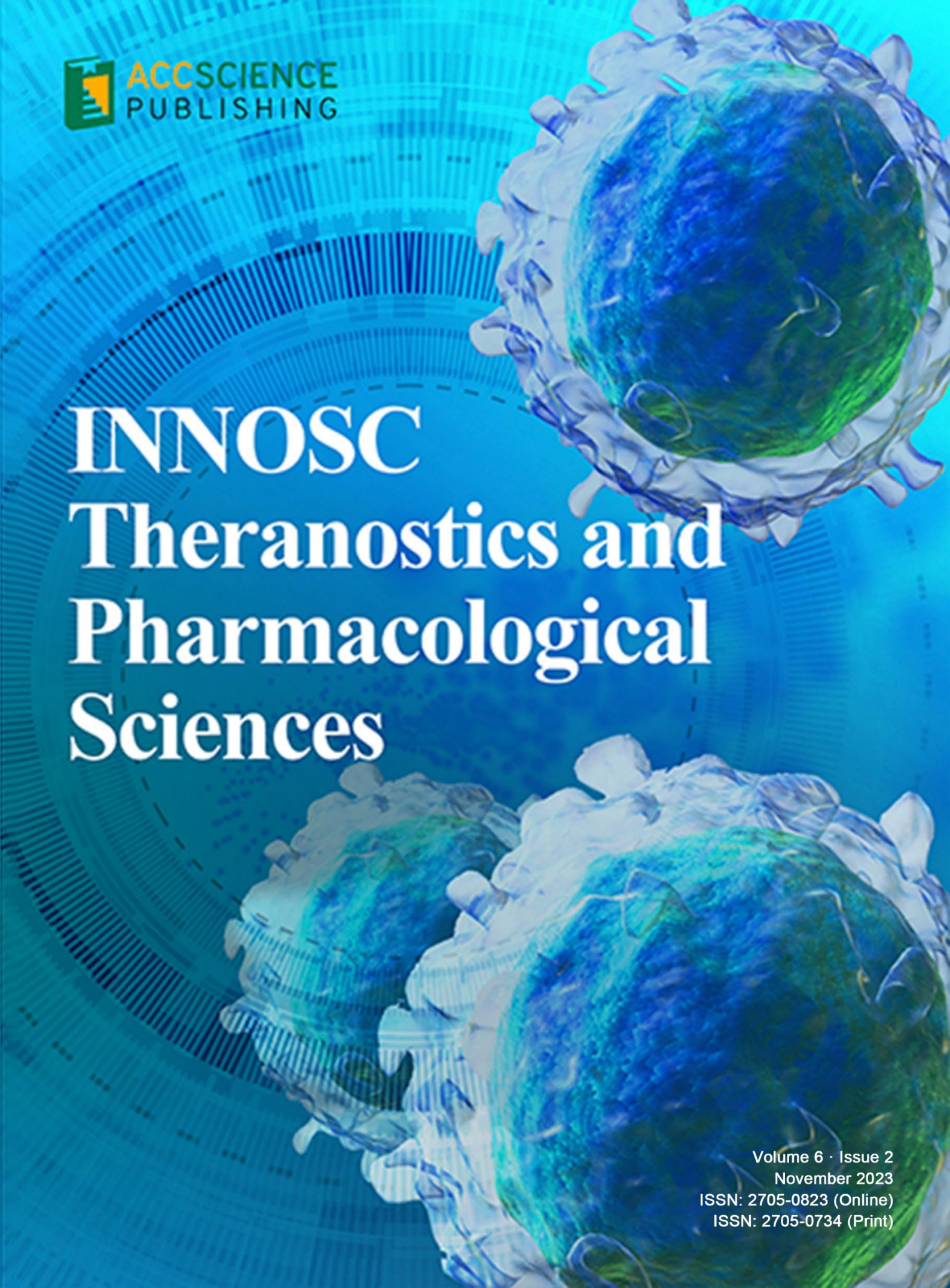

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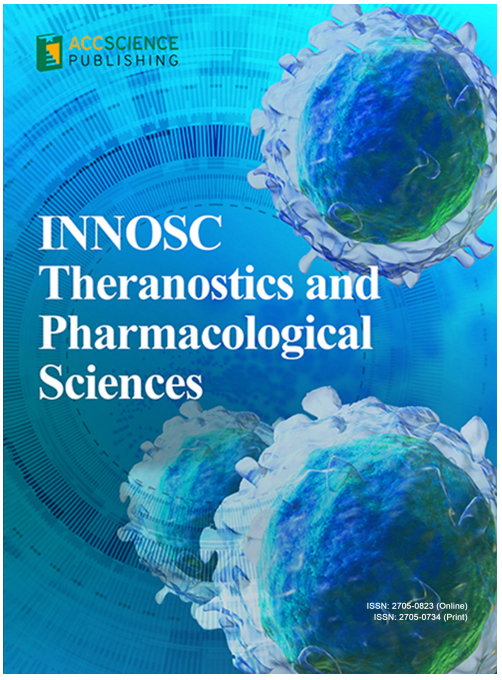

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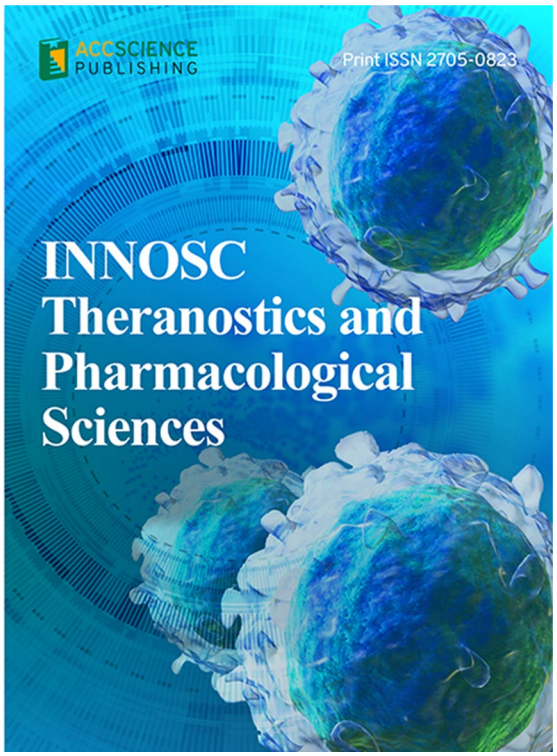

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REVIEW ARTICLE

Precision medicine in neurosurgery: The evolving role of theranostics

Drashti Patel, Andrew Nguyen, Chance Fleeting, Anjali B. Patel, Mohammed Mumtaz, and Brandon Lucke-Wold*

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Abstract

Theranostics in neurosurgery is a rapidly advancing field of precision medicine that combines diagnostic and therapeutic modalities to optimize patient outcomes. This approach has the potential to provide real-time feedback during therapy and diagnose a condition while simultaneously providing treatment. One such form of theranostics is focused ultrasound, which has been found to be effective in inducing neuroablation and neuromodulation and improving the efficacy of chemotherapy drugs by disrupting the blood–brain barrier. Targeted radionuclide therapy, which pairs positron emission tomography tracers with therapeutic effects and imaging modalities, is another promising form of theranostics for neurosurgery. Automated pathology analysis is yet another form of theranostics that can provide real-time feedback during the surgical resection of tumors. Electrical stimulation has also shown promise in optimizing therapies for patients with cerebral palsy. Overall, theranostics is a cost-effective way to optimize medical care for patients in neurosurgery. It is a relatively new field, but the advancements made so far show great promise for improving patient outcomes.

Keywords: Focused ultrasound; Radiopharmaceuticals; Electrical stimulation; Cerebral palsy; Intraoperative consultation; Automated pathology analysis

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1. Introduction

Neurosurgery is a complex field of medicine where individual differences can significantly impact patient outcomes. Recent advances in technology and the development of novel therapies have paved the way for precision medicine, which aims to personalize clinical care using a scientific framework[1]. Precision medicine accounts for biopsychosocial differences among patients to optimize standardized clinical procedures for each patient's unique prognosis. One such approach within precision medicine is theranostics. The term "theranostics" is a combination of diagnostics and therapy. It was coined by John Funkhouser in 2002, but despite being a relatively new term, the concept has been applied and revisited over many years[2,3]. When a patient requires a neurological procedure, diagnostic imaging is typically conducted beforehand to assess the patient's diagnosis before proceeding with surgery. Theranostics aims to combine these two objectives together to improve the accessibility of neurosurgery, as well as patient outcomes. There are two types of theranostics within neurosurgery: (i) Combined treatment and diagnostics within the same medium or (ii)

separate therapeutic and diagnostic modalities to achieve theranostic capabilities.

First, theranostics can be a modality that combines diagnostic and therapeutic capabilities within the same medium. Delivering treatment while the patient's condition is being evaluated has countless implications for improving the accessibility and cost-effectiveness of neurosurgical procedures. If treatment and diagnosis can be completed at the same time, fewer hospital visits and less overall technology would be required to provide care, reducing the need for multiple treatment regimens, which can improve patient adherence over time. One such example is focused ultrasound (FUS), which has therapeutic effects, such as neuroablation, while simultaneously existing as a useful diagnostic tool[4]. In addition, using radiopharmaceuticals for molecular imaging can treat neurological disorders while they are being diagnosed[5]. In both procedures, the diagnostic medium can also be used for the treatment of a condition. Second, theranostics can combine a diagnostic tool with a separate therapeutic modality. This approach to theranostics strives to collect diagnostic biofeedback during therapy to improve the evaluation of clinical outcomes and the management of treatment toxicities. For instance, automated pathology analysis and intraoperative consultation (IOC) enhance the surgical resection process for brain tumors[6]. In addition, diagnostic tools for cerebral palsy (CP) can be used to inform electrical stimulation therapies to improve their accuracy and precision[7]. In these approaches, diagnostic techniques are used to enhance the treatment process.

Theranostics has many clear advantages, including the improved prediction of toxicities and a real-time evaluation of patient responses, as discussed through the various technologies used[3]. One limitation of theranostics is the relatively recent development of theranostic tools, which leads to a highly variable sensitivity and specificity and results in the unintended exclusion of patients who would have otherwise benefited from these technologies. In recent years, theranostics research has focused on oncology and immunology[8]. The current review will further investigate its applicability in neurosurgery through FUS, molecular imaging using radiopharmaceuticals, automated pathology analysis of brain tumors, and electrical stimulation in CP.

2. FUS

FUS in neurosurgery has transformed the treatment procedures available for various neurological diseases. This technology focuses beams of energy on a single location to provide critical diagnostic and therapeutic care without harming the surrounding tissue[9]. Research has shown that a promising benefit from this technology has emerged in its ability to disrupt the blood–brain barrier (BBB)[10], which is a dynamic membrane that encapsulates various cells, proteins, and molecules to protect the brain from harmful substances. The permeability of the BBB is maintained by tight endothelial junctions created between neuroendothelial cells, pericytes, microglia, and astrocytes[11]. These cells have different functions. For instance, microglia play a role in immune response, while astrocytes contribute to the structure of the BBB[10]. Contrastingly, the blood tumor barrier (BTB) that forms in the brain manipulates this barrier to become more "leaky" and permeable[11]. The BTB, however, is not leaky enough to promote enhanced permeability of various chemotherapy molecules or other therapeutic drugs. FUS is utilized for its ability to disrupt the BBB and temporarily create gaps to allow various therapeutic agents to transfuse and cross through the membrane.

FUS can improve the administration of specific chemotherapeutic agents by increasing the permeability of BTBs. For instance, drugs such as doxorubicin have displayed significant improvements in tumors *in vitro*, but they cannot cross the BTB[10]. Research on FUS in disrupting the BTB was previously restricted by the fear of permanent tissue damage, but FUS is now being increasingly studied for this ability. The interaction of FUS with tissue is frequency-dependent. FUS with lower frequencies provides a greater level of penetration but with a lower resolution. Higher frequencies achieve the opposite effect, with higher resolution and lower levels of penetration[12]. To protect the extraneous healthy soft tissue, lower frequencies with penetration depths restricted to 1 cm are used[13]. Further, a study by Hynynen *et al.* showed that when specific low frequencies of FUS are used, permanent cranial damage can be minimized[14]. One method for administering the low frequencies is through the use of microbubbles that span from 1 to 5 μm in diameter[10]. These bubbles contain lipid-enveloped gas molecules that are administered episodically, and dosage delivery to the BBB can be monitored and modified as necessary[15,16]. The various oscillations and concentrations of the microbubbles have the potential to create mechanical forces to open the BBB[10,13].

One team led by Liu *et al.* monitored the effectiveness of FUS in delivering therapeutic agents to the brain using magnetic resonance imaging (MRI)[17]. Their studies indicated that FUS can help provide an enhanced image monitoring system and dramatically increase the delivery of epirubicin, among other chemotherapeutic agents[17]. Another study analyzed pembrolizumab, which is a commonly used chemotherapy drug that targets the immune system and prevents specific T-cells from being

inhibited by tumor cells[18]. Utilizing the patient's own immune system against the tumor prevents many side effects that other foreign agents could cause. FUS-directed chemotherapy has drastically improved clinical outcomes for patients with glioblastoma[16,17]. Many studies have observed increased inflammation and cranial edema after repeated FUS use, but this finding is dependent on the parameters of FUS used. For instance, one study by Choi *et al.* evaluating an animal model found that 0.25 MPa of pressure led to no cellular or tissue damage, whereas a pressure of 0.42 MPa induced an inflammatory response[19]. For this reason, more research should be catered toward the prevention of adverse reactions when utilizing FUS.

2.1. Neuromodulation

Neuromodulation, another therapeutic tool, involves artificially firing neurons to stimulate a response within a patient's brain[20]. In particular, neuromodulation was found to provide relief from pain and discomfort for individuals with mobility disorders, such as Parkinson's disease (PD) or Tourette syndrome[21]. Its effect can be extended to brain tumors through the use of FUS. Since FUS is non-invasive, it allows for the proper charting and mapping of specific areas of the brain for neuromodulation without significantly exposing other regions[22]. Applying this pair of theranostic tools allows for cellular killing, discharge, and necrosis in selected tissue locations; this discharge can lead to the release of molecules that positively impact the prognosis of brain tumors[22]. One predominant side effect of neuromodulation is the unintended stimulation of both sides of the cortex in unilateral stimulation. A study by Guo *et al.* also found that after widespread use of FUS, there was overstimulation of auditory responses in patients[23]. The research team concluded that the administration of longer single pulses instead of multiple shorter pulses decreases this side effect[23].

2.2. Neuroablation

Similar to neuromodulation, neuroablation is an innovative treatment that kills suspected tumor cells by combining various mediums, such as toxic chemicals and extreme temperatures[24]. This technique is combined with FUS to minimize the killing of healthy neuronal cells. High-intensity FUS uses a high temperature that allows targeted tumor cells to undergo thermal ablation, which causes coagulative necrosis within brain structures and decreases tumor viability[25,26]. Similar to neuromodulation, neuroablation has unintended consequences, such as head and leg tremors[27]. More research will allow us to develop better techniques to guard nearby tissue while providing therapeutic interventions to patients, as depicted in Figure 1.

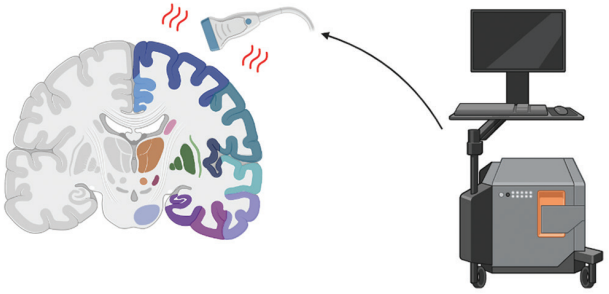

Figure 1. Guided neuroablation is a theranostic tool that combines the ability of focused ultrasound to visualize the various neurological fields while administering various substances, such as chemicals or heat, to destroy neurons. The guided probe provides specification while administering low-frequency ultrasound waves to minimize the killing of healthy tissue[28].

3. Radiopharmaceuticals and molecular imaging

Targeted radionuclide therapy (TRT) within neurosurgery is the theranostic application of radiopharmaceuticals[29]. This therapy involves the pairing of radioactive agents with imaging modalities, allowing for treatment decisions to be made while the condition is being diagnosed. The mechanism and action of radiopharmaceuticals are based on conjugating a radionuclide to carriers such as antibodies, peptides, or ligands. These carriers then enact a radiation effect on a tumor target or a specific tissue location. TRT emits energy signals in the form of β-particles or α-particles to effectively combat tumor progression or molecular pathologies[30]. Emitted when a neutron splits into an electron and proton, β-particles are energetic electrons that have negligible mass and carry a negative charge. They have a higher penetration power than α-particles. On the other hand, α-particles contain two protons and two neutrons, and they have a higher ionization power than β-particles. Nuclear imaging is used to monitor radioactive effects to ensure tissue specificity and minimal invasiveness in surrounding tissues. This duality that combines the diagnostic aspect of location selectivity and the therapeutic capability of radioactive particles provides a deep synergy that allows radionuclide therapy to be an effective theranostic tool, as shown in Figure 2[31]. TRT has been proven to be notably helpful in patients with other neurological comorbidities, where location specificity becomes a particularly relevant consequential factor when making treatment decisions.

3.1. Radiotracers in neuro-oncology

One example of TRT is the use of [^{68}Ga]Ga-DOTA-SSTR positron emission tomography (PET) tracers for their diagnostic and therapeutic capabilities in neuro-

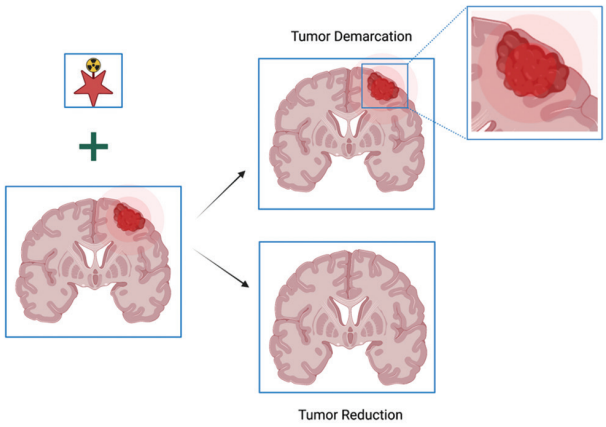

Figure 2. Targeted radionuclide therapy possesses both therapeutic and diagnostic capabilities in the form of size reduction and tumor demarcation, respectively. The incorporation of radiolabeled molecules safely allows for several routes of action as demonstrated.

oncology. A study by Palmisciano *et al.* found that [^{68}Ga]Ga-DOTA-SSTR PET tracers can provide accurate images for the clinical management of meningiomas and pituitary adenomas due to their strong binding to a pathological overexpression of somatostatin subtype receptors 2 (SSTR2)[32]. Accordingly, this tracer is employed in surgical resections to clearly demarcate tumor involvement and minimize damage to the surrounding tissue. Numerous studies have been conducted to assess the clinical applications of [^{68}Ga]Ga-DOTATOC-PET, which are outlined in Table 1. In one case report of a 68-year-old man with pituitary carcinoma, [^{68}Ga]Ga-DOTA-SSTR was combined with [^{177}Lu]Lu-DOTATATE and contributed to a decline in tumor progression over four years[33]. As [^{68}Ga]Ga-DOTA-SSTR imaged the tumor through its affinity for SSTR2, which was overexpressed in the pituitary gland by the tumor, [^{177}Lu]Lu-DOTATATE was able to exert its radioactive effects at binding sites to slow tumor progression[33]. Hence, diagnostic radiotracers, such as [^{177}Lu]Lu-DOTATATE, can be used to have a therapeutic effect on tumor progression, granting them excellent theranostic capabilities. Other forms of radionuclide therapy can be also used for the treatment of meningioma, such as ^{90}Y-DOTATOC. Studies evaluating the utility of this isotope are outlined in Table 2.

3.2. Radiotracers in neurodegenerative disorders

Radiopharmaceuticals can also be utilized to provide real-time feedback during neurosurgical interventions. For instance, [^{11}C]UCB-J in PET scans can be used to target synaptic vesicle protein A2 (SVA2) and subsequently measure synaptic density. SVA2 is a synaptic vesicle membrane protein involved in neurotransmitter release from neurons. Neurodegenerative disorders, such as PD or Alzheimer's disease (AD), are characterized by a decreased expression of SVA2 with a decreased synaptic density[42,43]. Various therapeutic modalities can be used to slow the progression of neurodegenerative disorders, such as electrical neural stimulation, and these forms of treatment can be paired with [^{11}C]UCB-J to provide active feedback while implementing therapeutic measures[44]. The measure of SVA2 by [^{11}C]UCB-J has a remarkable diagnostic capability in neurodegenerative diseases. It has long been and continues to be the standard radiotracer to diagnose different forms of dementia, as well as their severity and varying cognitive manifestations[42]. For instance, in one preclinical study by Bertoglio *et al.* using an animal model, PET scans were conducted for two groups of mice: One wild-type group and one group with AD[45]. Mice with AD showed a significantly lower uptake in the hippocampus. Similar studies were done in mouse models with PD and Huntington's disease, which showed diminished SV2A quantity on PET imaging, emphasizing that [^{11}C]UCB-J can be used to diagnose brain activity in different forms of dementia[45].

