Furthermore, at the tested concentrations, these compounds were also non-toxic to normal human cell lines. Mechanistic analysis revealed that they induce apoptosis (PCD) in trophozoites by increasing the level of ROS, without compromising the membrane integrity of trophozoites. This suggest that their use in the treatment of GAE infection would not provoke inflammation in human. This will offer a fast and cost-effective approach which will accelerate the treatment of an emerging GAE infection by leveraging known compounds with both stage potential and low or no toxicity.

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8. Data Availability: The key findings and data are presented completely in this report. Other correlated data will be provided if further invstigation required.
9. Conflict of interest: The authors affirm no probable interest to reveal in relation to these findings.

10. REFERENCES:

[1] X. T. Chen, Q. Zhang, S. Y. Wen, F. F. Chen, and C. Q. Zhou, “Pathogenic free-living amoebic encephalitis from 48 cases in China: A systematic review,” Front Neurol, vol. 14, no. 1, 2023, doi: 10.3389/fneur.2023.1100785.

[2] C. Bunsuwansakul et al., "Acanthamoeba in southeast Asia – Overview and challenges," Korean Journal of Parasitology, vol. 57, no. 4, pp. 341–357, 2019, doi: 10.3347/kjp.2019.57.4.341.
[3] S. Sangkanu et al., "Efficacy of propolis extract and eye drop solutions to suppress encystation and excystation of Acanthamoeba triangularis WU19001-T4 genotype," PeerJ, vol. 12, no. 4, pp. 1–23, 2024, doi: 10.7717/peerj.16937.
[4] R. Boonhok et al., "Amoebicidal activity of Cassia angustifolia extract and its effect on Acanthamoeba triangularis autophagy-related gene expression at the transcriptional level," Parasitology, vol. 148, no. 9, pp. 1074–1082, 2021, doi: 10.1017/S0031182021000718.
[5] D. Das et al., "Unfurling a case of encephalitis with Acanthamoeba after a near-drowning event," Rare, vol. 2, no. November 2023, p. 100035, 2024, doi: 10.1016/j.rare.2024.100035.
[6] Q. Jiang, Z. Zhang, Y. Cai, L. Chen, L. Deng, and Y. Xiong, "Disseminated Acanthamoeba castellanii infection in a patient with AIDS: a case report and literature review," Front Med (Lausanne), vol. 11, no. June, pp. 1–7, 2024, doi: 10.3389/fmed.2024.1377302.
[7] H. M. Elsheikha, R. Siddiqui, and N. A. Khan, "Drug discovery against acanthamoeba infections: Present knowledge and unmet needs," Pathogens, vol. 9, no. 5, pp. 1–17, 2020, doi: 10.3390/pathogens9050405.
[8] S. da L. Borges et al., "Experimental Granulomatous Amebic Encephalitis Caused by Acanthamoeba castellanii," Trop Med Infect Dis, vol. 9, no. 7, p. 145, 2024, doi: 10.3390/tropicalmed9070145.
[9] S. K. Kalra, P. Sharma, K. Shyam, N. Tejan, and U. Ghoshal, "Acanthamoeba and its pathogenic role in granulomatous amebic encephalitis," Exp Parasitol, vol. 208, p. 107788, 2020, doi: 10.1016/j.exppara.2019.107788.