Decreased concentrations of the [^{11}C]UCB-J in mice with AD were recently corroborated in clinical trials, which further emphasized the diagnostic potential of [^{11}C]UCB-J. For instance, one study by Wilson *et al.* compared a group of 12 patients with PD to 16 control patients[46]. The study found significantly lower [^{11}C]UCB-J uptake volume on PET scans in various brain regions in patients with PD, but it also found correlations between SVA2 and the severity of patient symptoms. In doing so, it highlighted the correlation between synaptic density and subsequent brain and cognitive damage[46]. In another comparative study by Chen *et al.*, ten patients with AD were compared to 11 control patients. The study found a 41% reduction in hippocampal [^{11}C]UCB-J in patients with AD, and it expanded on this finding by linking [^{11}C]UCB-J to cognitive ability[47]. Hence, in neurosurgical procedures, [^{11}C]UCB-J can be used as a form of biofeedback for cognitive ability when paired with therapeutic modalities, including but not limited to electrical stimulation.

In addition, 18F-FDG is a glucose analog that can be utilized to monitor cerebral glucose metabolism. It has similar implications to [^{11}C]UCB-J in classifying neurodegenerative diseases by severity or cognitive impacts. Because patients with dementia have decreased cerebral glucose metabolism, 18F-FDG with PET scanning allows for the differentiation of AD from the other classifications of dementia by highlighting specific brain regions[48]. In recent years, 18F-FDG is being increasingly investigated to replace [^{11}C]UCB-J in imaging for patients with AD due to its longer half-life advantage[47].

Table 1. Summary of studies assessing the clinical application of [^{68}Ga] Ga-DOTATOC-PET as a diagnostic measure

Study	Participants enrolled	Outcome
Milker-Zabel *et al.*, 2006[34]	Twenty-six patients with meningioma diagnosed with [^{68}Ga] Ga-DOTATOC-PET	[^{68}Ga] Ga-DOTATOC-PET provided additional information about tumor extension that was otherwise not seen in imaging modalities, such as CT/MRI. It also identified a tumor in 1 patient that was otherwise not visible on CT/MRI. The researchers concluded that [^{68}Ga] Ga-DOTATOC-PET improves target definition for intracranial meningiomas
Gehler *et al.*, 2009[35]	Twenty-six patients with skull base meningioma diagnosed with [^{68}Ga] Ga-DOTATOC-PET	[^{68}Ga] Ga-DOTATOC-PET data provided additional information about the tumor in 17 patients. There were major changes observed in clinical target volume in 14 patients. The researchers concluded that [^{68}Ga] Ga-DOTATOC-PET strongly complements CT/MRI data in cases of complex meningioma
Kowalski *et al.*, 2021[36]	Nineteen patients with meningioma diagnosed with MRI and [^{68}Ga] Ga-DOTATOC-PET	Utilizing [^{68}Ga] Ga-DOTATOC-PET resulted in changes in clinical management for 3 patients. Maximum total lesion activity was better identified with [^{68}Ga] Ga-DOTATOC-PET, but meningioma volumes did not change significantly from what was detected by MRI

Abbreviations: CT/MRI: Computed tomography/magnetic resonance imaging; PET: Positron emission tomography.

Table 2. Summary of studies evaluating the utility of ^{90}Y-DOTATOC in meningioma treatment

Study	Patients enrolled	Outcome
Marincek *et al.*, 2015[37]	Thirty-four patients with progressive, unresectable meningioma treated with [^{177}Lu] Lu-DOTATATE and ^{90}Y-DOTATOC	Twenty-three patients achieved stable disease. Three patients experienced severe hematotoxicity, and one patient experienced severe renal toxicity. The study concluded that [^{177}Lu] Lu-DOTATATE and ^{90}Y-DOTATOC are promising tools for treating progressive, unresectable meningioma
Gerster-Gilliéron *et al.*, 2015[38]	Fifteen patients with recurrent or progressive meningioma treated with ^{90}Y-DOTATOC	^{90}Y-DOTATOC is a promising second- or third-line treatment for complex meningiomas
Bartolomei *et al.*, 2009[39]	Twenty-nine patients with recurrent meningioma resistant to treatment treated with ^{90}Y-DOTATOC; grade I (n=14), grade II (n=9), grade III (n=6)	Nineteen patients achieved disease stabilization after 3 months, and 10 experienced disease progression. Better results were seen in grade1 compared to higher grades, with a mean time to progression of 61 months compared to 13
Otte *et al.*, 1999[40]	Twenty-nine patients with advanced SSTR-positive tumors treated with ^{90}Y-DOTATOC	Twenty patients showed disease stabilization, with four showing a reduction of tumor mass and two showing partial remission. Three patients experienced disease progression. Five patients experienced severe renal and/or hematotoxicity from the treatment. The study concluded that ^{90}Y-DOTATOC is a promising therapy if issues of toxicity can be resolved
Seystahl *et al.*, 2016[41]	Twenty patients with progressive meningioma treated with [^{177}Lu] Lu-DOTATATE; grade I (n=5), grade II (n=7), grade III (n=8)	50% of patients reached stable disease after treatment with [^{177}Lu] Lu-DOTATATE. No statistically significant differences were observed between grades

4. Automated pathology diagnosis in neurosurgery

The role of pathology within surgery has held invariable significance over time. This is particularly accurate in neurosurgery with regard to the treatment of brain tumors[49]. The final objective of neuro-oncological procedures is typically dependent on the extent of tumorous tissue resection, which affects the level of cure that can be provided. The intricacies of neuro-oncology treatment following pathological analysis consist of several key events. To date, tumor resection for tissue analysis has followed a traditional pipeline: (i) intraoperatively, tissue samples are acquired and sent to pathologic analysis before proceeding with tissue resection. (ii) This is swiftly followed by the preparation of the sample for histological analysis. (iii) A pathological diagnosis is delivered to the operative team, including the neurosurgeon. (iv) Ultimately, this diagnosis guides the intraoperative, and consequently, the post-operative decisions of those involved in the patient's treatment. Treatment can exist in the form of chemotherapy, radiotherapy, or further surgical intervention following proper post-operative appraisal of the situation[50]. The pipeline described within surgical resection of neoplasms is often labeled as IOC, which is an indispensable step within the operative sequence and has granted theranostics a timely invitation.

Theranostics within the surgical resection of brain tumors primarily emphasizes a means to supplement surgical resection with the intraoperative diagnosis of tissue samples. Characteristics granting speed and accuracy have been increasingly explored within the field, paving the way for deep learning. Deep learning is a form of machine learning and artificial intelligence that is giving way to the development of more diagnostic technology within automated pathology for neurosurgical resections. Notably, a step within IOC pertains to the preparation of tissue samples, which lends itself to successful and accurate diagnoses. Conventionally, tissue samples have been processed as frozen sections and subjected to processing and labeling. Processing involves three steps to ensure the tissue is suitable for fixation within supportive molds. The steps are: (i) Dehydration with agents such as ethanol or isopropanol, among other alcohols; (ii) clearing, involving agents, such as xylene; and (iii) infiltration with a medium of choice, such as paraffin[51,52]. The invasiveness of these steps may carry downstream consequences for the visualization and analysis of slide samples[53]. These consequences can manifest as structural variations, including tissue shrinkage, protein denaturation, the resolution of macromolecules, and the intended degree of staining[54,55]. Conventionally, the processed specimen is labeled with hematoxylin and eosin (H&E) staining[56]. More recently, two techniques have been explored which include stimulated Raman histology (SRH) and third harmonic generation (THG) microscopy[57,58].

4.1. SRH

SRH, a modality that allows for the analysis of tissue through an unlabeled and unprocessed method for preparation and staining, contributes to the greater preservation of important molecular structures, which supports downstream analyses. Developed in 2008, SRH is an infrared microscopical technique that utilizes the vibrational frequencies within the chemical bonds of proteins, lipids, and DNA; it generates images highly reminiscent of H&E-produced imaging[59,60]. SRH has footholds within spontaneous Raman scattering, which is its original predecessor. This method, however, is multiphotonic and utilizes two lasers to generate the desired emission signal through stimulated rather than spontaneous excitation[59]. Overall, the potent variation within the hydrocarbon bonds of the sample creates ample contrast for quicker image resolution and generation.

The capabilities of machine learning in the form of deep convolutional neural networks (DCNNs) have also been explored in the context of SRH. DCNNs utilize trainable features based on histological patterns to allow for automated analysis of processed images. Depending on the specific disorder and pathology, the capacity to train will vary. For example, pathologies with a higher prevalence of pathognomonic features will lend themselves to greater ease in distinction[61]. In a non-inferiority randomized controlled trial, Hollon *et al.* assessed SRH paired DCNN to conventional H&E pathological analysis across 278 cases[61]. Sister samples were generated and designated to one arm of the trial. The overall diagnostic accuracy was 94.6% for the DCNN arm and 93.9% for the control, suggesting that accuracy is not sacrificed at the expense of augmented diagnostic speed. While IOC may consume approximately 20 min in duration, DCNN is implemented on a nearly real-time scale, as shown in Figure 3[62-64]. Further, its potential has been illustrated in other studies that coupled this modality to other forms of image preparation, namely, THG microscopy. Hence, the adoption of machine learning within IOC serves utility in the face of efficiency.

4.2. THG microscopy

As discussed, the processing stage plays a salient role in the diagnostic ability provided by machine learning and this subset of neurosurgical theranostics. Similar to SRH, THG is a multiphotonic technique that utilizes a label-free application. It uses three photons to produce a single photon with the sum of their energy in a process known as photoconversion[65]. The produced images rely on the inherent contrast of the visualized material to create high resolution, bypassing the detriments of certain procedures, such as photobleaching or reactive oxygen species (ROS) production[66,67]. A study of 45 samples assessing THG efficacy in distinguishing gliomas from non-tumorous tissue (n = 37 glioma and n = 8 normal) was described by Blokker *et al.*[68] According to the study, THG has an imaging capture speed that is 8 times faster than SRH, yielding significant advantages regarding speed. The researchers applied fully convolutional networks (FCN), which is a form of deep learning, based on a set of image-level features determined by three pathologists. This was applied to assess the binary diagnostic ability to distinguish between glioma and non-tumor. Overall, the accuracy was 79% with a mean average precision of 0.83. The results of this study in addition to those of the Hollon *et al.*[61] corroborate evidence for the diagnostic efficacy of machine learning and artificial intelligence within IOC while still granting a superior diagnostic speed. Nonetheless, development in this field remains relatively nascent. The latter of the two studies solely assessed the diagnosis of gliomas without other intracranial tumors. The success is extrapolatable to other forms of tumors, such as meningiomas and glioblastomas, based on the former

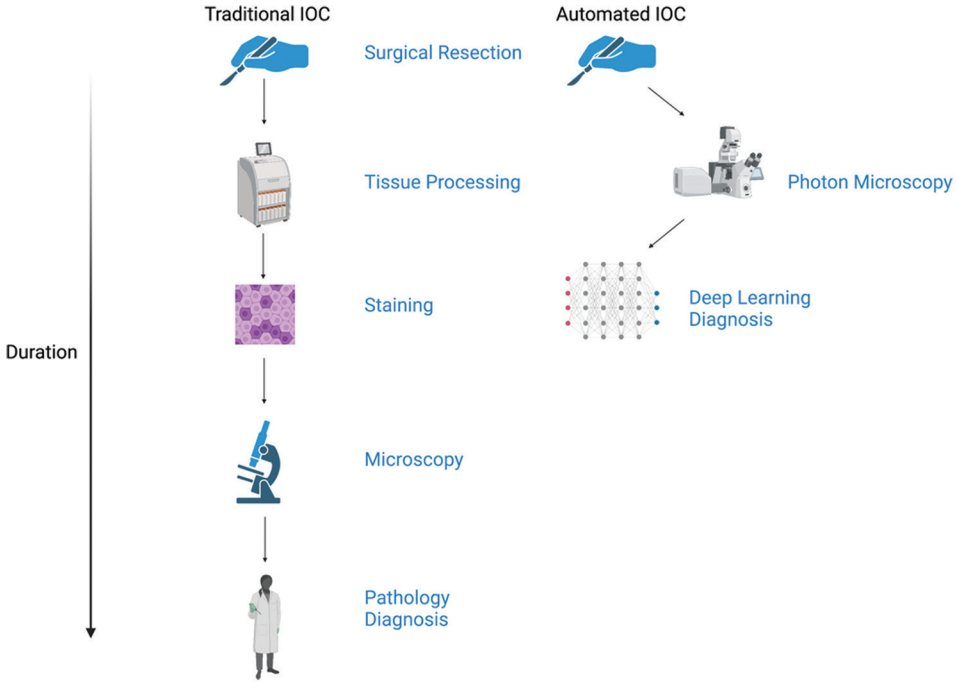

Figure 3. Intraoperative consultation pipeline for histological diagnosis in tumor resection.

study involving SRH and multiple forms of neoplasms, but few other studies have additionally assessed this.

4.3. Confocal laser endomicroscopy (CLE)

In a study by Izadyyazdanabadi *et al.*, CLE was assessed as a mode of imaging, similarly paired to DCNN[68]. The architecture of CLE involves a pen-sized device operated by the surgeon during a procedure requiring fluoroscopy for image generation[63]. This process occurs in real-time at a speed of 0.8 – 1.2 frames/s, as the surgeon navigates the nervous system. Further, these images can be adjusted in both depths without requiring resection, which is also controlled by the surgeon. There are two relevant disadvantages to using this imaging modality. First, as with any fluorophore dependency, there is a possibility of ROS production and damage to cellular structures[69,70]. Second, pertaining to CLE, the rapid generation of images creates a barrier in itself, as the interpretation and diagnosis of such images is a demanding task. Another limitation of CLE is its design – the motion in addition to blood flow and indiscernible tissue features ultimately produces images that are impractical for diagnosis. These images were described by Izadyyazdanabadi *et al.* as non-useful and occurred in nearly half of the 20,000 images generated in an *in vivo* application of CLE for intracranial neoplasms[71]. The DCNN model applied by Izadyyazdanabadi *et al.* displayed a tumoral diagnostic accuracy of 85% in comparison to a 75% and 67% accuracy, based on interpretations by two neurosurgeons, which still shows a significant benefit of utilization[68]. Overall, these recent advances suggest that machine learning within neurosurgery could play a significant role in theranostics and IOC.

5. Electrical stimulation in CP

CP is a group of neurological disorders that affect muscle tone, movement, and coordination; it is the most common cause of motor disability in children[72]. Historically, the diagnosis, prognosis, and management of CP have been challenging; however, with recent advances in technology, different treatments are becoming increasingly available. One such paradigm of treatment is through the use of electrical stimulation, which has emerged as a particularly effective mode of therapy for patients with CP, mirroring the success seen with other neuromuscular conditions. Within the scope of electrical stimulation, there are a few types that have predominated academic and clinical interest over the past decade, such as functional electrical stimulation (FES) and transcranial direct current stimulation (tDCS); both have shown efficacy in improving muscle function and movement. Along the theme of theranostic application, a notable benefit of utilizing electrical stimulation is its direct connection to the standard metric and methods used diagnostically to uncover and track the progression of neurological diseases, such as CP. This is congruent with the current drive within research to develop real-time biofeedback systems that can help patients during therapy,

which would automate and adapt treatment to the needs of each patient.

5.1. Functional electrical stimulation

Functional electrical stimulation is currently being studied in relation to its efficacy among patients with CP. Through this ongoing research, scientists and clinicians have found evidence of use-dependent muscle plasticity in patients with CP[73]. In many cases, this resulted in permanent improvements in voluntary ankle control after repetitive stimulation, implying a lasting therapeutic effect of FES, and providing a measurable metric of real-time efficacy. Trials conducted by Pool *et al.* have corroborated these findings by showing how FES can result in an increase in initial contact ankle angle, maximum dorsiflexion ankle angle in swing, normalized time in stance, and normalized step length in pediatric unilateral patients with CP[74]. Further, by combining adaptive ankle assistance with step-length biofeedback, lower-extremity gait mechanics in patients with CP can be monitored in real-time and immediately improved[75]. The expectation that this form of therapy will likely be beneficial is consistent with the understanding that an altered central drive to the ankle muscles and increased passive muscle stiffness may be the primary causes of foot drop and toe walking in patients with CP[76]. The feasibility and practical application of the mainstream application of this treatment are still being researched and established[77].

One specific form of FES, neuromuscular electrical stimulation (NMES), has shown particular promise in treating CP. When paired with electromyography or other acute, non-invasive measurements, NMES can act as a closed-loop system providing immediate feedback for therapeutic procedures. One specific example is the combination of NMES and robotic knee extension assistance as a theranostic modality. The combination of NMES and electromechanical feedback positively affects knee extension during stance with reliable, customized, and low-latency electrical stimulation[7]. Similarly, the knee-extension metric can be utilized as a prognostic metric to measure, recover, or diagnose the severity of CP symptoms. Continued work toward the use of diagnostics to synchronize NMES to gait cycles for improved effects is a current goal in the scope of rehabilitation medicine and medical research. Within that scope, the consideration of pathogenic physiology is still being explored[78].

5.2. Electromyography

Within neurosurgery, real-time biofeedback systems have garnered recent attention. Studies have shown that electromyography with high-quality biofeedback can encourage and support home-based therapy for pediatric patients with CP, particularly when coupled with some form of interactive medium such as a video game[79]. This mode of diagnostics provides a self-metric, which allows subjects and patients to adapt and adjust dynamically. In addition, motion in the upper extremities can be improved when robotic feedback therapy is utilized before conventional therapeutic methods; self-contained robotic systems are being developed to achieve this goal[80-82]. These cases highlight that modern therapies are not necessarily intended to replace conventional therapies, but instead to augment them or address special cases or gaps in previous methods. The current research has found that gait training is the most effective rehabilitation method for patients with CP, while strength training is negligible. Other methods, such as velocity training, electromyographic biofeedback training, and whole-body vibration, have appeared promising in individual cases, but further research is needed to prove that they are as effective as stand-alone therapies[83]. Gait training, among other methods, can be further optimized for each individual with CP using biofeedback.

5.3. Transcranial direct current stimulation

In a similar vein, trials are being conducted to establish the clinical utility of tDCS in treating CP[80,84,85]. Two such trials are currently evaluating the effects of combining tDCS with treadmill and mobility training on neuromuscular functionality in patients with CP[86,87]. In one of these studies, multiple non-invasive metrics were proposed for simultaneous tracking of the condition and visual feedback was provided to the subject through a gamified setup to allow for self-adjustment[87]. While this was not designed to generate an immediate closed loop, it should be noted that this form of feedback in training may be applicable to the general case of stimulation therapy. Ultimately, even in its current form, gait training was found to be more effective when using combined therapy. Scientific interest in tDCS seems to be leaning more toward hybrid applications than stand-alone therapy for CP. For instance, researchers are also currently investigating the combination of tDCS with hydrotherapy as a treatment for CP[88]. In this format, buoyancy would act as a form of instant adjustment and biofeedback in a corrective sense rather than a diagnostic sense. Conclusions on efficacy and function cannot yet be assumed, since this study is ongoing through December 2023, and there are no results as of yet. Safety standards will likely be discussed in the resulting paper.

Researchers have also found that anodal tDCS can result in an immediate improvement in the unimanual gross motor dexterity of the hemiplegic hand[89]. Under observation, this lasted for a minimum of 90 min,

Table 3. The post-operative effects of DBS on CP-based dystonia

Scale	Subscale	Pre-operative	3 months post-operative	6 months post- operative
Quality	Basal frequency (Hz)	192.11	212.77	219.38
	Jitter (%)	1.12	0.58	0.54
	Shimmer (dB)	4.948	3.39	2.988
	Noise-harmonic ratio	0.1822	0.114	0.0878
	Breath sound index	0.6	2.08	2.15
	Irregularity	1.72	1.4	0.81
	Maximum phonation time (s)	3.56	7.36	9.12
	DSI index	–0.2	0.8	2.9
Articulation	Jaw distance	550.73	535.92	508.96
	Tongue distance	1283.79	649.92	1783.17
	VSA	281914	278951	372958

Abbreviations: DBS: Deep brain stimulation; CP: Cerebral palsy; VSA: Vowel space area; DSI: Dysphonia severity index.

supporting its efficacy, even outside of the scope of gait correction. This conclusion, however, is not definitive. A literature review on tDCS interventions in pediatric motor disorders found that tDCS, while being a safe technique that likely improves gait measures and involuntary movements, has shown limited effectiveness in improving balance and upper extremity function overall[86]. Other studies have found that combining tDCS with bimanual training in children and young adults with unilateral CP showed inconsistent gains for objective measures of hand function[90]. The general and mechanistic effects of tDCS over the primary motor cortex, as well as when combined with functional training of the paretic limb, are currently under investigation[91]. Before considering the integration of tDCS into a brain-computer interface (BCI)-like loop or theranostic system, the mechanism must be isolated and defined.

5.4. Deep brain stimulation (DBS)

Along with FES and tDCS, DBS has rxxxecently been shown to be effective in treating CP-related pain and is currently being explored in terms of its capabilities to address motor symptoms[92]. In the clinic, stimulation of the superior cerebellar peduncles has shown efficacy in treating a subgroup of patients with CP-based dystonia and spasticity for whom stimulation of the dentate nuclei, which are deep cerebellar nuclei located within the white matter adjacent to the fourth ventricle, had been ineffective[93-95]. This finding coheres with other studies supporting the efficacy of DBS in improving motor symptoms in CP-based dystonia, as shown in Table 3[96]. Further research is still needed to determine the long-term effects of DBS on patients with CP, as DBS is still considered an experimental therapy for CP. Recent interest in developing a clinically viable bidirectional DBS-based neural interface projects a heavy implication that a real-time bidirectional form of DBS-based theranostic treatment may be just around the corner[97-99].

6. Conclusion

Theranostics in neurosurgery is an emerging field that combines diagnostic and therapeutic media in real-time to personalize medical care for each unique patient. FUS, radiopharmaceuticals, automated pathology analysis in tumor resection, and electrical stimulation for CP are four examples of innovative neurosurgical techniques within theranostics. Theranostics grants a new level of speed and accuracy to neurosurgical procedures, as seen in these techniques. Because these technologies are recent developments, more research is needed regarding their efficacy in different populations. Further, the accessibility of these technologies, particularly in rural areas, is largely unknown. These theranostic modalities are also likely applicable to many different neurological disorders, and future studies should focus on expanding their utility in various areas of neurosurgery. Despite these gaps in current research, theranostics permits a new level of precision medicine within neurosurgery, and its utility will only continue to increase as more research is conducted.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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PERSPECTIVE ARTICLE

Therapeutic drug monitoring of imipramine correlation with a case study

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Abstract

This article provides an overview of the pharmacokinetics, pharmacodynamics, contraindications, and precautions of imipramine, a tricyclic antidepressant (TCA) medication commonly used to treat depression and other mental health conditions. Imipramine's pharmacokinetic properties include rapid absorption, distribution to various tissues, metabolism in the liver, and elimination through the kidneys. Imipramine's pharmacodynamic effects are mediated through its actions on various neurotransmitters, including serotonin, norepinephrine, and dopamine. The article also discusses the contraindications and precautions associated with imipramine use. Imipramine is contraindicated in patients with a history of hypersensitivity to TCAs, recent myocardial infarction, and certain cardiac disorders. It should also be used with caution in patients with a history of seizures, urinary retention, glaucoma, and liver or kidney disease. In conclusion, imipramine is a medication with well-established pharmacokinetic and pharmacodynamic properties, but its use is associated with certain contraindications and precautions. Clinicians should carefully consider these factors when prescribing imipramine to patients with depression or other mental health conditions.

Keywords: Imipramine; Therapeutic drug monitoring; Case study; Adverse drug reactions; Contraindications

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1. Introduction

Therapeutic drug monitoring (TDM) is a crucial process in pharmacotherapy that involves measuring drug concentrations in the blood to optimize drug therapy. Imipramine, a tricyclic antidepressant (TCA), is widely used to treat major depression, anxiety disorders, and chronic pain. However, its narrow therapeutic index and potential for adverse effects can limit its clinical use. In this context, TDM emerges as a valuable tool to optimize imipramine dosing while minimizing the risk of side effects[1].

TDM of imipramine ensures that patients receive appropriate medication doses to achieve the desired therapeutic effect while minimizing the risk of side effects. This monitoring technique is particularly useful in cases involving concerns about drug interactions or individual variability in drug metabolism. For instance, in a case study, a patient taking imipramine for depression may experience side effects such as drowsiness or confusion. TDM can help determine whether the patient's blood levels of imipramine are excessively high, contributing to the side effects and whether dosage adjustments or a switch to a different medication would be appropriate[2,3].

The utility of TDM in optimizing imipramine therapy is demonstrated by a case study conducted by Yao *et al.* In the study, a 43-year-old woman with depression was initiated on imipramine at 50 mg orally twice daily. However, her serum imipramine concentration was found to be below the therapeutic range. Subsequent dose adjustments were made based on TDM, resulting in improved symptomatology and therapeutic serum levels. The case study highlights the value of TDM in identifying personalized dosage regimens for optimal treatment response[1-3].

Another case study conducted by Fong *et al.* involved a 47-year-old man with depression who experienced side effects such as drowsiness and dry mouth while taking imipramine at 75 mg orally once daily. TDM revealed a serum imipramine concentration above the therapeutic range. By reducing the dosage to 50 mg once daily based on TDM results, the patient achieved therapeutic levels and experienced an improvement in side effects[1,2].

Moreover, a retrospective study by Shen *et al.* explored the association between imipramine TDM and clinical outcomes in 82 patients with major depressive disorder. The study indicated that patients with imipramine concentrations within the therapeutic range had higher response rates and a lower incidence of adverse effects compared to those with concentrations below or above the therapeutic range. This suggests that imipramine TDM can effectively optimize therapy and improve clinical outcomes in patients with major depressive disorder[1-3].

Furthermore, pharmacogenetic studies have provided valuable insights into the interpatient variability in imipramine metabolism and its impact on therapeutic outcomes. For example, genetic variations in the cytochrome P450 isoenzymes, particularly CYP2C19 and CYP2D6, have been associated with variable imipramine metabolism and individual responses to treatment. TDM combined with genotyping can assist in identifying patients who may require dose adjustments based on their genetic profiles[4].

TDM of imipramine plays a critical role in optimizing pharmacotherapy by tailoring dosage regimens to individual patient characteristics. By monitoring imipramine blood levels and considering genetic factors, clinicians can adjust the dosage to achieve therapeutic levels while minimizing the risk of side effects. The correlation between imipramine TDM and specific case studies depends on the clinical context and the purpose of TDM. While TDM provides valuable insights, it should not be the sole method for monitoring imipramine therapy. Comprehensive clinical monitoring, including symptom assessment and consideration of patient-specific factors, remains important for safe and effective imipramine use[2,3].

2. Clinical pharmacology

Imipramine is a TCA that is commonly used to treat psychiatric disorders such as depression, panic disorder, anxiety, obsessive-compulsive disorder (OCD), and enuresis in children. The primary mechanism of action of imipramine is the blockade of the high-affinity reuptake mechanism of norepinephrine (NE), serotonin (5HT), and to a lesser extent, dopamine (DA) receptors. On acute administration, imipramine leads to an increased concentration of NE, 5HT, and DA in the synapse[4].

Chronic administration of imipramine can lead to a decrease in beta-adrenergic receptor density, which can cause decreased heart rate and blood pressure. Moreover, chronic administration can also result in a decrease in 5HT density, which can cause adverse effects. Imipramine primarily blocks the reuptake of NE and 5HT, leading to increased concentrations of these neurotransmitters in the synapse. Imipramine also has some affinity for DA receptors, but less than NE and 5HT. The clinical effects of imipramine are thought to be mediated through its actions on these neurotransmitter systems[4].

Overall, imipramine has been found to be an effective treatment for various psychiatric disorders, particularly depression. However, its use is associated with several adverse effects, including anticholinergic effects, sedation, and cardiovascular effects. Furthermore, taking other drugs with imipramine may affect the metabolism of imipramine or cause severe or fatal drug interactions[4].

TDM can be a valuable tool in optimizing the use of imipramine and minimizing the risk of adverse effects. Through TDM, the concentration of imipramine in the blood can be measured and adjusted the dose of medication to achieve therapeutic levels while minimizing the risk of adverse effects. Moreover, TDM can be particularly useful in cases where there is a concern about drug interactions or individual variability in drug metabolism[3,4].

3. Therapeutic range

The recommended therapeutic range for imipramine in the treatment of depression is generally considered to be between 150 and 300 ng/mL. However, due to significant interindividual variability in imipramine pharmacokinetics, some patients may require doses outside of this range to achieve therapeutic concentrations. Imipramine is metabolized to its active compound, desipramine, but there is currently no evidence of a therapeutic difference between the two[5,6].

4. Pharmacokinetics

This study focuses on the pharmacokinetics of imipramine, which includes its absorption, distribution, metabolism,

and elimination. Imipramine is available in oral forms such as tablets, capsules, oral solutions, and parenteral dosage forms. There may be differences in bioavailability between generic and branded formulations, which can lead to a decrease in plasma concentration. Imipramine has a half-life of around 12 – 24 h, is highly protein-bound, and has a large volume of distribution. Age, liver function, and drug interactions can affect its pharmacokinetics[6,7].

1. Bioavailability (F): The bioavailability of imipramine ranges from 20% to 70%. Absorption through the gastrointestinal tract is almost complete, but the drug undergoes first-pass metabolism in the liver, which reduces its bioavailability to 50% in most patients. The rate of absorption is variable, and it reached the peak concentrations at 2 – 8 h after a single dose. In general, different dosage forms of imipramine are bioequivalent. Parenteral administration results in less first-pass metabolism or none at all, which is important for drugs that produce active metabolites that have different therapeutic effects. There is little information suggesting that drug interactions or food alter imipramine absorption[6,7].
2. Clearance (Cl): Imipramine is cleared exclusively by the liver, with <5% eliminated by the kidney. Active metabolites of imipramine should be considered when monitoring plasma levels of imipramine. The estimated clearance for imipramine varies from 3- to 5-fold, with a mean clearance of 10 mL/kg/min (0.6 mL/kg/h). This wide range is due to the intra- and inter-individual variations in hepatic metabolism and drug reactions. Some compounds and liver diseases can increase imipramine plasma levels, but it is difficult to estimate the clinical significance of these drug-drug interactions when drugs are added or deleted from the therapy regimen. Differences in intrinsic metabolism among patients and difficulties in predicting the magnitude of drug interactions are also factors that should be carefully monitored in patients[6,7].
3. Volume of distribution (Vd): Imipramine is widely distributed throughout the body due to its extensive binding to plasma proteins and tissues. The estimation of imipramine's Vd varies, but the most common range is 15 – 20 L/kg. This pharmacokinetic parameter is not used clinically since loading doses of imipramine are not administered. Due to its large Vd and high protein binding, it is unlikely for hemodialysis to remove significant amounts of the drug from the body[6,7].
4. Half-life ($t_{1/2}$): Imipramine has an average half-life of around 20 h due to its increased Vd and Cl. However, its half-life ranged widely from 9 to 50 h depending on the changes in Vd and Cl. It is unclear whether $t_{1/2}$ of imipramine would be used clinically to establish a dosing interval, since there may be a lag phase between the onset of clinical response and the plasma concentration of imipramine. The plateau of the dose-responsive curve reflects the association of therapeutic response with either a slower equilibrating deep tissue compartment, or some other biochemical change, or both. Daily changes in blood levels may not be important for the therapeutic control of depression but could be an issue regarding imipramine's side effects[6-8].

The wide range of pharmacokinetic parameters for imipramine can be attributed to two factors: either true alternation in these parameters, or limitations in estimating a drug's half-life within an individual for the dosing interval establishment; it is difficult to determine which of the two factors is responsible for the wide range. As imipramine requires 1 – 3 weeks of treatment for apparent therapeutic effectiveness, clinicians typically wait at least 2 weeks for plasma samples collection, unless overt signs of toxicity were shown in patients. Samples obtained before the steady state are inaccurate and not recommended. The determination of trough and peak concentrations of imipramine within the dosing interval is challenging as the rate of absorption is dependent on individuals. It is often inconvenient to collect samples for trough concentration, and collection for peak concentration a few hours after the dose administration is likely inaccurate. Yet, adjustments in dosing regimens can only be made in proportion to the changes in plasma concentrations. Therefore, clinicians standardized the dosing of imipramine at bedtime and sample collection in the morning (approximately 12 h after the oral dose). The mid-interval plasma concentrations observed would approximate the steady-state concentrations of imipramine, and any revisions to pharmacokinetic parameters would focus on estimating clearance and bioavailability[8].

4.1. Key parameters

Key parameters that should be monitored during TDM of imipramine include the patient's clinical response to therapy, serum levels of imipramine, and the occurrence of any adverse effects. The goal of TDM is to optimize dosing to achieve therapeutic concentrations while minimizing the risk of side effects. Imipramine is available in tablets of 10 mg, 25 mg, and 50 mg, as well as capsules of 75 mg, 100 mg, 125 mg, and 150 mg. Injectable imipramine is available in a concentration of 12.5 mg/mL[9].

The key parameters to be considered during TDM of imipramine are as follows[9]:

1. Therapeutic range: The optimal serum concentration of imipramine for therapeutic efficacy is typically

between 180 μg/L and 350 μg/L.
2. Free fraction: The unbound or free fraction of imipramine in the bloodstream should be <10%.
3. Bioavailability: The bioavailability of imipramine is approximately 40%.
4. Volume of distribution: The volume of distribution of imipramine is about 20 L/kg.
5. Clearance: The clearance of imipramine from the body is approximately 0.9 L/kg/h.
6. Half-life: The half-life of imipramine is approximately 20 h.

Imipramine undergoes extensive hepatic metabolism and its clearance is solely dependent on hepatic metabolism. Only 5% of the drug is excreted unchanged through urine. The major metabolic pathways for imipramine are demethylation and hydroxylation followed by glucuronide conjugation, while the minor pathways include N-oxidation and dealkylation. The metabolites of imipramine undergo further metabolism, including ring hydroxylation of the parent compound or N-demethylation of the side chain before being excreted in urine or bile[10].

Imipramine is a highly lipophilic basic compound that is ionizable at stomach pH. Its rate of absorption is rapid with maximum plasma concentration occurring 2 – 8 h after administration. The effect of food has no significant impact on the absorption of imipramine. However, the first-pass effect decreases its bioavailability up to 20 – 70%, leading to decreased clearance and plasma concentration. Imipramine has an extraction ratio of up to 0.3 – 0.75, and changes in hepatic blood flow can affect its clearance. Imipramine follows a non-linear kinetics[11].

Imipramine has a large volume of distribution of up to 3 – 63 L/kg, and its highest concentration is found in the lung, kidney, brain, liver, and skeletal muscle. The lowest concentration is found in plasma and adipose tissues. Imipramine has a partition coefficient of 1000 – 100000[11].

4.2. Special populations

Certain populations require different imipramine doses due to the reasons listed as follows:
1. Pediatrics: Pediatrics patients have a higher proportion of lean body mass than fatty tissues, which leads to altered tissue stores. Furthermore, due to the increase in hepatic area, they show an increase in metabolism. In neonates, a higher unbound fraction of imipramine is observed at 26% which may lead to toxicity[12].
2. Geriatrics: Geriatric patients may show reduced hepatic blood flow leading to a slower clearance, and changes in the volume of distribution lead to low clearance. They may also show a decreased half-life with no change in clearance. Decreased renal flow leads to the accumulation of metabolites which can lead to adverse drug reactions[13].
3. Patients with hepatic diseases: Hepatic impairment results in the implication of cytochrome P450 isoenzyme. Alteration in clearance and plasma concentration, reduction in the first-pass effect, and prolongation of the elimination half-life can be observed[14].
4. Patients with renal failure: Renal failure can result in the accumulation of the metabolite desipramine leading to toxicity[15].
5. Patients with cardiovascular diseases: A decrease in cardiac output results in reduced hepatic blood flow with an increase in bioavailability[16].

5. Pharmacodynamics

The pharmacodynamics of imipramine included:
1. Dose-response relationship: The relationship between the concentration of imipramine and its response is sigmoidal with a therapeutic threshold of 180 ng/mL. Concentrations below 150 ng/mL show no response, whereas concentrations above 450 ng/mL show toxicity. Higher doses lead to seizures and OCD, thus requiring dose adjustment[17].
2. Concentration and toxicity: Mainly anticholinergic and cardiovascular side effects can be observed. Delirium can be observed at concentrations of 450 ng/mL, seizures at 745 ng/mL, and overdose may lead to death[18].

5.1. Factors affecting the dose–response relationship

There are several factors affecting the relationship between the concentration and response of imipramine:
1. Active metabolite: Hepatic metabolism produces active metabolites which have longer half-lives. Monitoring of hydroxy metabolite may be necessary to avoid toxicity[19,20].
2. Protein binding: Imipramine binds to alpha-1 acid glycoprotein, lipids, and cholesterol. The unbound fraction for imipramine is 4.2% to 10.9%. Methods for TDM include ultrafiltration and equilibrium dialysis-free drug concentration assays[20,21].
3. Drug interactions: Drug interactions may occur on the levels of pharmacodynamic and pharmacokinetic. Enzyme induction, increased hepatic clearance, cigarette smoking, inhibition of cytochrome P450 isoenzymes, and use of antihistamines, selective serotonin reuptake inhibitors, other psychotropic drugs, and alcohol are some known factors that can lead to drug interactions with imipramine[22,23].

Imipramine is contraindicated in patients with a history of hypersensitivity to TCAs, recent myocardial infarction,

Table 1. Tricyclic antidepressants' therapeutic range[31]

Drugs	Therapeutic range	Comments
Imipramine+Desipramine	150 – 240 μg/L	Most clinicians prefer a wider range of 180 – 350 μg/L
Amitriptyline+Nortriptyline	Not clearly established: 120 – 250 μg/L	The concentration of >450 μg/L does not increase response in patients who failed to develop a response at a lower concentration but may develop anticholinergic side effects

and in those with certain cardiac disorders. Caution should also be exercised in patients with a history of seizures, urinary retention, glaucoma, and liver or kidney disease[9,24].

6. Discussion

The TCA imipramine has been widely used for the treatment of depression and other mood disorders since its introduction in the 1950s. Over the years, extensive research has been conducted on the pharmacokinetics and pharmacodynamics of imipramine, as well as its contraindications and precautions.

Imipramine is highly lipophilic, with a rapid rate of absorption and maximum plasma concentration occurring 2 – 8 h after administration. Its distribution throughout the body is extensive, with a large volume of distribution, and it is mainly metabolized in the liver before being excreted. However, it is inadvisable for patients with a history of TCAs hypersensitivity, recent myocardial infarction, and certain cardiac disorders to use imipramine. Patients with a history of seizures, urinary retention, glaucoma, and liver or kidney disease should use imipramine with care. The therapeutic range for imipramine in the treatment of depression is generally considered to be between 150 and 300 ng/mL[25]. This range is consistent with previous studies that have established a therapeutic threshold of 180 ng/mL[17]. Imipramine concentrations below 150 ng/mL show no response, while concentrations above 450 ng/mL can lead to toxicity, including anticholinergic and cardiovascular side effects[18]. The recommended therapeutic range for imipramine and its active metabolite desipramine is between 150 and 240 μg/L, although some clinicians prefer a wider range of 180 – 350 μg/L[25]. It is important to consider these therapeutic ranges during TDM of imipramine to ensure optimal treatment response and minimize the risk of adverse effects (Table 1).

A study by Leinonen *et al.* (1980) found that cigarette smoking can affect the metabolism of imipramine in humans, suggesting that smoking should be avoided by patients taking imipramine[26]. In addition, imipramine has been found to have a therapeutic threshold of 180 ng/mL, with no response found at concentrations below 150 ng/mL, and toxicity including anticholinergic and cardiovascular side effects occurring at concentrations above 450 ng/mL.

In terms of special populations, pediatric patients have a higher proportion of lean body mass than fatty tissues, leading to altered tissue stores and increased metabolism due to increased hepatic area[27,28]. Geriatric patients, on the other hand, have low hepatic blood flow, leading to decreased clearance and increased adverse drug reactions. Patients with hepatic or renal impairment may also experience altered clearance and plasma concentrations of imipramine[29-32].

Overall, the pharmacokinetics and pharmacodynamics of imipramine should be carefully considered when prescribing this medication to patients. The contraindications and precautions should be taken into account, and special attention should be paid to patients with hepatic or renal impairment, as well as geriatric and pediatric patients. Patients should also be advised to avoid cigarette smoking while taking imipramine.

In conclusion, imipramine remains an important medication for the treatment of depression and other mood disorders, but it is important to understand its pharmacokinetics and pharmacodynamics, as well as its contraindications and precautions, to ensure its safety and effective use in patients. Further research may shed more light on the optimal dosing and management of patients taking imipramine, particularly in special populations.

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ORIGINAL RESEARCH ARTICLE

Antimalarial potential of five Nigerian medicinal plants: Repository versus curative activities

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Abstract

The conventional management of malaria in most endemic areas is based on phytomedicine. It is commonly believed that prevention is better than cure when it comes to disease management. Therefore, medicinal plants commonly used for treatment in herbal medicine are also used for prevention purposes. Hence, it is important to investigate the efficacy of medicinal plants in relation to the timing of their use. To document the medicinal plants used for treating malaria, a structured questionnaire-based ethnobotanical survey was conducted in Omu-Aran, Kwara State, Nigeria. The survey revealed the use of 31 plant species from 24 families, with origin in Omu-Aran, in herbal antimalarial recipes. Some of the identified plants were subjected to *in vivo* antimalarial bioassays. The aqueous and dichloromethane: Methanol (1:1) extracts of the leaves and stem bark of *Morinda lucida* and *Nauclea latifolia*, as well as the leaf extracts of *Chromolaena odorata*, *Tithonia diversifolia*, and *Lawsonia inermis* were tested at doses of 100, 250 and 400 mg/kg against *Plasmodium berghei* Anka. The repository and curative tests were conducted to assess the residual and curative abilities of the extracts, respectively, with chloroquine as a reference drug. The tested extracts demonstrated better antiplasmodial activities in the repository tests, particularly the aqueous extracts. Only the organic extract of *T. diversifolia* at 100 mg/kg exhibited a high antiplasmodial activity with a percentage chemosuppression (PCS) value of 66.13%, while other extracts showed moderate (PCS: 30 – 60%) to no activity (PCS: <0%) in curative tests. In repository tests, only *N. latifolia* showed high activity with percentage chemoprophylaxis (PCP) values ranging from 61.51% to 81.69%, while other extracts generally showed moderate activities. Chloroquine showed strong chemosuppression (92.74 ≤ PCS ≤ 98.77) in curative tests but weak chemoprophylaxis (PCP <30%). The efficacy tests showed that most of the investigated medicinal plants were more effective for prevention rather than for curative purposes.

Keywords: Medicinal plants; Antiplasmodial; Curative test; Repository test; Chloroquine

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1 Introduction

The global impact of malaria on human health and social well-being is detrimental, with particular emphasis on its effect on children under the age of five. According to

the World Health Organization (WHO) in 2022, this specific age group accounted for 77% of all malaria-related deaths worldwide[1]. In 2020, the number of malaria cases increased to 241 million, an increase from 228 million cases in 2018, with 627,000 of these cases resulting in fatalities. Notably, 96% of these deaths occurred in the African region[1]. The WHO identified twenty countries in the African region, along with India, as the locations where approximately 85% of all global malaria deaths occur.