[10] B. E. Gidal, T. Resnick, M. C. Smith, and J. W. Wheless, "Zonisamide: A Comprehensive, Updated Review for the Clinician.," Neurol Clin Pract, vol. 14, no. 1, p. e200210, 2024, doi: 10.1212/CPJ.0000000000200210.
[11] M. Essam, E. Hamid, E. Abushady, M. El-Balkimy, A. Antonini, and A. Shalash, "Role of zonisamide in advanced Parkinson's disease: a randomized placebo-controlled study," Neurological Sciences, vol. 45, no. 4, pp. 1725–1734, 2024, doi: 10.1007/s10072-024-07396-w.
[12] M. Murata et al., "Effect of zonisamide on parkinsonism in patients with dementia with Lewy bodies: A phase 3 randomized clinical trial," Parkinsonism Relat Disord, vol. 76, no. November 2018, pp. 91–97, 2020, doi: 10.1016/j.parkreldis.2019.12.005.
[13] A. Lavu et al., "Efficacy and safety of perampanel in epilepsy: A systematic review and meta-analysis of randomised controlled trials," Seizure, vol. 102, no. June, pp. 54–60, 2022, doi: 10.1016/j.seizure.2022.09.020.
[14] P. Liu, Z. Zhu, and H. Wu, "The safety of perampanel in different disorders and doses: A meta-analysis," Seizure, vol. 106, no. 348, pp. 22–28, 2023, doi: 10.1016/j.seizure.2023.01.013.
[15] C. Y. Lin et al., "Therapeutic drug monitoring of perampanel: Clinical utility and impact of co-medication on pharmacokinetic variability," Heliyon, vol. 10, no. 1, p. e23962, 2024, doi: 10.1016/j.heliyon.2023.e23962.
[16] D. J. Cada, T. L. Levien, and D. E. Baker, "Perampanel," Hosp Pharm, vol. 48, no. 4, pp. 321–331, 2013, doi: 10.1310/hpj4804-321.
[17] A. Anwar, K. Rajendran, R. Siddiqui, M. Raza Shah, and N. A. Khan, "Clinically Approved Drugs against CNS Diseases as Potential Therapeutic Agents to Target Brain-Eating Amoebae," ACS Chem Neurosci, vol. 10, no. 1, pp. 658–666, 2019, doi: 10.1021/acschemneuro.8b00484.
[18] U. Ahmed et al., "Malabaricones from the fruit of Myristica cinnamomea King as potential agents against Acanthamoeba castellanii," Acta Trop, vol. 248, no. October, p. 107033, 2023, doi: 10.1016/j.actatropica.2023.107033.
[19] R. L. Rodríguez-Expósito et al., "Induction of Programmed Cell Death in Acanthamoeba culbertsoni by the Repurposed Compound Nitroxoline," Antioxidants, vol. 12, no. 12, 2023, doi: 10.3390/antiox12122081.
[20] K. M. Zamli et al., "Synthesis, anti-amoebic activity and molecular docking simulation of eugenol derivatives against Acanthamoeba sp.," Saudi Pharmaceutical Journal, vol. 31, no. 9, p. 101703, 2023, doi: 10.1016/j.jsps.2023.101703.
[21] N. Spottiswoode et al., "Challenges and advances in the medical treatment of granulomatous amebic encephalitis," Ther Adv Infect Dis, vol. 11, pp. 1–14, 2024, doi: 10.1177/20499361241228340.
[22] S. P. S. Rao, U. H. Manjunatha, S. Mikolajczak, P. G. Ashigbie, and T. T. Diagana, "Drug discovery for parasitic diseases: powered by technology, enabled by pharmacology, informed by clinical science," Trends Parasitol, vol. 39, no. 4, pp. 260–271, 2023, doi: 10.1016/j.pt.2023.01.010.