On the global scale, Nigeria bears a very high malaria burden, with the highest cases of malaria-related death (24%), followed by the Democratic Republic of Congo, with an estimated occurrence of about 11% of malaria-related death. The economic and social effects of malaria should not be overlooked, as it leads to a reduction in the Gross Domestic Product of Nigeria by approximately 1% annually and is the primary cause of absenteeism[2].

The increasing incidence of malaria, a rise in malaria-related death, and the growing resistance of the malaria parasite to synthetic drugs have sparked interest in alternative treatment strategies. Traditional medicinal plants have historically played a significant role in the development of novel antimalarial drug therapies. For instance, compounds such as quinine and its derivatives are primarily derived from the Cinchona plant, while artemisinin is derived from *Artemisia annua*[3,4]. These discoveries highlight the potential of medicinal plants to provide valuable insights into effective anti-parasitic compounds. Consequently, it is important to properly document and scientifically investigate the diverse range of medicinal plants that abound in Nigeria to validate their traditional usage.

Various medicinal plants folklorically used for the treatment of malaria have been scientifically confirmed. Some of these plants include *Chromolaena odorata*, *Nauclea latifolia*, *Lawsonia alba lam.*, *Morinda lucida*, and *Tithonia diversifolia.*

C. odorata (L.) is a perennial shrub that belongs to the *Asteraceae* plant family. In Nigeria, the plant is known for its antimicrobial and wound-healing properties and is commonly used in the treatment of various ailments, such as diarrhea, malaria fever, toothache, diabetes, skin diseases, dysentery, and colitis[5,6].

N. latifolia Smith (*Rubiaceae* family) is a plant used in Africa for treating various ailments, including malaria[7,8].

Lawsonia inermis L. (*Lythraceae* family), commonly called Henna, is widely spread and cultivated in North Africa, Australia, Asia, and the Northern and Southern parts of Nigeria[9,10]. According to the World Flora Online (WFO) database, *L. inermis* is synonymous with *Lawsonia speciosa L.*, *Lawsonia purpurea Lam.*, *Lawsonia coccinea Sm*, *Lawsonia alba Lam.*, and *Lawsonia spinosa L.* (WFO, 2022)[11]. Its plant parts, including the leaves, flowers, seeds, stem bark, and roots, have been reported to exhibit many biological activities, which include wound healing, antimicrobial, antioxidant, anticancer, antidiabetic, antimalarial, hepatoprotective, and hypoglycemic[12-14].

M. lucida (L.), also commonly called the Brimstone tree, is a medicinal plant that belongs to the family of *Rubiaceae* and is located in the tropical West African rainforest[15]. Conventionally, the leaves are used as an analgesic, antipyretic, laxative, and antimalaria[16]. Different parts of the plant have also been reported to possess strong trypanocidal, aortic vasorelaxant, anticancer, hepatoprotective, antispermatogenic, anti-inflammatory, hypoglycemia, and antidiabetic activities[14,17-19].

T. diversifolia (Hemsl.) A. Gray, commonly called the Mexican sunflower, belongs to the family of *Asteraceae*. It is used folklorically to treat ailments, including malaria. It has scientifically been reported to possess antiplasmodial activity[13].

This report focuses on the ethnobotanical survey conducted in Kwara State, Nigeria, to identify local plants used to treat malaria. In addition, it presents the *in vivo* antiplasmodial activity of aqueous and organic extracts of leaves of *T. diversifolia*, *C. odorata*, and *L. inermis*, as well as the leaves and stem bark extracts of *N. latifolia* and *M. lucida*. The present study investigated the effects of administration timing of these extracts on mice infected with *Plasmodium berghei* ANKA strain, a rodent malaria parasite.

2 Materials and methods

2.1. Description of the study area

Omu-Aran is the headquarter of Irepodun Local Government Area, one of the 16 Local Government Areas in Kwara State. It is located at coordinates 8°2'17" N, 5°10'30" E at 80 km from the state capital, Ilorin. The region is home to Igbomina ethnic group, and the major spoken language is Yoruba (Figure 1).

2.2 Ethnobotanical data collection

The ethnobotanical survey was conducted among traditional healers and users of traditional medicine. Socio-demographic data of respondents were collected. Ethnobotanical data were collected mainly through oral interviews using a structured questionnaire. Information on local plant names, useful plant parts, preservation of plant materials, methods of preparation, application mode, dosage, and duration of treatment were recorded during

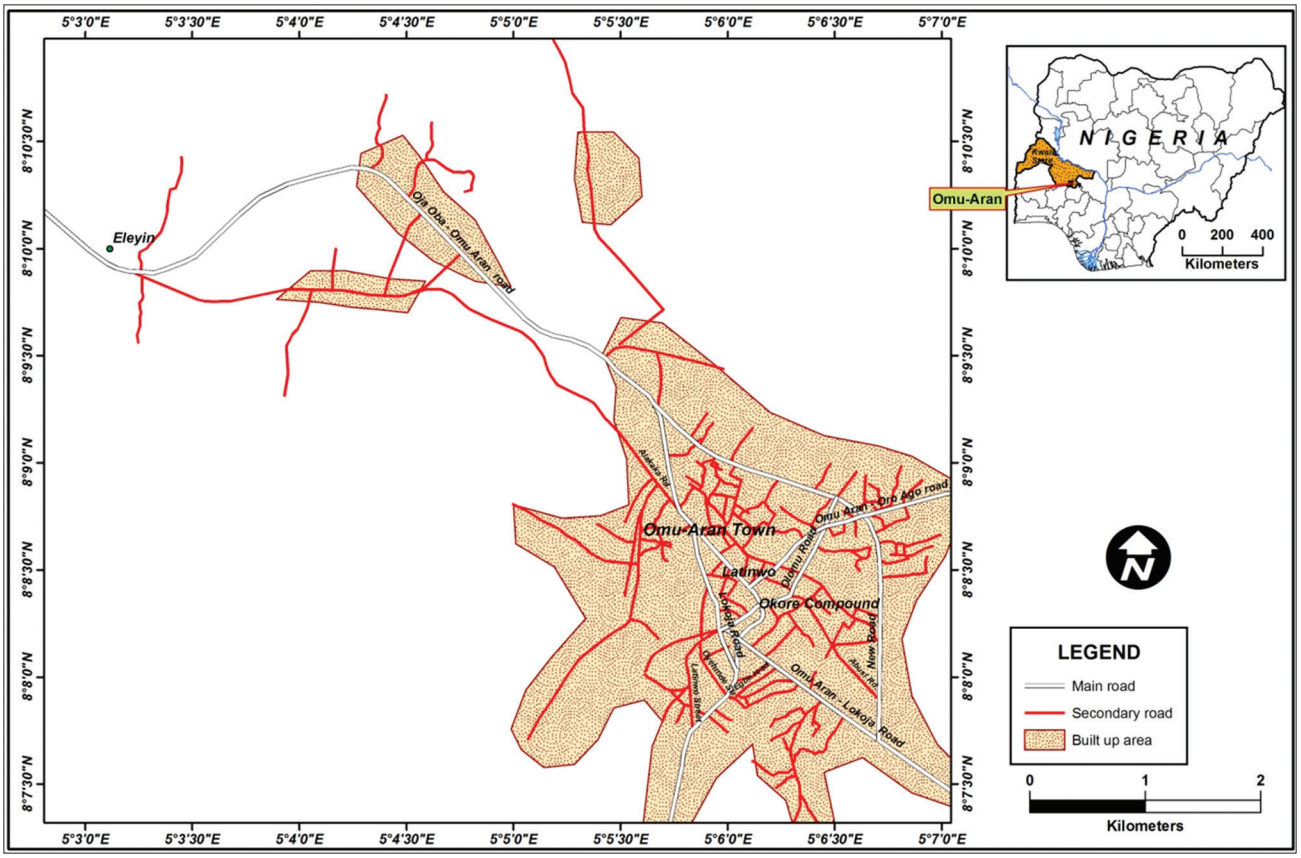

Figure 1. Map of the study site, Omu-Aran, Kwara State, Nigeria.

the interviews and the plants' names were validated and verified with information available on the website (http://www.theplantlist.org).

2.3. Collection, authentication, processing, and extraction of plant materials

Fresh leaves of *T. diversifolia*, *C. odorata*, and *L. inermis* and stem bark and leaves of *M. lucida* and *N. latifolia* were collected, identified, authenticated, processed, and extracted as previously described in[9,10]. The following voucher numbers were given during the authentication and identification of the plants at the Forestry Research Institute of Nigeria: FNI 108427 (*C. odorata*), FNI 108428 (*T. diversifolia*), FNI 108429 (*L. inermis*), FNI 108430 (*N. latifolia*), and FNI 108431 (*M. lucida*).

2.4. Experimental animals

In this study, 6 – 8 weeks old healthy Swiss albino mice, weighing 20 ± 2 g, were obtained from Kenya Medical Research Institute (KEMRI), Nairobi, Kenya. The mice were fed on commercial rodent food and water *ad libitum* in standard Makrolon type II cages in air-conditioned rooms at 22°C and 50 – 70% relative humidity, complying with the internationally accepted principles for laboratory animal use and care as found in the WHO guidelines. The Animal Care and Use Committee (ACUC) guidelines of KEMRI were followed.

2.5. Parasite species, preparation of inocula, and inoculation of animals

Chloroquine-sensitive *P. berghei* ANKA strain was used for *in vivo* antiplasmodial study. The parasite was obtained from the Centre for Traditional Medicine (CTMDR), KEMRI, Nairobi, Kenya. The parasite was maintained through weekly passaging. The preparation of inocula and inoculation of animals were carried out according to a previous study[13].

2.6. Experimental design and drug administration

The assay protocols used were Rane's (curative) and repository (prophylactic) tests, as previously described[20-22]. A total of 25 mice per test (curative and repository) were used for the study. In each test, the mice were inoculated with 0.2 mL of blood containing 1.2×10^7 parasitized red blood cells. The animals were divided into five groups ($n = 5$). Three of the groups were orally administered 100 mg/kg, 250 mg/kg, or 400 mg/kg of the extract with the aid of a cannula, and the other two groups were the

negative and positive controls which were given the vehicle (tween 80/distilled water) and chloroquine diphosphate, respectively. Mice were treated with the extracts, vehicle, and standard drug once daily, and the level of parasitemia was examined following the treatment. The animals were given their doses of extract on day 4 after the inoculation of parasites on D_0, then once daily for 2 more days (D_4 – D_5) for the curative test and before inoculation for the repository test.

2.7 *In vivo* plasmodial bioassay

2.7.1 Evaluation of antimalarial properties of the extract on established infection (curative test)

The curative potential of the test plants was evaluated using the method described in a previous study[21]. Twenty-five mice were divided into five groups (n = 5). Each mouse was inoculated intraperitoneally on the 1st day (D_0) with infected blood containing *P. berghei*-parasitized red blood cells and left untreated. On day 3 (D_2) of the experiment, a pretreatment blood smear of each mouse was made to confirm the infection. Animals were treated daily for 3 days (D_3 – D_5). Blood smears were collected on day 7 post-infection (D_6), the percentage of parasitemia was determined, and the percentage chemosuppression (PCS) was calculated using the formula below:

$$A = \frac{B - C}{B} \times 100 \quad (1)$$

where:

A=Average PCS, B=Average percentage of parasitemia in the negative group, and C=Average percentage of parasitemia in the test group[23].

2.7.2 Evaluation of antimalarial properties of extract on the residual infection (repository test)

The residual infection protocol was a modification of the procedure described in a previous study[24] to evaluate the prophylactic activity of the extract. A set of mice in various groups categorized as above were used. Animals were pre-treated before infection for 3 days. Treatment was initiated on D_0 and continued till D_2, and inoculated on D3 with the parasite. On days 3 and 5 (D_6 and D_8) post-infection, blood smears were obtained from each mouse. The percentage of parasitemia and chemoprophylaxis were then determined. The chemoprophylaxis was also calculated using Equation 1.

2.7.3 Evaluation of parasitemia

Thin blood films were prepared by collecting blood from the tail of each mouse and smeared on a properly cleaned and well-labeled microscopic slide. The thin blood films were fixed in methanol, stained with 10% Giemsa at pH 7.2 for 20 min, and then microscopically examined (×100 magnification).

2.8 Statistical analysis

The results were expressed as mean ± SD. Descriptive statistics were used to present the results and Student's *t*-test was used to compare the treated and negative control groups.

3 Results

3.1 Medicinal plants used in the management of malaria in Omu-Aran

A total of 22 respondents were interviewed: Two traditional healers and twenty individual users of herbal medicine. They were aged between 34 and 91 years, and their knowledge of herbal medicine was acquired from relatives, spouses, parents, and friends. The ethnobotanical survey revealed a total of 31 plant species belonging to 24 plant families, with the highest number of antimalarial plants from *Rubiaceae*, followed by *Asteraceae*, *Anacardiaceae*, *Poaceae*, and *Musaceae* (Table 1).

The leaf was the most frequently used part of the plant for medicinal preparations, followed by the stem barks, while two to three parts of the same plant are sometimes used. Medicinal plants were dried and kept aerated until used. Some respondents indicated that the lack of moisture content in the dried forms facilitates the preservation process, which they considered superior to using fresh materials.

The medicinal plants were prepared using various methods, including boiling the material in water (decoction), soaking the plant material in water (infusion), or soaking the plant in alcohol, aqueous extract from fermented maize (pap water), or other solvents such as a carbonated drink or non-alcoholic beverages (tincture). It was indicated that most of these medicinal plants are consumed orally in the form of decoction. For specific cases, steam baths and oral consumption of remedies were recommended, particularly when the materials were prepared with decoction.

According to the respondents, all medicinal plants were considered collectible throughout the year and at any time during the day, with the exception of the period between 6 pm and 7 am. In addition, many respondents indicated that they avoided collecting plants on the town's market days due to their superstitious beliefs. The collection of plants was not associated with any specific manner or the need for incantations.

The dosage for most of the remedies was usually half of a small glass cup or stainless cup (approximately 100 mL),

Table 1. Plants used in the management of malaria in Omu-Aran, Nigeria

Species name	Family	Common name	Local name	Part used
Tithonia diversifolia (Hemsl) A. Gray	*Asteraceae*	Tree marigold	June 12/Jogbo	Leaves
Chromolaena odorata (L.) R.M king & H. Rob	*Asteraceae*	Siam weed	Akintola	Leaves
Magnifera indica J.F.Macbr	*Anacardiaceae*	Mango	Mongoro	Leaves and stem bark
Lawsonia inermis var. spinosa (L.) pers.	*Lythraceae*	Henna plants	Laali	Leaves
Azadirachta indica A. Juss	*Meliaceae*	Neem	Dongoyaro	Leaves and stem bark
Psidium guajava	*Myrtaceae*	Guava	Gilofa	Leaves
Carica papaya L.	*Caricaceae*	Pawpaw	Ibepe	Leaves, root, fruits
Gossypium barbadense L.	*Malvaceae*	Cotton	Owu	Leaves
Senna siamea (Lam.) H.S irwin and Barneby	*Caesalpiniaceae*	Senna	Kasia	Leaves and stem bark
Citrus aurantifolia f. aurantifolia (christm) M.Hiroe	*Rutaceae*	Lime	Osanwewe	Leaves and fruits
Alstonia boonei De wild	*Apocynaceae*	Stool wood	Ahun	Root, stem bark and leaves
Anacardium occidentale L.	*Anacardaceae*	Cashew nut tree	Kasu	Leaves
Cymbopogon citratus stap f.	*Poaceae*	Lemon grass	Ewe tea	Leaves
Enantia polycarps Engl.&Diels	*Annonaceae*		Dokita Igbo	Stem bark
Aloe vera (L.) Burnm. F	*Aloeaceae*	Aloe	*Aloe vera*	Gel
Ocimum basilicum subsp. gratissimum	*Labiatae*	Sweet and hairy basil	Ejinrinwewe	Whole plant
Axonopus compressus P. Beauv.	*Poaceae*	Tropical carpet grass	Idi	Leaves
Cocos nucifera L.	*Palmae*	Coconut palm	Agbon	Shaft
Ananas comosus (L.) Merr.	*Bromeliaceae*	Pineapple	OpeOyinbo	Unriped fruit
Nauclea latifolia Blanco	*Rubiaceae*	Nauclea African peach	Ogbesi	Leaves, stem bark and root
Nauclea diderrichii merr.	*Rubiaceae*	Opepe African peach	Pepe	Leaves
Khaya grandifoliola A.Juss	*Maliaceae*	African Mahogany	Oganwo	Stem bark
Morinda lucida A.Gray	*Rubiaceae*	Brimstone tree	Oruwo	Leaves and stem bark
Allium sativum L.	*Liliaceae*	Garlic	Ayuu	Bulb
Kigelia africana (Lam.) Benth.	*Bignoniaceae*	Sausage tree	Pandoro	Leaves
Aldama excelsa (wills) E.E school.&panero	*Moraceae*	Iroko	Iroko	Stem bark and leaves
Corchorus olitorus L.	*Tiliaceae*	Jute plant	Ewedu	Leaves
Ficus opposita Car.opposita now.	*Labiatae*	Hoshindia	Efinrin	Whole plant
Musa paradisiaca L.	*Musaceae*	Plantain	OgedeAgbagba	Leaves, roots, fruit
Musa sapientum L.	*Musaceae*	Banana	Ogedeomini	Withered leaves
Vernonia amygdalina Del.	*Compositae*	Bitter leaf	Ewuro	Leaves

and the duration of treatment usually lasted for 3 – 5 days or until the symptoms disappeared. Most respondents further explained that in cases where two or more plants or plant parts were combined, the synergistic or additive effects of their constituents were exploited (Table 2).

According to the responses, some of these medicinal plants were not only used for treating malaria but also for addressing various other diseases or purposes. For example, *N. latifolia* was used for diabetes, pile, back pain, and measles. *L. inermis* was used for hypertension, hernia, and measles, and used as cosmetics. *M. lucida* was used for jaundice, hernia, and yellow fever. *C. odorata* was used for skin diseases and wounds. *T. diversifolia* was used for managing typhoid.

3.2 Antimalarial activity of medicinal plants

3.2.1 Curative test

Parasitemia was detected in the blood on day 2 post-infection, and subsequent monitoring revealed an increase in parasitemia until day 9 post-infection in both the experimental groups and negative controls. Out of the 42

Table 2. Medicinal plant used in combinations in the treatment of malaria in Omu-Aran, Kwara State

No	Plants in combinations
1	*Citrus aurantifolia* (leaves and root bark), *Lawsonia inermis*
2	*Magnifera indica* (stem bark), *Chromolaena odorata*
3	*Chromolaena odorata, Senna siamea, Tithonia diversifolia*
4	*Azadirachta indica, Carica papaya, Chromolaena odorata*
5	*Azadirachta indica, Carica papaya, Nauclea latifolia*
6	*Tithonia diversifolia, Ocimum basilicium, Chromolaena odorata*
7	*Magnifera indica, Psidium guajava, Chromolaena odorata*
8	*Magnifera indica* (male), *Carica papaya* (withered leaves), *Chromolaena odorata*
9	*Magnifera indica, Anarcadium occidentale, Chromolaena odorata*
10	*Magnifera indica, Anarcadium occidentale, Lawsonia inermis*
11	*Lawsonia inermis, Magnifera indica, Psidiumguajava*
12	*Anarcadium occidentale, Azardirachta indica, Carica papaya, Chromolaena odorata*
13	*Magnifera indica, Anarcadium occidentale, Azadirachta indica, Chromolaena odorata*
14	*Magnifera indica, Anarcadium occidentale, Azadirachta indica, Morinda lucida*
15	*Magnifera indica, Anarcadium occidentale, Citrus aurantifolia, Lawsonia inermis*

doses administered in the curative tests, one dose exhibited high activity (PCS >60%), 6 showed moderate activity, 22 displayed weak activity, and 13 showed no activity (PCS ≤0) (Figure 2). The dichloromethane-methanol (DCM-MeOH) (1:1) extracts generally displayed greater antiplasmodial potential compared to the aqueous extracts. Two of the aqueous groups exhibited moderate activity, 12 had weak activity, and 13 showed no activity, while 1 DCM-MeOH (1:1) dose displayed high activity, 4 showed moderate activity, 10 exhibited weak activity, and 6 had no activity (PCS ≤0). However, despite the observed activity, there was no significant ($P < 0.05$) increase in survival days. Notably, most of the chloroquine-treated (positive) groups exhibited significant survival days (Tables 3-7). The activities elicited by selected medicinal plants are further elaborated below.

3.2.1.1 M. lucida

The curative activity of *M. lucida* leaves extracts ranged from inactivity (PCS ≤0%) to weak activity (PCS <30%), while the stem bark aqueous and DCM-MeOH (1:1) extracts demonstrated moderate activity with PC of 43.45% and 51.14%, respectively, at doses of 100 mg/kg and 400 mg/kg (Table 3). In terms of survival days, the highest recorded value among *M. lucida*-treated infected mice was 10.60 ± 1.95, while positive and negative control groups had values of 18.20 ± 4.82 and 11.20 ± 2.75, respectively.

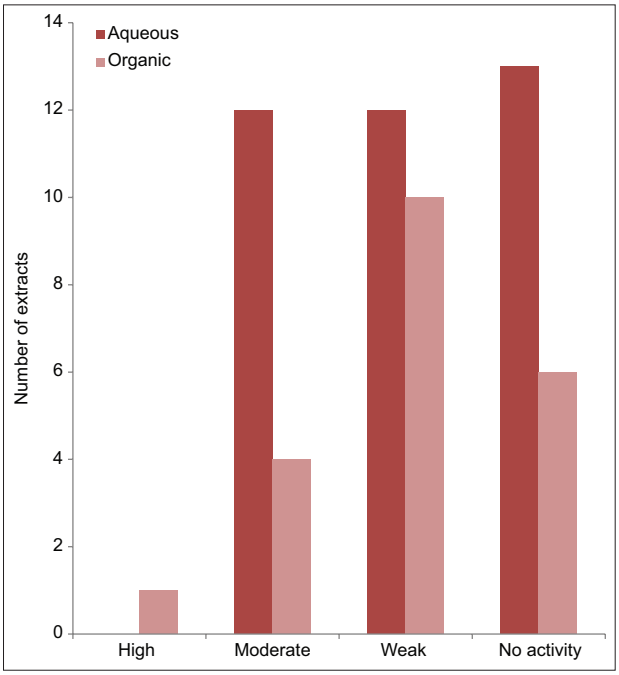

Figure 2. The curative antimalarial activity of the selected medicinal plant (aqueous and organic extracts).

3.2.1.2 N. latifolia

The curative antimalarial activity of the aqueous and DCM-MeOH (1:1) extracts from both the leaves and stem bark of *N. latifolia* was found to be very poor, ranging from weakly active (16.51%) to inactive (−56.93%) (Table 4). The mean survival time (MST) of *P. berghei*-infected mice treated with *N. latifolia* ranged from 6.80 ± 1.09 to 10.20 ± 1.10 days, while the groups treated with chloroquine and the vehicle had MTS between 13.00 ± 4.55 and 13.75 ± 1.89 days and 7.60 ± 0.55 to 9.80 ± 1.64 days, respectively (Table 4).

3.2.1.3 T. diversifolia

The organic (DCM-MeOH [1:1]) extract of *T. diversifolia* leaves exhibited high activity against established infection, with PCS of 66.13%. The aqueous extract also displayed moderate activity at the highest tested dose. However, in the 400 mg/kg organic group, most of the mice died before the end of the experiment, making it impossible to determine the PCS at this concentration (Table 5). The MST in the *T. diversifolia*-treated group ranged from 7.25 ± 0.5 to 13.00 ± 4.24 days. The positive and negative control groups had MST between 14.00 ± 2.45 to 14.67 ± 1.15 days and 7.40 ± 0.55 to 9.50 ± 1.73 days, respectively (Table 5).

3.2.1.4 L. inermis

L. inermis exhibited curative activity ranging from weak to moderate. The DCM-MeOH (1:1) extract at 400 mg/kg showed a PCS of 34.79% (Table 6). The activity was

Table 3. *In vivo* curative antimalarial activity of the aqueous and organic extracts of *Morinda lucida* in *Plasmodium berghei*-infected mice

Plant part	Extract	Dose (mg/kg)	Parasite density (%)	Chemosuppression (%)	Mean survival time (days)
Leaves	H_2O	100	76.70±3.37	−11.45	10.20±1.64
		250	55.98±0.41	18.66	9.40±1.52
		400	77.89±2.00	−13.17	10.60±1.95
Positive control	Chloroquine	5	2.01±1.75*	97.08	18.20±4.82*
Negative control	Distilled H_2O	0.2 mL	68.83±3.68	0.00	11.20±2.75
Leaves	Organic	100	81.93±1.45	−7.70	9.0±1.87
		250	74.08±2.24	2.61	8.60±1.34
		400	65.13±1.5	14.38	9.75±4.19
Positive control	Chloroquine	5	6.24±0.66*	91.80	14.8±2.59*
Negative control	Tween 80	0.2L	76.07±0	0.00	9.8±2.68
Stem bark	H_2O	100	49.67±2.54*	43.45	9.40±2.19
		250	80.86±0.46	7.93	8.00±0.00
		400	68.79±22.75	21.67	9.00±1.87
Positive control	Chloroquine	5	0.96±1.17*	98.91	14.20±4.09*
Negative control	Distilled H_2O	0.2 mL	87.82±8.67	0.00	8.20±1.64
Stem bark	Organic	100	N/A	17.79	7.20±1.10
		250	81.35±2.77	0.55	8.20±1.64
		400	28.88±7.51*	51.14	8.00±0
Positive control	Chloroquine	5	1.06±0.80*	98.71	11.80±1.30*
Negative control	Tween 80	0.2 mL	81.79±13.42	0.00	7.80±0.45

Notes: *Indicates significant difference from control, $P \leq 0.05$. Chemosuppression: ≥60%, high activity; 30 – 60%, moderate activity; <30%, weak activity; 0%, inactivity[13].

found to be dose-dependent. Among the *L. inermis*-treated groups, the highest MST recorded was 10.50 ± 2.3 days, while the positive and negative control groups had MST of 14.80 ± 2.95 and 9.00 ± 0.00 days, respectively (Table 6).

3.2.1.5 C. odorata

C. odorata exhibited activity ranging from weak and moderate. In the 400 mg/kg group of the aqueous extract, most of the mice died before the end of the experiment (Table 7). The MST in the *C. odorata*-treated *P. berghei*-infected mice ranged from 8.00 ± 0 to 10.50 ± 1.75 days, while the positive and negative control groups had MST between 14.67 ± 1.15 to 14.80 ± 2.95 days and 8.00 ± 0.0 to 8.67 ± 0.58 days, respectively (Table 7).

3.2.2 Repository test results

The residual antimalarial effects of the extracts were assessed on day 7 (D_6) and 9 (D_8) of the experiments. On D_6, out of the 42 experimental groups (21 aqueous and 21 DCM-MeOH [1:1]), 3 groups exhibited high activity (PCS>60%), 19 showed moderate activity, 17 groups had weak activity, and 3 were inactive. However, on D_8, the activities decreased, with 16 groups displaying moderate activity, 21 showing weak activity, and 5 being inactive (Tables 8-12). Although the survival time in most of the experimental groups was higher than that of the negative controls, the differences were not statistically significant ($P < 0.05$) (Figure 3). In contrast to the suppressive and curative tests, chloroquine did not demonstrate a high percentage chemoprophylaxis (PCP). Its activity ranged from weak to inactive on D_8, which was reflected in the survival days of the group. Aqueous extracts exhibited better antimalarial prophylactic properties, with 2 out of the 21 doses showing high activity, 12 being moderate, and 7 displaying weak activity. On the other hand, the DCM-MeOH (1:1) extracts showed a dose with high activity, 7 with moderate activity, 10 with weak activity, and 3 without activity (Figure 3). The activities elicited by selected medicinal plants are further elaborated below.

3.2.2.1 M. lucida

On D_6, both the DCM-MeOH (1:1) and aqueous leaf extracts of *M. lucida* displayed activity ranging from weak (PCP = 18%) to moderate (PCP = 41.05%). While the activity of the DCM-MeOH (1:1) extract groups decreased on D_9, the activity of the aqueous extract groups increased,

Table 4. *In vivo* curative antimalarial activity of the aqueous and organic extracts of *Nauclea latifolia* in *Plasmodium berghei*-infected mice

Plant part	Extract	Dose (mg/kg)	Parasite density (%)	Chemosuppression (%)	Mean survival time (days)
Leaves	H_2O	100	64.16±5.75	0.43	7.60±0.55
		250	64.02±11.25	0.66	9.60±1.95
		400	53.80±6.57	16.51	9.80±1.10
Positive control	Chloroquine	5	4.19±1.87*	93.50	13.00±4.55*
Negative control	Distilled H_2O	0.2 mL	64.44±7.10	0.00	8.80±2.05
Leaves	Organic	100	52.49±7.74	6.17	7.00±0
		250	67.13±6.69	−20.02	7.60±0.55
		400	54.73±8.73	2.15	6.80±1.09
Positive control	Chloroquine	5	4.54±2.09*	91.89	13.60±0.89*
Negative control	Tween 80	0.2 mL	55.9±36.62	0.00	7.60±0.55
Stem bark	H_2O	100	64.66±5.65	−5.43	9.20±1.64
		250	57.28±7.39	6.61	8.80±2.05
		400	62.34±2.43	−1.63	10.20±1.10
Positive control	Chloroquine	5	5.77±1.64*	90.59	15.00±3.32*
Negative control	Distilled H_2O	0.2 mL	61.33±6.27	0.00	9.80±1.64
Stem bark	Organic	100	35.50±7.87	1.66	10.0±1.87
		250	56.65±7.95	−56.93	9.0±1.87
		400	51.97±8.81	−43.95	10.00±2.45
Positive control	Chloroquine	5	5.12±4.73*	85.83	13.75±1.89*
Negative control	Tween 80	0.2 mL	36.10±16.83	0.00	8.80±2.05

Notes: *Indicates significant difference from control, $P \leq 0.05$. Chemosuppression: ≥60%, high activity; 30 – 60%, moderate activity; <30%, weak activity; 0%, inactivity[13].

Table 5. *In vivo* curative antimalarial activity of the aqueous and organic extracts of *Tithonia diversifolia* in *Plasmodium berghei*-infected mice

Plant part	Extract	Dose (mg/kg)	Parasite density (%)	Chemosuppression (%)	Mean survival time (days)
Leaves	H_2O	100	52.39±3.67	23.44	10.25±2.06
		250	57.80±0.74	15.53	10.50±1.73
		400	39.06±5.60*	42.91	13.00±4.24
Positive control	Chloroquine	5	2.65±1.24*	96.12	14.67±1.15
Negative control	Distilled H_2O	0.2 mL	68.43±12.14	0.00	9.50±1.73
Leaves	Organic	100	28.41±7.84*	66.13	7.33±0.58
		250	68.80±13.89	17.98	8.25±1.89
		400	N/A	N/A	7.25±0.5
Positive control	Chloroquine	5	3.03±2.58*	96.39	14.00±2.45*
Negative control	Tween 80	0.2 mL	83.88±0.52	0.00	7.40±0.55

Notes: *Indicates significant difference from control, $P \leq 0.05$. Chemosuppression: ≥60%, high activity; 30 – 60%, moderate activity; <30%, weak activity; 0%, inactivity[13].

although the differences were not statistically significant ($P > 0.05$) (Table 8). Unlike the DCM-MeOH (1:1) extract groups, which showed no activity at any dose levels, the aqueous extract of *M. lucida* stem bark exhibited moderate activity at all dose levels on D_6. However, the activity decreased on D8. The MST of the *M. lucida*-treated mice in this experiment ranged from 10.00 ± 0.82 to 12.50 ± 1.73 days, when the positive and negative control groups had MST between 10.75 ± 0.96 to 12.50 ± 2.38 days and 10.50 ± 1.00 to 11.25 ± 1.89 days, respectively (Table 8).

Table 6. *In vivo* curative antimalarial activity of the aqueous and organic extracts of *Lawsonia inermis* in *Plasmodium berghei*-infected mice

Plant part	Extract	Dose (mg/kg)	Parasite density (%)	Chemosuppression (%)	Mean survival time (days)
Leaves	H_2O	100	61.24±5.14	3.45	10.50±1.73
		250	49.64±6.29	21.73	10.50±2.3
		400	N/A	9.07	9.0±0
Positive control	Chloroquine	5	2.65±1.24*	95.82	14.67±1.15*
Negative control	Distilled H_2O	0.2 mL	63.43±2.87	0.00	9.00±0
Leaves	Organic	100	64.38±7.17	14.81	8.60±1.34
		250	66.35±4.18	12.19	7.80±0.45
		400	49.28±3.77*	34.79	8.33±0.58
Positive control	Chloroquine	5	1.24±0.70*	98.36	14.80±2.95*
Negative control	Tween 80	0.2 mL	75.56±5.23	0.00	8.00±0

Notes: *Indicates significant difference from control, $P \leq 0.05$; chemosuppression: ≥60%, high activity; 30 – 60%, moderate activity; <30%, weak activity; 0%, inactivity[13].

Table 7. *In vivo* curative antimalarial activity of the aqueous and organic extracts of *Chromolaena odorata* in *Plasmodium berghei*-infected mice

Plant part	Extract	Dose (mg/kg)	Parasite density (%)	Chemosuppression (%)	Mean survival time (days)
Leaves	H_2O	100	58.19±1.08	14.96	10.50±1.73
		250	54.04±5.70	21.02	8.00±0.00
		400	N/A	N/A	8.33±0.58
Positive control	Chloroquine	5	2.65±1.24*	96.12	14.67±1.15*
Negative control	Distilled H_2O	0.2 mL	63.43±2.87	0.00	8.67±0.58
Leaves	Organic	100	67.14±8.00	11.15	8.00±0.00
		250	50.42±8.38*	33.28	10.20±3.49
		400	43.83±2.78*	42	10.20±4.66
Positive control	Chloroquine	5	1.24±0.70*	98.36	14.80±2.95*
Negative control	Tween 80	0.2 mL	75.57±3.70	0.00	8.00±0

Notes: *Indicates significant difference from control, $P \leq 0.05$. Chemosuppression: ≥60%, high activity; 30 – 60%, moderate activity; <30%, weak activity; 0%, inactivity[13].

3.2.2.2 N. latifolia

On day 7 of the experiment, when blood smears were examined and percentage parasitemia was determined, both the DCM-MeOH (1:1) and aqueous leaf extracts of *N. latifolia* exhibited moderate antiplasmodial activity (Table 9). However, this activity decreased on D_8. In contrast, the DCM-MeOH (1:1) and aqueous stem bark extract of *N. latifolia* demonstrated high activity, with PCP ranging from 61.51% to 81.69%, which then decreased to moderate activity by day 9. The mean survival days in *N. latifolia*-treated groups ranged from 10.25 ± 0.05 to 13.25 ± 2.22, with the highest MST observed in the aqueous stem bark extract group at 250 mg/kg. The highest MST in the positive and negative control groups were 12.50 ± 2.38 days and 11.25 ± 1.89 days, respectively (Table 9).

3.2.2.3 T. diversifolia

On D_7, the repository activity of *T. diversifolia* ranged from very weak (9.3%) to moderate (54.59%). However, by D_9, the activity observed in most of the groups had decreased (Table 10). The MST of the *T. diversifolia*-treated mice ranged from 10.25 ± 0.50 to 15.00 ± 3.08 days, while the highest MST observed in the chloroquine and vehicle-treated mice were 12.00 ± 4.69 days and 12.20 ± 1.79 days, respectively (Table 10).

3.2.2.4 L. inermis

The assessment of the *in vivo* antiplasmodial prophylactic ability of *L. inermis* revealed a range of very weak (4.65%) to moderate (34.78%) activity on D_6. However, on D_9, the results for PCP were inconsistent, as some groups showed a decrease in activity while others displayed an

Table 8. ***In vivo*** **repository antimalarial activity of the aqueous and organic extracts of** ***Morinda lucida*** **in** ***Plasmodium berghei*****-infected mice**

Plant part	Extract	Dose (mg/kg)	D_6 Parasite density (%)	D_6 Chemoprophylaxis (%)	D_8 Parasite density (%)	D_8 Chemoprophylaxis (%)	Mean survival time (days)
Leaves	H_2O	100	7.18±1.09*	35.33	31.70±4.68*	39.96	11.25±1.89
		250	8.97±3.14	19.12	41.05±0.86	22.27	10.75±0.5
		400	6.54±0.48*	41.05	27.50±4.95*	47.92	12.50±1.73
Positive control	Chloroquine	5	8.76±2.47	21.02	52.98±3.51	−0.32	12.25±2.06
Negative control	Distilled H_2O	0.2 mL	11.10±4.99	0.00	52.81±3.53	0.00	10.75±0.5
Leaves	Organic	100	7.90±2.70	18.46	53.84±4.45	9.87	11.0±1.73
		250	7.57±1.57	21.9	52.73±2.74	11.72	11.40±3.21
		400	7.05±1.69	27.26	48.21±5.25	19.3	11.20±1.64
Positive control	Chloroquine	5	7.34±4.62	24.25	67.48±3.28	−12.97	10.80±0.45
Negative control	Tween 80	0.2 mL	9.69±3.28	0.00	59.73±3.14	0.00	11.25±1.89
Stem bark	H_2O	100	3.84±0.69*	39.36	38.15±2.88	20.19	10.0±0.82
		250	3.47±1.30*	45.26	37.56±1.15	21.42	10.33±0.58
		400	2.82±0.72*	55.52	32.48±2.97*	32.06	10.75±0.96
Positive control	Chloroquine	5	4.40±1.05*	30.52	42.77±4.56	10.53	10.75±0.96
Negative control	Distilled H_2O	0.2 mL	6.33±1.30	0.00	47.80±2.42	0.00	10.50±1.00
Stem bark	Organic	100	13.45±3.51	−18.12	60.07±6.30	−0.88	11.75±2.87
		250	12.09±6.71	−6.1	66.60±2.57	−11.86	10.25±0.5
		400	13.49±2.49	−18.44	82.85±1.74	−39.14	10.33±0.58
Positive control	Chloroquine	5	9.85±1.89	13.56	58.34±4.71	2.02	12.50±2.38
Negative control	Tween 80	0.2 mL	11.39±0.75	0.00	59.54±3.56	0.00	11.25±1.89

Notes: *Indicates significant difference from control, $P \leq 0.05$. Chemosuppression: ≥60%, high activity; 30 – 60%, moderate activity; <30%, weak activity; 0%, inactivity[13].

insignificant increase ($P > 0.05$) (Table 11). The MST in *L. inermis*-treated group ranged from 10.50 ± 0.58 to 12.00 ± 1.83 days, while the positive and negative control groups had MST ranging from 10.75 ± 0.96 to 12.20 ± 1.64 days and 10.50 ± 1.00 to 11.60 ± 0.55 days, respectively.

3.2.2.5 C. odorata

On D_7, the extracts of *C. odorata* extracts exhibited prophylactic activity ranging from weak (13.57%) to moderate (56.21%), with the DCM-MeOH (1:1) extract groups showing better activity (Table 12). The MST ranged from 10.00 ± 0.82 to 12.60 ± 2.19 days for the mice treated with *C. odorata* extracts. The positive and negative control groups had MST ranging from 10.75 ± 0.96 to 12.20 ± 1.64 days and 10.50 ± 1.00 to 11.60 ± 0.55 days, respectively (Table 12).

3.2.3 Comparisons of curative and repository test results

Generally, better *in vivo* antiplasmodial activity was observed when extracts were administered before infection (Repository test) than after the establishment of infection (curative test). This is observed in all the extracts tested in Figure 4.

4 Discussion

The ethnobotanical survey conducted in Omu-Aran showed that a variety of medicinal plants are traditionally used in the community for treating malaria. These plants are mostly consumed orally in the form of decoction. Thirty-one species from 24 plant families were documented during the survey conducted in this study, indicating the relevance of the medicinal flora in the day-to-day management of malaria in the area. Furthermore, most of the mentioned medicinal plants have been reported in other parts of Nigeria and Africa, and studies have investigated their efficacy, yielding positive results. This confirms the validity of the gathered information[25,26].

In the traditional medicine system, certain herbal medicines are usually used for prevention before the onset of disease, while others are used after an infection has already occurred. It is noteworthy that the same plants often serve both preventive and curative purposes. Therefore, this study aimed to evaluate the antimalarial

Table 9. *In vivo* repository antimalarial activity of the aqueous and organic extracts of Nauclea latifolia in *Plasmodium berghei*-infected mice

Plant part	Extract	Dose (mg/kg)	D_6 Parasite density (%)	D_6 chemoprophylaxis (%)	D_8 parasite density (%)	D_8 chemoprophylaxis (%)	Mean survival time (days)
Leaves	H_2O	100	5.02±1.34*	54.78	28.84±1.83*	45.39	12.25±2.06
		250	6.00±1.14*	45.92	29.94±4.26*	43.3	12.25±2.06
		400	9.44±3.45	14.92	48.60±1.94	7.97	11.50±1.73
Positive control	Chloroquine	5	8.76±2.47	21.02	52.98±3.51	−0.32	12.25±2.06
Negative control	Distilled H_2O	0.2 mL	11.10±4.99	0.00	52.81±2.54	0.00	10.75±0.5
Leaves	Organic	100	7.63±2.05*	33.04	47.78±3.50	27.22	11.50±1.73
		250	4.89±3.98*	57.1	35.20±3.02*	46.38	10.25±0.5
		400	7.60±4.40*	33.3	48.44±4.90	26.21	12.75±2.75
Positive control	Chloroquine	5	9.85±1.89	13.56	63.64±1.74	3.06	12.50±2.38
Negative control	Tween 80	0.2 mL	11.39±0.75	0.00	66.65±3.57	0.00	11.25±1.89
Stem bark	H_2O	100	8.72±3.72	21.23	42.56±1.84	19.41	11.67±2.08
		250	4.27±2.27*	61.51	26.32±0.56*	50.16	13.25±2.22
		400	4.01±1.52*	63.9	26.22±2.74*	50.35	12.67±2.31
Positive control	Chloroquine	5	8.76±2.47	21.02	49.16±1.48	6.9	11.67±2.08
Negative control	Distilled H_2O	0.2 mL	11.10±4.99	0.00	52.81±1.56	0.00	10.75±0.5
Stem bark	Organic	100	7.32±2.85	24.5	58.90±2.41	2.21	10.40±0.55
		250	7.64±2.87	21.13	48.61±2.89	19.30	11.00±1.73
		400	1.78±1.90*	81.69	30.40±2.56*	49.53	11.40±1.67
Positive control	Chloroquine	5	7.34±4.62	24.25	67.48±3.28	−12.02	10.50±0.45
Negative control	Tween 80	0.2 mL	9.69±3.28	0.00	60.23±3.93	0.00	11.25±1.89

Notes: *Indicates significant difference from control, $P \leq 0.05$. Chemosuppression: ≥60%, high activity; 30 – 60%, moderate activity; <30%, weak activity; 0%, inactivity[13].

activity of *N. latifolia*, *L. inermis*, *T. diversifolia*, *C. odorata*, and *M. lucida*. The evaluation was conducted both before the establishment of infection and after using *P. berghei*, which causes a disease similar to human plasmodium infections[27].