[23] A. Abdullah Hamad, "In vitro Evaluation the Efficacy of Some Nnew Plant Extracts and Biocides on the Viability of Acanthamoeba castellanii," Protist, vol. 174, no. 3, p. 125966, 2023, doi: 10.1016/j.protis.2023.125966.
[24] J. Tatsuoka et al., "Anti-tumor effects of perampanel in malignant glioma cells," Oncol Lett, vol. 24, no. 6, pp. 1–9, 2022, doi: 10.3892/ol.2022.13541.
[25] H. B. Newton and J. Wojkowski, "Antiepileptic Strategies for Patients with Primary and Metastatic Brain Tumors," Curr Treat Options Oncol, vol. 25, no. 3, pp. 389–403, 2024, doi: 10.1007/s11864-024-01182-8.
[26] E. K. Avila et al., "Brain tumor-related epilepsy management : A Society management," vol. 26, no. September, pp. 1–18, 2023.
[27] I. E. Leppik, "Zonisamide: Chemistry, mechanism of action, and pharmacokinetics," Seizure, vol. 13, no. SUPPL. 1, Dec. 2004, doi: 10.1016/j.seizure.2004.04.016.
[28] S. Haapanen, H. Barker, F. Carta, C. T. Supuran, and S. Parkkila, "Novel Drug Screening Assay for Acanthamoeba castellanii and the Anti-Amoebic Effect of Carbonic Anhydrase Inhibitors," J Med Chem, vol. 67, no. 1, pp. 152–164, Jan. 2024, doi: 10.1021/ACS.JMEDCHEM.3C01020.
[29] R. Siddiqui and N. A. Khan, "Biology and pathogenesis of Acanthamoeba," Parasit Vectors, vol. 5, no. 1, pp. 1–13, Jan. 2012, doi: 10.1186/1756-3305-5-6/FIGURES/6.
[30] A. Tacic, V. Nikolic, L. Nikolic, and I. Savic, "Antimicrobial sulfonamide drugs," Advanced technologies, vol. 6, no. 1, pp. 58–71, 2017, doi: 10.5937/SAVTEH1701058T.
[31] A. Kamalova and T. Nakagawa, "AMPA receptor structure and auxiliary subunits," J Physiol, vol. 599, no. 2, pp. 453–469, Jan. 2021, doi: 10.1113/JP278701.
[32] M. A. Rogawski and T. Hanada, "Preclinical pharmacology of perampanel, a selective non-competitive AMPA receptor antagonist," Acta Neurol Scand Suppl, vol. 127, no. 197, pp. 19–24, 2013, doi: 10.1111/ANE.12100.
[33] F. Brockhaus and B. Brüne, "U937 apoptotic cell death by nitric oxide: Bcl-2 downregulation and caspase activation," Exp Cell Res, vol. 238, no. 1, pp. 33–41, Jan. 1998, doi: 10.1006/EXCR.1997.3778.
[34] A. Manna et al., "Malabaricone-A Induces A Redox Imbalance That Mediates Apoptosis in U937 Cell Line," PLoS One, vol. 7, no. 5, p. e36938, May 2012, doi: 10.1371/JOURNAL.PONE.0036938.
[35] U. Ahmed, A. Anwar, S. K. Ong, A. Anwar, and N. A. Khan, "Applications of medicinal chemistry for drug discovery against Acanthamoeba infections," Med Res Rev, vol. 42, no. 1, pp. 462–512, Jan. 2022, doi: 10.1002/MED.21851.
[36] U. Ahmed et al., "Effect of embelin on inhibition of cell growth and induction of apoptosis in Acanthamoeba castellanii," Arch Microbiol, vol. 205, no. 12, Nov. 2023, doi: 10.1007/S00203-023-03698-3.
[37] U. Ahmed et al., "Alpha-Mangostin and its nano-conjugates induced programmed cell death in Acanthamoeba castellanii belonging to the T4 genotype," Int Microbiol, vol. 27, no. 4, pp. 1063–1081, Aug. 2024, doi: 10.1007/S10123-023-00450-1.
[38] S. Elmore, "Apoptosis: A Review of Programmed Cell Death," Toxicol Pathol, vol. 35, no. 4, p. 495, 2007, doi: 10.1080/01926230701320337.
[39] A. M. Rieger, K. L. Nelson, J. D. Konowalchuk, and D. R. Barreda, "Modified Annexin V/Propidium Iodide Apoptosis Assay For Accurate Assessment of Cell Death," J Vis Exp, no. 50, p. 2597, 2011, doi: 10.3791/2597.
[40] C. Fleury, B. Mignotte, and J. L. Vayssière, "Mitochondrial reactive oxygen species in cell death signaling," Biochimie, vol. 84, no. 2–3, pp. 131–141, 2002, doi: 10.1016/S0300-9084(02)01369-X.