The antiplasmodial activities varied depending on the type of plant, plant part, method of extraction, and parasite. Earlier researchers have reported the antimalarial activities of these plants, although they were tested using different methods[13,28-36]. However, there was a difference in the *in vivo* antiplasmodial activity of the extracts, with aqueous extracts appearing to be more effective than organic extracts.

In this study, the efficacy of the extracts was measured based on the percentage of chemo suppressions/ chemoprophylaxis against parasitemia in mice and the MST of the animals. The *in vivo* antimalarial activity of the extracts exhibited a significant difference in parasite density reduction and survival days ($P < 0.05$) between the extract-treated groups and the group treated with the vehicle (negative control). The observed reduction in parasitemia in the treated groups, compared to the negative control groups, indicates the antimalarial potential of the extracts.

In this study, it was observed that the extracts *M. lucida* generally exhibited between moderate and weak activity in the tests, despite being reported as one of the five most frequently used antimalarial herbs in folk medicine, especially in South-west Nigeria[36,37]. The moderate activity of *M. lucida* against *P. falciparum* was also observed in

Table 10. *In vivo* repository antimalarial activity of the aqueous and organic extracts of *Tithonia diversifolia* in *Plasmodium berghei*-infected mice

Plant part	Extract	Dose (mg/kg)	D_6 Parasite density (%)	D_6 Chemoprophylaxis (%)	D_8 Parasite density (%)	D_8 Chemoprophylaxis (%)	Mean survival time (%)
Leaves	H_2O	100	3.81±0.61*	30.65	34.22±2.71	10.96	14.4±1.67
		250	2.50±1.16*	54.59	25.12±4.38*	34.64	12.2±4.09
		400	3.31±3.00*	39.73	26.88±0.99*	30.06	15.0±3.08
Positive control	Chloroquine	5	3.32±0.63*	39.61	41.40±5.45	−7.74	12.0±4.69
Negative control	Distilled H_2O	0.2 mL	5.50±1.48	0.00	38.43±3.18	0.00	12.2±1.79
Leaves	Organic	100	4.45±2.25*	33.78	36.0±2.19*	32.97	13.75±3.86
		250	5.75±1.39	14.51	48.47±4.80	9.76	10.25±0.50
		400	6.10±0.67	9.3	39.76±3.44	25.97	12.50±1.73
Positive control	Chloroquine	5	6.46±2.85	3.92	46.84±4.98	12.79	10.75±0.50
Negative control	Tween 80	0.2 mL	6.72±1.47	0.00	53.71±1.92	0.00	10.75±0.50

Notes: *Indicates significant difference from control, $P \leq 0.05$. Chemosuppression: ≥60%, high activity; 30 – 60%, moderate activity; <30%, weak activity; 0%, inactivity[13].

Table 11. *In vivo* repository antimalarial activity of the aqueous and organic extracts of *Lawsonia inermis* in *Plasmodium berghei*-infected mice

Plant part	Extract	Dose (mg/kg)	D_6 parasite density (%)	D_6 Chemoprophylaxis (%)	D_8 Parasite density (%)	D_8 Chemoprophylaxis (%)	Mean survival time (%)
Leaves	H_2O	100	5.36±1.79	15.41	38.33±1.58	19.81	12.00±1.83
		250	5.47±0.98	13.57	47.40±2.40	0.85	10.50±0.58
		400	4.13±1.27*	34.78	35.18±7.64	26.41	10.50±1.73
Positive control	Chloroquine	5	4.40±1.05*	30.52	42.77±4.56	10.53	10.75±0.96
Negative control	Distilled H_2O	0.2 mL	6.33±1.30	0.00	47.80±2.42	0.00	10.50±1.00
Leaves	Organic	100	8.11±1.71	4.65	38.29±1.84	15.68	12.00±1.73
		250	5.84±2.57*	31.35	26.99±2.63*	40.57	11.80±1.79
		400	7.86±1.69	27.26	29.97±0.68*	34.01	11.60±1.95
Positive control	Chloroquine	5	5.87±1.59*	30.92	38.53±5.11	15.15	12.20±1.64
Negative control	Tween 80	0.2 mL	8.50±1.00	0.00	45.41±3.07	0.00	11.60±0.55

Notes: *Indicates significant difference from control, $P \leq 0.05$. Chemosuppression: ≥60%, high activity; 30 – 60%, moderate activity; <30%, weak activity; 0%, inactivity[13].

the unpublished work by Makinde and colleagues, cited in[38]. This study demonstrated an interplay between host immunity and the activity of *M. lucida*. In adults, *M. lucida* (leaves extract) showed 100% parasite clearance, whereas no such clearance was observed in children, who have a less well-developed immune response. Meanwhile, children are the group at greatest risk of mortality, making efficacy particularly important in this population.

The leaves and stem bark of *N. latifolia* demonstrated relatively good activity in this study, particularly in the repository test. The aqueous stem bark extract exhibited high activity at 250 and 400 mg/kg, while the organic extract showed high activity at 400 mg/kg. Earlier studies conducting preliminary phytochemical screening of the leaf and stem bark extracts of this plant reported the presence of bioactive compounds such as alkaloids,

Table 12. ***In vivo*** **repository antimalarial screening of the aqueous and organic extracts of** ***Chromolaena odorata*** **in** ***Plasmodium berghei*****-infected mice**

Plant part	Extract	Dose (mg/kg)	D_6 Parasite density (%)	D_6 Chemoprophylaxis (%)	D_8 Parasite density (%)	D_8 Chemoprophylaxis (%)	Mean survival time (%)
Leaves	H_2O	100	2.78±2.67	15.41	36.52±2.76	23.61	11.75±0.96
		250	5.47±0.98	13.57	40.06±2.40	16.19	10.50±0.58
		400	4.13±1.27*	34.78	49.40±7.64	−3.34	10.00±0.82
Positive control	Chloroquine	5	4.40±1.05*	30.52	42.77±4.56	10.53	10.75±0.96
Negative control	Distilled H_2O	0.2 mL	6.33±1.30	0.00	47.80±2.42	0.00	10.50±1.00
Leaves	Organic	100	7.23±1.26	14.91	29.59±4.72	15.68	12.60±2.19
		250	4.21±2.12*	50.47	28.31±3.86*	40.57	12.20±1.64
		400	3.72±2.03*	56.21	36.17±1.40*	34.01	12.20±1.64
Positive control	Chloroquine	5	5.87±1.59*	30.92	38.53±5.11	15.15	12.20±1.64
Negative control	Tween 80	0.2 mL	8.50±1.00	0.00	45.41±3.07	0.00	11.60±0.55

Notes: *Indicates significant difference from control, $P \leq 0.05$. Chemosuppression: ≥60%, high activity; 30 – 60%, moderate activity; <30%, weak activity; 0%, inactivity[13].

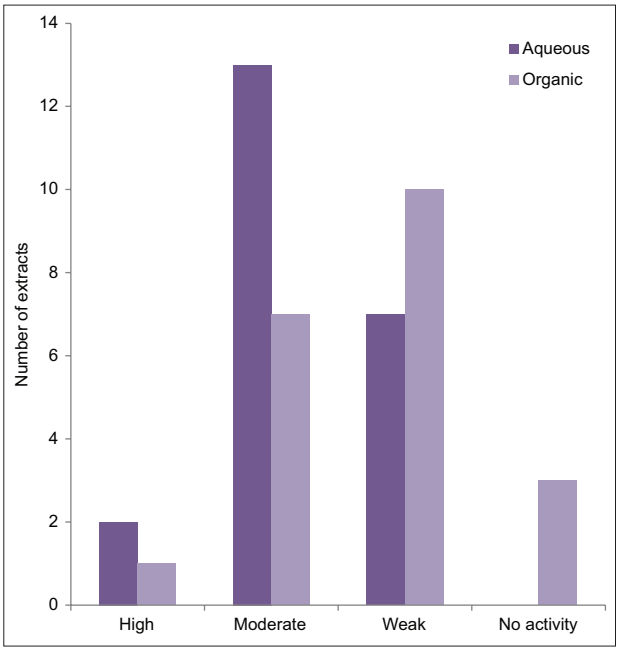

Figure 3. *In vivo* antiplasmodial activities of the selected medicinal plant extracts in repository and curative tests.

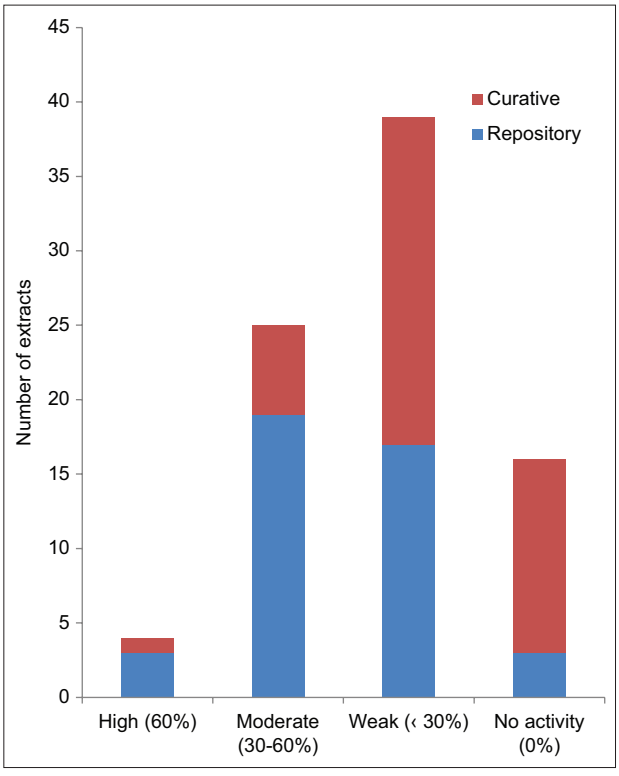

Figure 4. *In vivo* antiplasmodial activities of the selected medicinal plant extracts in repository and curative tests.

saponins, flavonoids, tannins, and terpenes[39-41]. These phytochemical compounds increase the level of oxidative molecules in the cells, thereby inhibiting protein synthesis in the parasites[42]. This mechanism could nullify the oxidative damage induced by the malarial parasite. It also suggests that the antiplasmodial activity of the ethanolic leaf extract of *N. latifolia* may be attributed to the antioxidant and antiplasmodial effects of these phytochemicals[43]. These findings corroborate the reported antiplasmodial activity of the plant and support further evidence for its traditional

use. Furthermore, other studies[31,35] have demonstrated good *in vivo* antiplasmodial activity of *N. latifolia*, with the highest activity observed in the prophylactic test.

Repository experiments revealed that chloroquine is a very weak prophylactic agent, which can be attributed to its mechanism of action as a blood schizontocidal agent. Chloroquine, like other quinine derivatives, requires the presence of free heme to act. The free heme produced during the hydrolysis of hemoglobin is toxic to the parasite and, therefore, must be removed or detoxified. However, the malaria parasite lacks enzymes for detoxification, and therefore, heme is detoxified by converting it into a nontoxic malaria pigment called hemozoin. Chloroquine inhibits the detoxification reaction by binding to heme, hence preventing the destruction of parasites in liver cells where no hemoglobin digestion takes place[44]. Consequently, the singular use of chloroquine in the prevention and treatment of malaria has led to the widespread distribution of chloroquine-resistant strains. The discontinuation of chloroquine usage has reduced the drug pressure, resulting in a decrease in chloroquine-resistant *Plasmodium* parasites[45]. It was observed that after 12 years of chloroquine withdrawal from use in Malawi, its efficacy in the treatment of malaria has shown signs of returning[46]. Similar observations of chloroquine efficacy returning against *Plasmodium vivax* malaria in Ethiopia and the Saharan Zone in Mauritania after the use of artemisinin-based combination therapy have been reported[47,48]. Moreover, chloroquine has shown a greater chemo suppressive activity than the extracts and fractions of the plants used in this study in the curative test. However, due to resistance, chloroquine is no longer used as an antimalarial agent in humans. The advantage that these plants have over chloroquine is that they are new, effective, and not associated with known resistance.

The extracts generally exhibited weak to moderate activity in the repository test, with *N. latifolia* showing high activity at certain concentrations. However, in the curative test, the majority of the extracts, especially *N. latifolia*, displayed between no to weak activity. The antiplasmodial effect of *M. lucida* on established malaria infection in mice showed significant malaria suppression compared to the other extracts. These findings suggest that *M. lucida* could be a better curative agent rather than a prophylactic agent, while other extracts, especially *N. latifolia*, could be considered prophylactic agents.

5 Conclusion

The study investigated the plants used for treating malaria in Omu Aran Kwara State, Nigeria, and revealed that the extracts of *M. lucida*, *N. latifolia*, *C. odorata, T. diversifolia*, and *L. inermis* have both curative and repository effects in the mice infected with *P. berghei*. However, the antimalarial effect of the extracts was found to be higher when administered before infection rather than after infection. These results agree with the traditional use of these plants and provide a scientific basis for their continuous use. The findings in the present study encourage further research on these plants, which have demonstrated antiplasmodial activity, with the view to developing new antiplasmodial drugs.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

Conceptualization: Funmilayo I. D. Afolayan
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All authors read and approved the final manuscript.

Ethics approval and consent to participate

The respondents to the ethnobotanical survey questionnaires verbally consented to participate in the survey. The guidelines provided by the WHO and the ACUC of KEMRI were adhered to, which outline the internationally recognized principles for the use and care of laboratory animals.

Consent for publication

Not applicable.

Availability of data

Supporting data can be obtained from the corresponding author following formal request.

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ACCSCIENCE PUBLISHING

INNOSC Theranostics and Pharmacological Sciences

ORIGINAL RESEARCH ARTICLE

Evaluation of genotoxicity of (4-fluorophenyl) thiazolidin-4-one in CHO-K1 cells

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Abstract

4-thiazolidinones are five-membered heterocyclic ring compounds with diverse pharmacological impacts. In a previous study, we reported a series of newly synthesized derivatives of 4-thiazolidinones with different functional groups, which exhibited anticancer activity against ovarian (SKOV3) and cervical (HeLa) cancer cell lines. Among these derivatives, (4-fluorophenyl) thiazolidin-4-one (4-TH) demonstrated potent cytotoxic activity against SKOV3, with an IC_{50} value of 12.3 μM. However, it was also found to be extremely toxic to normal cells (CHO-K1) with an IC_{50} of 7.5 μM. Before considering its use in cancer research, it is crucial to gain a comprehensive understanding of its potential genotoxic effects on normal cells. In this study, we aimed to assess the *in vitro* cytogenetic toxicity of 4-TH using normal Chinese hamster ovary cells (CHO-K1). Referring to the IC_{50} of 4-TH, we selected three sub-lethal concentrations (2, 5, and 7.5 μM) and treated CHO-K1 cells for 24 h (one cell cycle duration) to estimate its dose-dependent induction of chromosome aberrations, and examine the effect of 4-TH on cell division, micronucleus induction potential and cell cycle arrest properties following standard protocols. The results showed that 4-TH was highly toxic to normal cells, as all three sublethal concentrations caused a statistically significant increase in the number of chromosomal aberrations ($P < 0.001$), formation of micronuclei ($P < 0.01$), and changes in the rate of cell division (mitotic index) ($P < 0.05$) compared to control. In addition, there was a significant increase in the number of cells in the G1 phase, indicating that all concentrations of 4-TH tested induced apoptosis. The evaluation of the cytotoxic, clastogenic, and aneugenic properties of 4-TH, a potent cytotoxic agent, will undoubtedly provide critical information for determining its safety and potential as an anticancer drug.

Keywords: Cytotoxicity; Chromosome aberration; Mitotic index; Micronucleus; Cell cycle

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1. Introduction

Heterocyclic compounds hold significant pharmacological importance[1,2]. Recently, there has been increasing industrial and biological interest in the chemistry of 4-thiazolidinones due to their diverse bioactive properties[3]. Thiazolidinones are five-membered heterocyclic compounds with a sulfur atom at position 1, a nitrogen atom at position 3, and a carbonyl group

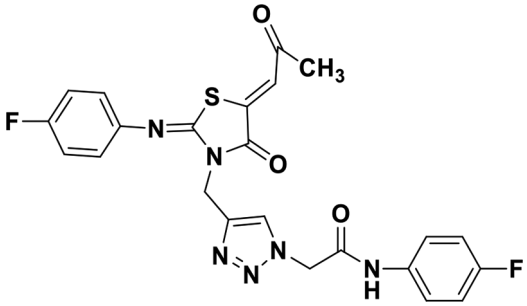
at position 2, 4, or 5. Depending on the position of the oxo group or keto group, they are classified as 2-thiazolidinones, 4-thiazolidinones, and 5-thiazolidinones[4]. The applications of 4-thiazolidinone compounds are particularly diverse and can be found in a variety of clinically employed drugs. These compounds exhibit antitubercular[4], antimicrobial[5,6], anticonvulsant[7-9], anti-inflammatory and analgesic[10], and anti-cancer[11-13] properties. In addition, they have been reported as cox-1 inhibitors[14], bacterial enzyme inhibitors, and non-nucleoside inhibitors of HIV Type 1 reverse transcriptase[15-20]. Some synthetic 4-thiazolidinone (2-thioxo-4-thiazolidinone) derivatives have demonstrated potent cytotoxic against renal cancer, non-small cell lung cancer, breast cancer, melanoma, and ovarian cancer cell lines[21]. The versatility of 4-thiazolidinone derivatives has made them widely utilized pharmaceuticals and agrochemicals[22]. They have also been identified as optical brighteners[23], antioxidants[24], corrosion inhibitors[25], and additives with various additional properties. The popularity of heterocyclic molecules can be attributed to their ease of molecular structural modification, allowing for specific customization[26]. The reason these heterocyclic compounds perform so well is that they can act as molecules that can control protein synthesis, transmission of energy of nerve impulses, sight, and metabolism, all of which depend on chemical involvement of heterocyclic molecules in the form of vitamins, enzymes, co-enzymes, nucleic acids, ATP, and serotonin[27].

Earlier toxicological assessments were performed on various synthetic 4-thiazolidinone derivatives employing different methodologies, including acute toxicity, analgesic, and anti-inflammatory studies using mice models, which reported that the compounds did not cause any morbidity and mortality[28]. In line with this, we previously synthesized novel thiazolidine-4-one derivatives that demonstrated significant anticancer and antibacterial activities[29]. Among these derivatives, (4-fluorophenyl) thiazolidin-4-one (4-TH) showed potent anticancer activity against ovarian cancer cells (SKOV3) as well as toxicity to normal Chinese hamster ovary cells (CHO-K1). However, further investigation is needed to fully explore the safety and possible toxic effects of these compounds, particularly in the context of their use as chemotherapeutic agents. Hence, in this study, we employed 4-TH to assess its detailed genotoxic potential (clastogenic, mitotoxic, and aneugenic potentials) as well as its impact on the cell cycle in normal CHO-K1 cells.

2. Materials and methods

2.1. Test chemical

(4-fluorophenyl) thiazolidin-4-one (4-TH) was used in this study to assess its possible genotoxic potential *in vitro*. Its chemical structure and IUPAC name are as follows:



Molecular weight: 496.49

IUPAC name: N-(4-fluorophenyl)-2-(4-(((2Z,5Z)-2-((4-fluorophenyl)imino)-4-oxo-5-(2-oxopropylidene) thiazolidin-3-yl)methyl)-1H-1,2,3-triazol-1-yl)acetamide (4-TH)

2.2. DPPH assays

2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) is an electron transfer-based free radical technique that creates a violet solution in methanol. This experiment was carried out following our prior published work[30]. The DPPH assay is a simple and rapid approach to investigate antioxidant properties. The violet color of the DPPH solution changes to yellow as the molar absorptivity of DPPH radicals at 517 nm decreases from 9660 to 1640. During the assay, the odd electron of the DPPH radical is coupled with a hydrogen atom from a free radical scavenging antioxidant, resulting in the formation of the reduced DPPH-H. The subsequent decolorization of the solution is stoichiometric in terms of electron capture.

2.3. Cell line

The CHO-K1 cell line, a normal mammalian cell line, was obtained from ATCC (Bethesda, MD, USA). It was maintained in DMEM medium supplemented with 10% FBS, 2 mM L-glutamine, 100 µg/ml penicillin, and 100 µg/ml streptomycin, incubated at 37°C in a 5% CO_2 incubator. Cell viability, chromosome aberration, mitotic index, and micronucleus (MN) assays were conducted following the Organisation for Economic Co-operation and Development guidelines and our earlier published work[31,32].

2.4. Cell assays

2.4.1. Cytotoxicity and in vitro cytogenetic toxicity

MTT assay is a cell viability test commonly used to determine the cytotoxicity potential of chemicals. In the present study, CHO-K1 cells were seeded in 96-well culture plates. Once all of the cells attached to the plate surface, different concentrations of 4-Thiozolidone, ranging from

1 to 20 μM, were added in triplicates and incubated for 24 h. The media was discarded carefully to avoid cell dislodgement, and serum-free media containing MTT was added. The plate was then kept in a CO_2 incubator for 2 – 3 h to allow drying. Next, 100 μl of dimethylsulfoxide (DMSO) was added to each well to dissolve the crystals, and OD readings were taken at 570 nm to calculate the percentage of cell viability.

Based on the cytotoxicity results from the MTT assay, three sub-lethal concentrations of the (4-TH) (2, 5, and 7.5 μM) were selected for further cytogenetic toxicity studies, including chromosome aberration, mitotic index, and MN assays. All these tests were performed following our previously published work[31,33].

2.4.2. Chromosomal aberration assay

Chromosome aberration assay is performed to assess the clastogenic effects of compounds. In the present study, mitomycin-C and DMSO were used as the positive and vehicle controls, respectively, for comparison. The assay procedure followed our earlier work with some minor modifications, which are described briefly in the following. Cells were cultured and categorized into five different test groups. One group was treated with only DMSO as a vehicle control, and another with mitomycin-C (2.5 μM) as a positive control. The remaining three groups were treated with three different concentrations of 4-TH. All the treated cells were incubated for 24 h in 5% CO_2 at 37°C, then transferred to fresh media, and treated with 0.02% (w/v) colchicine for 30 – 40 min to arrest the cells in metaphase. The collected cells were washed with phosphate-buffered saline (PBS) and trypsinized. After hypotonic treatment with 0.5% KCl, cells were incubated in a 37°C incubator for 20 – 30 min and then centrifuged at 2000 rpm for 5 min. The cell pellet was collected and fixed with a fixative (1:3 v/v methanol and acetic acid). Chromosome slides were prepared using the flame drying method and stained with 10% Giemsa. At least 100 well-spread chromosome spreads in triplicates were scored from each concentration of 4-TH and from both control groups. During the count, each metaphase spread was screened to determine the clastogenic effects of the chemical by observing chromatid and chromosome aberrations, such as gaps, breaks, fragments, minutes, pulverization, and translocations.

2.4.3. Mitotic index assay

Mitotic index assay is performed to assess the effect of a compound on the proliferation rate of the cells and provides information on the rate of cell division in the presence of a toxicant. In the present study, slides were prepared following a similar procedure to the chromosome aberration assay, and the mitotic index was obtained from the ratio of the number of dividing cells (prophase to telophase) to the total number of cells observed. At least 2000 cells per test concentration were screened in triplicates.

2.4.4. MN assay

MN assay is a method used to assess both the clastogenic and aneugenic potency of a test chemical. In this study, CHO-K1 cells were treated with three different concentrations of 4-TH for 24 h. After treatment, the cells were washed with PBS and treated with cytochalasin-B (3 μg/ml) for another 24 h to obtain bi-nucleated cells. Subsequently, the cells were collected and incubated with 0.9% (v/v) sodium citrate for 10 min at 8°C. Following this, the cells were centrifuged at 1200 rpm for 5 min, and the pellet was collected. To this pellet, 0.5 ml of fresh sodium citrate was added, and smears were prepared on clean, grease-free glass slides. The slides were then kept overnight at 37°C in an incubator under 85% relative humidity. Finally, the slides were stained with 0.5% of Giemsa stain to visualize and score the presence of MN. At least 2000 cells were scored in triplicate to calculate the percentage of MN for each concentration, which was then compared with the control groups.

2.4.5. Cell cycle analysis

The effect of 4-TH on different phases of the cell cycle was assessed using flow cytometry analysis, following our previously reported protocol[33]. Briefly, CHO-K1 cells were treated with three different concentrations of 4-TH (2, 5, and 7.5 μM). Following treatment, the cells were trypsinized, collected in ice-cold 1 × PBS, and then washed twice. Subsequently, they were fixed overnight in 70% ethanol at −20°C. The following day, the sample was washed with 1×PBS, and the cells were stained using a propidium iodide (PI) solution supplemented with RNAse and triton-X for 45 min at room temperature. After staining, the cells were washed with 1 × PBS to remove any unbound PI, and cell cycle analysis was performed using the BD fluorescence-activated cell sorting (FACS) Canto system (USA).

2.5. Statistical analysis

Each experiment was carried out in triplicate. The results were analyzed using Student's *t*-test, followed by one-way analysis of variance tests (Dennett's post-test was performed using GraphPad Prism version 8, GraphPad Software, San Diego, CA). The mean differences were compared with the corresponding control samples to determine the level of significance.

3. Results and discussions

Despite the numerous bioactive properties reported for 4-thiazolidinone derivatives, a comprehensive

safety assessment is essential for their further clinical development[34]. Therefore, in this study, we conducted a detailed cytogenetic investigation on 4-TH using a normal mammalian cell line.

3.1. Cytotoxic and antioxidant effects of 4-TH

The cytotoxicity of 4-TH was tested at various concentrations ranging from 0.1 µM to 100 µM on the CHO-K1 cell line, and 50% cell death was observed at 7.5 µM concentration (Figure 1), indicating a high cytotoxic nature of the compound. Using this IC_{50} value as a reference, we selected three sub-lethal concentrations and conducted tests on normal CHO-K1 cells to assess their cytogenetic toxicity and effects on the cell cycle. In addition, we investigated whether the toxic effects were due to radical generation or direct interaction with the genetic material by evaluating the antioxidant properties of 4-TH using the DPPH assay.

The DPPH assay conducted on 4-TH demonstrated significantly lower antioxidant activity compared to the standard compound butylated hydroxytoluene. These observed antioxidant properties of 4-TH were consistent with the previous results on various thiazolidinone derivatives of 1,3-thiazole and 1,3,4-thiadiazole[35]. In general, the phenolic hydroxyl group is associated with antioxidant activities due to hydrogen donation and free radical stabilization through resonance. The results showed that the scavenging effect increased with increasing 4-TH concentrations, with the EC_{50} value found to be 50 µM (Figure 2). The antioxidant activity could be attributed to the presence of phenolic hydroxy groups in the structures. However, IC_{50} in CHO-K1 cells revealed that even at 7.5 µM, it induced 50% cell death. This high toxicity in the presence of 4-TH could be due to an increase in oxidant radical generation, even at low concentrations. These radicals may interact with cell biomolecules and genetic material, causing more cytotoxic effects in CHO-K1 cells, even at very low concentrations.

3.2. Effect of 4-TH on cell cycle phases

We performed FACS to evaluate changes in cell cycle phases and understand the toxic effect of 4-TH on CHO-K1 cells. As shown in Figure 3, all three tested concentrations of 4-TH (2.5, 5, and 7.5 µM) induced apoptotic cells in the sub-G1 phase, with percentages of 8.7%, 5.9%, and 7.5%, respectively. In addition, a higher accumulation of cells was observed in G0/G1 phase. This arrest of cells at the G0/G1 phase could be attributed to the DNA repair process, indicating that the cells experienced a highly toxic exposure to the compound and required time to pass through S-phase. Thereby there was a reduction in cell population in the S phase. Moreover, during the G2/M

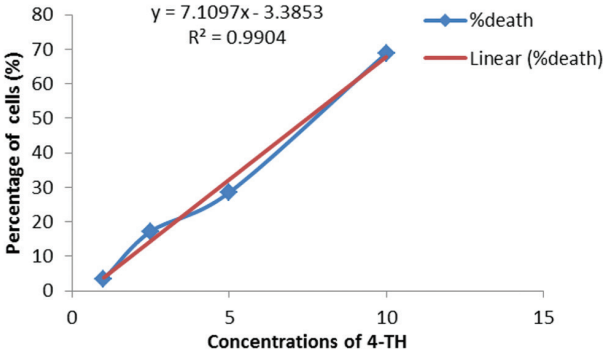


Figure 1. Percentage of cytotoxic effect (MTT assay) of 4-TH on CHO-K1 cell line.

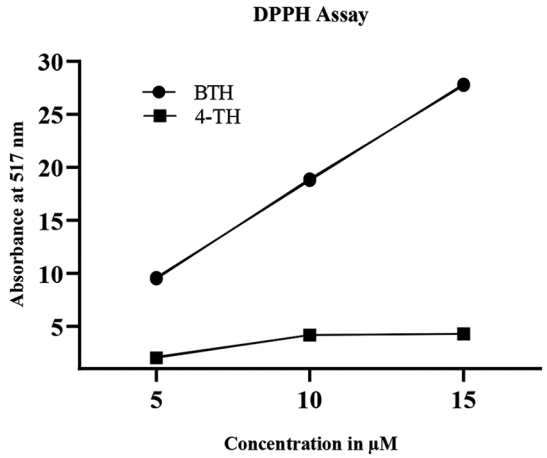


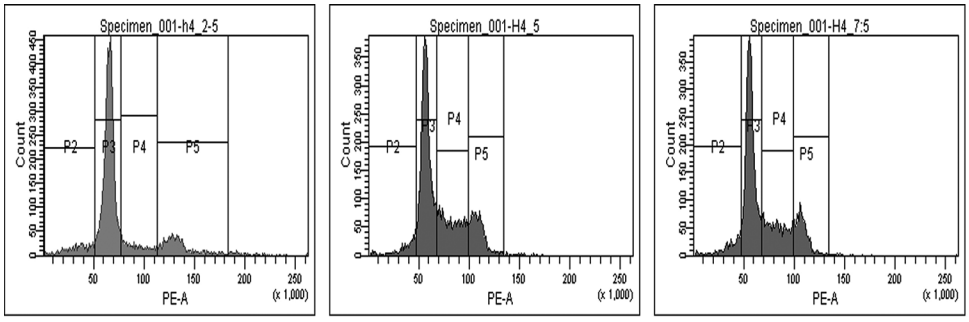
Figure 2. Antioxidant activity of 4-TH. Data are presented as the mean of two independent replications ± standard error of the mean (SE); EC_{50} value of 4-TH = 50 µM.

phase, a higher number of cells accumulated compared to other phases, suggesting that cells were undergoing DNA repair during mitotic cell division. This study provides evidence that 4-TH has the ability to induce DNA damage, leading to the activation of apoptosis pathways in the cells. It is also assumed that the unrepaired DNA-damaged cells from the G1 phase finally arrest at the G2/M phase for further DNA repair before entering the next cell division. Hence, 4-TH is likely to induce cell death, possibly through apoptosis, and the toxicity caused is independent of the S phase.

3.3. Cytogenetic toxicity of 4-TH

3.3.1. Chromosomal aberration assay

Chromosomal aberrations were assessed in CHO-K1 cell lines after 24 h of post-treatment with DMSO, mitomycin-C, and 4-TH at different concentrations (2.5, 5, and 7.5 µM). DMSO-treated cells exhibited 26



Test chemical	Concentration (µM)	Cell cycle distribution (%)			
		Sub-G1	G1/G0	S	G2/M
4-TH	2.5	8.7	64.4	9.5	15.3
	5	5.9	51.2	25.8	16.5
	7.5	7.5	52.8	23.5	15.8

Figure 3. The effect of 4-TH on the cell cycle distribution in CHO-K1 cells.

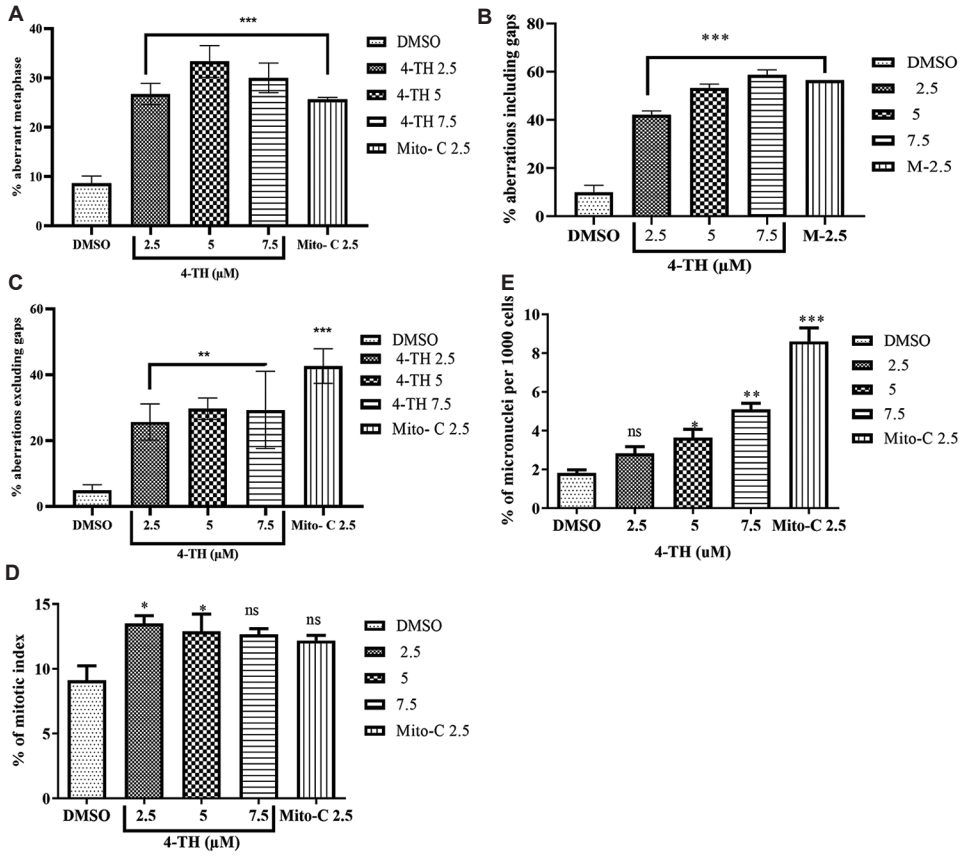


Figure 4. Cytogenetic toxicity of 4-TH in different concentrations on Chinese hamster ovary cells. (A) Percentage of aberrant metaphases; (B) total number of aberrations including gaps; and (C) total number of aberrations excluding gaps. (D) Changes in mitotic index and (E) induction of micronucleus. ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$ compared to dimethylsulfoxide (vehicle control) using Dunnett's multiple comparison test.

aberrant metaphases (8.63%) out of 301 metaphases, with 9.95% of aberrations including gaps and 4.9% excluding gaps. Mitomycin-C induced 90 aberrant metaphases (25.63%) out of 351 metaphases, with 56.41% of aberrations including gaps, and 40.48% excluding gaps. During the evaluation, mitomycin-C treated cells induced a greater number of complex chromosomal abnormalities like triradial and dicentric chromosomes. The induction of chromosomal aberrations was found to be statistically significant at $P < 0.001$ level compared to the group treated with DMSO (Table 1). 4-TH compound at 2.5 µM induced 73 aberrant metaphases (26.76%) out of 270 metaphases, with 42.22% of aberrations including gaps, which were statistically significant compared to DMSO-treated cells ($P < 0.001$). Moreover, the percentage of aberrations excluding gaps was 26.29%, which showed statistical significance when compared with the vehicle control ($P < 0.01$). At an intermediate concentration of 5 µM, 101 aberrant metaphases (33.33%) were observed out of 301 metaphases, with 53.15% aberrations including gaps ($P < 0.001$), and 29.90% of aberrations excluding gaps ($P < 0.01$) compared to DMSO-treated cells. The highest concentration of 7.5 µM induced 90 aberrant metaphases (30%), with 58.66% aberrations including gaps, which were statistically significant when compared to the vehicle control ($P < 0.001$). In addition, the total number of aberrations excluding gaps (29.33%) was statistically significant ($P < 0.01$) when compared to the vehicle control (Figure 4A-C).

DNA strand-breaks induced by the toxic agents can manifest as various forms of chromosome aberrations, depending on the phase of the cell cycle. For example, DNA damage occurring during the G1 phase mostly leads to chromosome aberrations such as chromosome gaps, chromosome breaks, dicentric chromosomes, or ring chromosomes. On the other hand, when the agent causes DNA damage during the S-phase of the cell cycle, it primarily results in chromatid aberrations, including chromatid gaps, breaks, fragments, or minutes[31,36]. Among all the tested concentrations of 4-TH, the highest concentration (7.5 µM) induced a higher number of translocations, ring chromosomes, and other complex chromosomal aberrations compared to other concentrations. In addition, this chromosome aberration test confirms that 4-TH at all three tested concentrations exhibits highly clastogenic properties (Table 1).

3.3.2. Mitotic index

To understand the rate of cell division in the presence of different concentrations of 4-TH, the mitotic index was analyzed. The cells treated with DMSO exhibited 277 dividing cells (9.12%) out of a total of 6039 cells observed. In contrast,

Table 1. Frequency of chromosome aberrations induced by 4-TH at three different concentrations in CHO-K1 cells 24-h post-treatment

Compound	Dose (µM)	No. of metaphases observed	No. of aberrant metaphases	Types of aberrations									% of aberrant metaphase	Total no. of aberrations	% of aberrations including gaps	% of aberrations excluding gaps
				Chromatidtype of aberrations		Chromosome type of aberrations		F	M	Tr	Qr	Dic				
				G	B	G	B									
DMSO (vehicle control)	1%	301	26	15	13	0	1	0	0	0	0	0	8.63±0.85	30	9.95±1.69	4.96±0.96
4-TH	2.5	270	73	33	17	5	3	2	4	1	1	16	26.76±1.24***	114	42.22±0.93***	26.29±0.96**
	5	301	101	48	26	11	5	2	2	1	0	24	33.33±1.85***	160	53.15±1.01***	29.90±1.87**
	7.5	300	90	44	14	22	8	8	1	2	0	20	30.00±1.73***	176	58.66±1.20***	29.33±6.76**
Mitomycin-C (positive control)	2.5	351	90	34	46	11	5	4	7	9	0	25	25.63±0.23***	198	56.41±3.53***	40.48±3.03***

Notes: DMSO and mitomycin-C as vehicle and positive controls, respectively. Types of chromosomal aberrations: G: Gaps; B: Breaks; F: Fragment; M: Minute; Tri: Triradial; Qr: Quadra radial; Dic: Dicentric. The level of significance determined in the treated group was compared with the vehicle control using Dunnett's multiple comparison test. ***P<0.001; **P<0.01; ±: Mean±SEM.

Table 2. Changes in the mitotic index observed after 24-h post-treatment with three different concentrations of 4-TH in CHO-K1 cells

Compound	Dose (μM)	Total number of cells	Total number of dividing cells observed	Percentage of mitotic index
DMSO	--	3035	277	9.12±1.12
4-TH	2.5	3007	406	13.50±0.60*
	5	3027	390	12.88±1.33*
	7.5	3028	383	12.64±0.45 (ns)
Mitomycin- C	2.5	3051	371	12.15±0.42 (ns)

Notes: DMSO and mitomycin-C as vehicle and positive controls, respectively. ±: SEM; ns: Not significance and *P<0.05 compared to DMSO (vehicle control) using Dunnett's multiple comparison test

Table 3. Induction of micronuclei in CHO-K1 cells after treatment with three different concentrations of 4-TH for 24 h

Chemical used	Dose (μM)	Total number of cells scored	Number of micronucleus observed	Percentage of micronuclei/1000 cells
DMSO	-	6039	11	1.82±0.16
4-TH	2.5	6016	17	2.82±0.34 (ns)
	5	6048	22	3.63±0.43*
	7.5	6077	31	5.10±0.31**
Mitomycin-C	2.5	6046	52	8.60±0.70***

Notes: DMSO and mitomycin-C as vehicle and positive controls, respectively. ±: SEM; ns: Not significant, ***P<0.001, **P<0.01, *P<0.05 compared to DMSO (vehicle control) using Dunnett's multiple comparison test.

the positive control, the cells treated with mitomycin-C, showed 371 (12.15%) dividing cells out of 3051 cells, and the mitotic index was statistically not significant (Table 2). Similarly, in CHO-K1 cells treated with 4-TH at 2.5 μM and 5 μM concentrations, the rate of dividing cells was significantly higher compared to the vehicle control ($P < 0.05$). However, no significant difference in the rate of dividing cells was observed when the cells were treated with 4-TH at a concentration of 7.5 μM. The lowest and intermediate concentrations of 4-TH induced a significant increase in cell division compared to control DMSO ($P < 0.05$). The highest concentration of 4-TH induced a lower percentage of dividing cells, and it was not statistically significant compared to DMSO-treated cells (Figure 4D). Interestingly, all these three concentrations of 4-TH induced a greater number of dividing cells compared to the positive control group. These results are also correlated with the cell cycle study, where a greater number of cells accumulated in the G/M phase.

3.3.3. Micronuclei

In the DMSO-treated CHO-K1 cells, 11 micronuclei (1.82%) were observed out of 6039 cells. However, cells treated with mitomycin-C (2.5 μM) had a significantly higher percentage of MN (8.60%), with 51 micronuclei observed out of 6046 cells ($P < 0.001$). Among the 4-TH treatment groups, cells treated with 2.5μM concentration exhibited 17 MN (2.82%) out of 6016 cells, 5 μM concentration exhibited 22 micronuclei (3.63%) out of 6048 cells, and 7.5 μM concentration exhibited 31 MN (5.10%) out of 6077 cells. The lowest concentration of 2.5 μM did not show any statistical significance, while the intermediate concentration of 5 μM showed statistical significance at $P < 0.05$ level, and the highest concentration of 7.5 μM showed statistical significance at $P < 0.01$ level when compared to the vehicle control. Among all the tested concentrations, 7.5 μM showed the highest percentage of MN induction (Table 3). The formation of a greater number of MN is also correlated with the observed chromosome aberrations, such as breaks, fragments, and minutes, which could not participate during the anaphase stage and ultimately form MN[37,38]. Hence, based on this MN test, it is confirmed that all the tested concentrations of 4-TH exhibit a highly aneugenic nature.

4. Conclusion

Our *in vitro* genotoxicity assessments on the mammalian cell line system revealed that 4-TH possesses high clastogenic and aneugenic properties, induces apoptosis, and significantly affects the proliferation rate of normal cells. These findings raise concerns about the potential carcinogenic and mutagenic effects of 4-TH on normal cells, which could pose health risks, including the recurrence of secondary cancers post-treatment with this compound as a drug. Therefore, further investigations are imperative to ensure the safety of 4-TH.

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Conflict of interest

All the authors declare that they have no competing interests.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Supporting data can be obtained from corresponding author following formal request.

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ACCSCIENCE PUBLISHING

INNOSC Theranostics and Pharmacological Sciences

ORIGINAL RESEARCH ARTICLE

Evaluation of the microbial quality of commercial liquid herbal preparations on the Ghanaian market

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Abstract

Conventionally, the microorganisms in liquid herbal mixtures are curtailed due to the fresh preparation before the administration to patients. Prolonged storage of liquid herbal preparations (due to commercialization) coupled with primeval routine production processes may increase the potential of microbial contamination in liquid herbal preparations. This study aims to analyze the microbial quality of 15 selected commercial liquid herbal preparations on the Ghanaian market. The samples were obtained from accredited pharmacies and herbal outlets in the Greater Accra region of Ghana, specifically Central Accra, between November 2019 and January 2020. The selected samples were coded HM1 to HM15. The effectiveness of the primary package of all samples was determined using the seal integrity test. The presence of microorganisms in the sampled brands was determined using nutrient agar. Isolated microorganisms from the sampled herbal mixtures were then identified using various selective media. All 15 samples (100%) passed the seal integrity test. Ten (67%) out of the 15 samples were contaminated with various microorganisms, whereas the remaining 5 samples (33%) were devoid of microorganisms. Eight (53%) out of the 15 samples were contaminated with fungi, with 3 (20%) being above the pharmacopeial limit. Six (40%) out of the 15 samples showed the presence of *Escherichia coli*. Out of the 15 sampled products, only HM11 contained *Staphylococcus aureus*. Similarly, only one sampled product (HM15) contained *Salmonella* typhi. None of the sampled products was contaminated with *Pseudomonas aeruginosa*. Ultimately, this study revealed that commercialized liquid herbal preparations in Ghana are likely to be contaminated with pathogenic microorganisms. Good manufacturing practices must therefore be strictly adhered to bring out the best in local herbal manufacturing industries.

Keywords: Herbal medicines; Microbial quality; Good manufacturing practices

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1. Introduction

Traditional medicine is described by the World Health Organization (WHO) as the total knowledge, skills and practices based on the theories, beliefs, and experience indigenous to different cultures, whether explicable or not, that are used to maintain health as well as to prevent, diagnose, improve, or treat physical and mental illness[1]. Thus, African traditional medicine is indigenous to African culture and well patronized by the general populace. According to the WHO, the use of traditional medicine has increased exponentially over the past decade[1,2].

Traditional medicine plays a crucial role in healthcare all over the world, especially in developing countries. In Ghana, traditional medicines form an integral component of the health care system and due to cultural diversity in Ghana; herbal medicines are also highly diverse. Herbal medicines may contain either single or multiple herbs in a single drug preparation. Due to this reason, herbal medicines contain many chemical compounds that have therapeutic benefits in a wide variety of diseases[3]. The perception that herbal medicines are relatively safe and harmless is one of the key reasons for the recent increase in its usage among Ghanaians. However, the quality and safety of herbal products have become a matter of public interest due to their commercialization, global market expansion, and universality[2,4]. Studies carried out by Abba *et al.* in 2009 indicated that 46.67% of sampled herbal products in Kaduna were contaminated with *Salmonella* typhi, while 58.67% and 65.33% were contaminated with *E. coli* and *Staphylococcus aureus*, respectively[5]. Concomitantly, studies carried out by Enayatifard *et al.* in 2010 revealed that all the sampled herbal products (n = 20) in Sari were contaminated with *Salmonella* spp.[6]. These studies have revealed the potential serious health implications of herbal products and raise a serious public health concern about the paradoxical notion of the safety of herbal medicinal products in general.

In Ghana, approximately 65% of the population relies on herbal medicine alone to meet their basic health-care needs[7,8]. The majority of these herbal products, which have been licensed by the Food and Drugs Authority (FDA) of Ghana for sale on the Ghanaian market, are mostly in liquid dosage forms. Studies carried out by Agyeman-Duah *et al.* in 2017 indicated that herbal powders marketed in Ghana were contaminated with microorganisms[7]. Herbal liquid preparations are known to be more susceptible to microbial growth due to varied reasons, such as presence of water or moisture, contamination in raw materials, and primeval routine production processes. These microorganisms can make the product esthetically unpleasant, change the color of the preparation, and if they are pathogenic, cause serious infections in the patient with a resultant decrease in patronage and usage by clinicians and patients. It is therefore very crucial to perform assessment of the level of microbial contamination in herbal liquid mixtures after preparation as a routine post market surveillance activity[9,10]. In view of this, our study sought to evaluate the microbial quality of 15 selected commercial liquid herbal preparations on the Ghanaian market.

2. Materials and methods

2.1. Materials

All culture media, such as nutrient agar, MacConkey Agar, cetrimide nutrient Agar, Sabouraud dextrose agar, mannitol salt agar, and bismuth sulfite agar, were provided by Central University Microbiology Laboratory stores and sourced from Merck chemical company limited.

2.2. Methods

2.2.1. Sample collection

The samples were obtained from the accredited pharmacies and herbal medicine outlets in the Greater Accra region of Ghana, specifically Central Accra, between November 2019 and January 2020. The shelf life of all samples was longer than a year from the date of purchase.

2.2.2. Seal integrity test

The seal integrity test was done by inverting and clamping each sampled herbal product onto a ring stand. The herbal product was then immersed into a glass beaker full of water containing dye with the cap completely submerged. For prevention of any leakage, the setup was monitored for 30 min[11-13].

2.2.3. Preparation of media for microbial analysis

The various media, such as nutrient agar, cetrimide agar, bismuth sulphite agar, mannitol salt agar, MacConkey agar, and Sabouraud dextrose agar, were prepared according to the International Pharmacopeia 2019 and British Pharmacopeia 2018 standards[14,15].

2.2.4. Microbial analysis

2.2.4.1. Presence of microorganisms

Nutrient agar was used in ascertaining the presence of microorganisms in all the 15 samples (HM1 to HM15) using the method described by Esimone *et al.* and Okunlola *et al.*[16,17]. The procedure was carried out in triplicates for each sample.

2.2.4.2. Test for fungi (yeast or molds)

One milliliter of each sample was pipetted and subjected to a 10-fold serial dilution to 10^{-6}. An inoculum of 0.1 mL

of the 10^{-6} dilution was spread-plated on the well-dried surface of Sabouraud dextrose agar and incubated at 25°C (room temperature) for 4 days to allow for possible growth of fungi[2,15,18,19]. Each sample was inoculated in triplicates.

2.2.4.3. Test for E. coli

One milliliter of each sample was pipetted and subjected to a 10-fold serial dilution to 10^{-6}. An inoculum of 0.1 mL of the 10^{-6} dilution was spread-plated on the well-dried surface of MacConkey agar. The Petri dishes containing the spread-plated MacConkey agar were inverted and incubated at 37°C for 24 h to allow for possible growth of *E. coli*[2,15,18,19]. Each sample was inoculated in triplicates.

2.2.4.4. Test for S. typhi

One milliliter of each sample was pipetted and subjected to a 10-fold serial dilution to 10^{-6}. An inoculum of 0.1 mL of the 10^{-6} dilution was spread-plated on the well-dried surface of bismuth sulfite agar in a petri dish. The petri dishes containing the spread-plated bismuth sulfite agar were inverted and incubated at 37°C for 24 h to allow for possible growth of *S.* typhi[2,15,18,19]. Each sample was inoculated in triplicates.

2.2.4.5. Test for Pseudomonas aeruginosa

One milliliter of each sample was pipetted and subjected to a 10-fold serial dilution to 10^{-6}. An inoculum of 0.1 mL of the 10^{-6} dilution was spread-plated on the well-dried surface of cetrimide agar. The petri dishes containing the spread-plated cetrimide agar were inverted and incubated at 37°C for 24 h to allow for possible growth of *P. aeruginosa*[2,15,18,19]. Each sample was inoculated in triplicates.

2.2.4.6. Test for S. aureus

One milliliter of each sample was pipetted and subjected to a 10-fold serial dilution to 10^{-6}. An inoculum of 0.1 mL of the 10^{-6} dilution was spread-plated on the well-dried surface of mannitol salt agar. The petri dishes containing the spread-plated mannitol salt agar were inverted and incubated at 37°C for 24 h to allow for possible growth of *S. aureus*[2,15,18,19]. Each sample was inoculated in triplicates.

3. Result

Manufacturing dates, expiry dates, and batch numbers obtained from the sampled liquid herbal products together with their designated codes are summarized in Table 1. The shelf life of all sampled products was longer than 1 year from the date of purchase.

Results of the seal integrity test on the primary package of the sampled herbal liquid preparations are summarized in Table 2. None of the sampled herbal liquid preparations failed the sealed integrity test.

Table 1. Information of selected samples

Code	Manufacturing date (month/year)	Expiry date (month/year)	Batch number
HM1	06/19	06/21	LVCM 000290 LHC
HM2	05/19	05/22	HL-0015V
HM3	06/18	06/21	001
HM4	06/19	06/22	00G05
HM5	03/19	03/22	0319
HM6	08/18	08/22	FPVL0018
HM7	11/19	11/21	TX97
HM8	10/19	10/21	00342
HM9	02/19	02/21	MAMI 01/2019
HM10	10/19	10/21	0052
HM11	01/19	01/24	004
HM12	11/17	11/22	004
HM13	08/19	7/21	MC/08/19
HM14	03/19	03/21	ADT/1116
HM15	10/19	10/21	589

Table 2. Seal integrity test results for sampled herbal preparations

Sample code	Observation
HM1	No leak observed
HM2	No leak observed
HM3	No leak observed
HM4	No leak observed
HM5	No leak observed
HM6	No leak observed
HM7	No leak observed
HM8	No leak observed
HM9	No leak observed
HM10	No leak observed
HM11	No leak observed
HM12	No leak observed
HM13	No leak observed
HM14	No leak observed
HM15	No leak observed

The results of the presence or otherwise of specific microorganisms in the sampled liquid herbal preparations are shown in Table 3. HM1, HM2, HM3, HM8, and HM10 were not contaminated with microorganisms based on the nutrient agar results, and these results were confirmed by the absence of specific microorganisms in the various selective media used. Samples HM4, HM5, and HM9 were contaminated with both fungi and *E. coli*, while samples

HM6, HM7, and H13 contained only fungi. HM11 was contaminated with both fungi and *S. aureus*, while HM12 and HM15 were contaminated with *E. coli*. Fungi, *E. coli,* and *S.* typhi were found to be present in HM14.

The level of microbial contamination in the sampled herbal preparations is recorded in Table 4. Samples HM4, HM5, and HM12 had their *Escherichia coli* counts beyond pharmacopeial limits whiles HM9, HM14, and HM15 had *Escherichia coli* levels within pharmacopeial limits[20]. Samples HM 7, HM11, and HM14 had fungi counts beyond pharmacopeial limits whiles HM4, HM5, HM6, HM9, and HM13 had their counts being within pharmacopeial limits[15,19-21]. *S.* typhi counts in HM 14 were above pharmacopeial limits[15,19-21]. None of the sampled herbal liquid preparations contained *P. aeruginosa*. HM11

Table 3. Results of microbial analysis

Sample	Nutrient agar	Fungi	*E. coli*	*S.* typhi	*P. aeruginosa*	*S. aureus*
HM1	No growth	No growth	No growth	No growth	No growth	No growth
HM2	No growth	No growth	No growth	No growth	No growth	No growth
HM3	No growth	No growth	No growth	No growth	No growth	No growth
HM4	Growth	Growth	Growth	No growth	No growth	No growth
HM5	Growth	Growth	Growth	No growth	No growth	No growth
HM6	Growth	Growth	No growth	No growth	No growth	No growth
HM7	Growth	Growth	No growth	No growth	No growth	No growth
HM8	No growth	No growth	No growth	No growth	No growth	No growth
HM9	Growth	Growth	Growth	No growth	No growth	No growth
HM10	No growth	No growth	No growth	No growth	No growth	No growth
HM11	Growth	Growth	No growth	No growth	No growth	Growth
HM12	Growth	No growth	Growth	No growth	No growth	No growth
HM13	Growth	Growth	No growth	No growth	No growth	No growth
HM14	Growth	Growth	Growth	Growth	No growth	No growth
HM15	Growth	No growth	Growth	No growth	No growth	No growth

Abbreivations: *E. coli: Escherichia coli*; *S.* typhi: *Salmonella* typhi; *S. aureus*: *Staphylococcus aureus*; *P. aeruginosa*: *Pseudomonas aeruginosa.*

Table 4. Level of microbial contamination in sampled herbal preparations

Sample	*E. coli* (cfu/mL)	Fungi (cfu/mL)	*S.* typhi (cfu/mL)	*S. aureus* (cfu/mL)	*P. aeruginosa* (cfu/mL)
HM1	-	-	-	-	-
HM2	-	-	-	-	-
HM3	-	-	-	-	-
HM4	TNC	$<1\times10^1$	-	-	-
HM5	TNC	$<1\times10^1$	-	-	-
HM6	-	$<1\times10^1$	-	-	-
HM7	-	TNC	-	-	-
HM8	-	-	-	-	-
HM9	$<1\times10^1$	$<1\times10^1$	-	-	-
HM10	-	-	-	-	-
HM11	-	TNC	-	TNC	-
HM12	TNC	-	-	-	-
HM13	-	$<1\times10^1$	-	-	-
HM14	$<1\times10^1$	TNC	$<1\times10^1$	-	-
HM15	$<1\times10^1$	-	-	-	-

Abbreviations: TNC: Too numerous to count; *E. coli*: *Escherichia coli*; *S.* typhi: *Salmonella* typhi; *S. aureus*: *Staphylococcus aureus*; *P. aeruginosa*: *Pseudomonas aeruginosa.*

was contaminated with *S. aureus* above pharmacopeial limits[15,19-21].

4. Discussion

The safety of herbal medicinal products has been a significant concern for health agencies, pharmaceutical industries, and the general public[20]. More frequently than not, primary sources of raw materials for herbal products are tainted by several microorganisms from soils and the environment. Harvesting, handling and production activities also frequently contribute to additional microbial contamination. Commonly reported microorganism that may be present in liquid herbal preparations includes *E. coli*, fungi, *S.* typhi, *S. aureus,* and *P. aeruginosa*. Microbial contamination of liquid herbal preparations may potentially endanger health or cause severe infection if administered orally or through any means by which the organism may have exposure to the body[3,4,16,22]. A total of 15 samples consisting of herbal blood tonics and herbal preparations for piles, typhoid, and sexually transmitted infections were randomly chosen for this study (Table 1) and assessed for the presence and levels of microbial contamination.

Primary packages are expected to offer finished medicinal products a barrier to the contamination with microbes, which can be found in the natural environment and atmosphere. All the sampled herbal products (100%) passed the seal integrity test (Table 2). This indicates that their primary packages serve as a good barrier for preventing the ingress of microbes from the immediate environment and atmosphere into the finished herbal products; therefore, any microorganisms that could be found in the herbal product could have been introduced during the production processes or contaminated from its source, the starting raw materials[11,12].

Microbial contamination limits in herbal medicinal products set by the European Pharmacopoeia (EP), British Pharmacopoeia (BP), United States Pharmacopoeia (USP), and WHO help in the maintenance of product safety and efficacy, ultimately safeguarding the health of the consumer. A majority (67%) of the sampled herbal products were contaminated with microorganisms, whereas the remaining 33% showed no growth of any microorganism (Table 3). All the sampled herbal products (100%) were not contaminated with *P. aeruginosa* (Table 4) and hence passed the microbial limit of *P. aeruginosa* contamination in herbal medicinal products as specified by the British Pharmacopoeia[15,19-21]. *P. aeruginosa* is an opportunistic pathogen and a major cause of nosocomial infections; therefore, their absence in all the sampled herbal products is a desirable attribute. According to the British Pharmacopoeia, *S.* typhi should not be present in oral herbal preparations[23]. According to our findings, only one product (HM14) was contaminated with *S.* typhi (Table 4). This has grave consequences on the consumers of this product (HM14) since *S.* typhi is known to cause typhoid fever with debilitating effects. However, 93% of the sampled herbal products did not contain *S.* typhi and complied with the specifications set by the British Pharmacopoeia[15,19-21]. While the British Pharmacopoeia stipulates that oral herbal preparations should not contain *E. coli,* the WHO recommends a limit of not more than 1×10^1 Cfu/mL. Based on the limits set by the British Pharmacopoeia[15,19,21], 40% of the sampled products were contaminated with *E. coli*. Conversely, 20% of the herbal products exceeded the recommended WHO limits for *E. coli* (Table 4)[20]. *E. coli* has been implicated in common bacterial infections, including cholecystitis, cholangitis and urinary tract infections. This may result in a blind spot in the clinics, where clinicians may attribute complication of traditional medicine consumption to be the cause of such disease conditions while the reason could be microbial contamination. Their presence in herbal medicines may exacerbate the disease conditions of consumers with dire consequences. Only one sampled product (HM11) was contaminated with *S. aureus* (Table 4) and failed the specifications set by the British Pharmacopoeia and the WHO[15,19-21]. *S. aureus* has the potential of causing a wide range of conditions and life-threatening diseases, including skin infections, abscesses, pneumonia, and meningitis. Thus, the presence of this microorganism in HM11 can potentially aggravate the existing pathological condition(s) of the consumer or result in the development of life-threatening complications. Eight (53%) out of the 15 samples showed fungal growth, with 5 (33%) of the infected samples (HM4, 5, 6, 9, and 13) showing colony forming units which complied with the British Pharmacopoeia and the WHO specifications while the remaining infected samples (HM7, 11, and 14) exceeded the set limits[15,19-21]. High levels of fungi in oral preparations can cause candidiasis of the mouth, throat, and esophagus. They are also known to cause skin infections and, in extreme cases, pneumocystis pneumonia[24]. Thus, high levels of fungi in these herbal medicinal products (HM7, 11, and 14) will have negative health effects on the consumers, which may manifest as new disease condition(s), such as pyelonephritis and gastrointestinal hemorrhage. Ultimately, patients taking two or more of the sampled herbal products (contaminated with microorganisms) for the treatment of different ailments may be at risk of contracting serious infections and complicating their disease conditions due to the presence of these pathogenic microorganisms in their gastrointestinal tract and eventually in their blood.

5. Conclusion

The microbial quality of the sampled herbal formulations was evaluated and the microbes found to be present in the various herbal samples included *E. coli*, fungi, *S.* typhi, *P. aeruginosa,* and *S. aureus*. Only 33% of the sampled herbal products were not contaminated with microorganisms. Producers of herbal liquid preparations in Ghana should be educated on the need to enforce good manufacturing practices, effective harvesting practices, and safe handling and storage of liquid herbal medicinal products. Periodic sanitization and education courses should be carried out by various regulatory and research agencies, such as the FDA of Ghana and the Ghana standards authority, using easy-to-interpret flyers as well as online and in-person refresher courses.

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

The data used to support the findings of this study are included in the article and also available from the corresponding author on reasonable request.

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ACCSCIENCE PUBLISHING

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COMMENTARY

Tryptophan metabolism in schizophrenia

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This commentary aims to emphasize the molecular mechanisms involved in the pathophysiology of schizophrenia and in the onset of the disease addressed in an article recently published by Carvalho *et al.*[1] In addition, it is necessary to gather recent and updated information on the role of tryptophan and its metabolites, emphasizing the relationship of this amino acid and its metabolites in the pathophysiology of schizophrenia, with a focus on the kynurenine pathway and, above all, its possible associations with the mental illnesses[2].

Carvalho *et al.* reported that when tryptophan crosses the blood–brain barrier, its metabolites exert different actions on the central nervous system (CNS)[1]. Important neuroactive metabolites resulting from tryptophan metabolism, including serotonin, melatonin, 3-hydroxykynurenine (3-HK), quinolinic, and kynurenic acid (KYNA), have been associated with neuropsychiatric diseases such as schizophrenia[2].

Schizophrenia is a disorder that shows a functional imbalance of the dopaminergic system, causing cognitive and emotional functions to be altered due to changes in the dopaminergic response. These changes can be divided into two subgroups of symptoms: positive and negative[3]. Positive symptoms, such as delusions, hallucinations, psychoses, paranoia, disordered thinking, and disorganized speech, occur as a result of dopaminergic hyperactivity in the mesolimbic area. Negative symptoms, such as demotivation, violent emotional behavior, social isolation, cognitive impairment, and slow speech, occur due to dopaminergic hypoactivity in prefrontal cortex projections[3].

Tryptophan is an essential amino acid that is not synthesized by the body and, therefore, needs to be obtained from the diet; it is present in the vast majority of foods. About 90% of ingested tryptophan is bound to plasma albumin and only 10% is found in free form in the bloodstream[4]. Non-esterified fatty acids are essential for the balance between the tryptophan in free form and the tryptophan bound to albumin, as they compete with this amino acid for binding to plasma albumin, consequently increasing the level of free tryptophan in plasma[5].

This acid amine is a substrate for the production of several neuroactive molecules with biological activities, such as serotonin (5-hydroxytryptamine [5-HT]), melatonin, 3-HK, quinolinic acid (QA), and KYNA, among others[6]. The two main known pathways of tryptophan metabolism are 5-HT and kynurenine. About 95% of tryptophan obtained from the diet is metabolized through the *kynurenine* pathway, and only about 1% of tryptophan is converted to 5-HT in the CNS, while the rest is converted to proteins and melanin[6].

As shown in Figure 1, tryptophan is a substrate for the synthesis of 5-hydroxytryptamine, also known as serotonin (5-HT), in the CNS. Tryptophan

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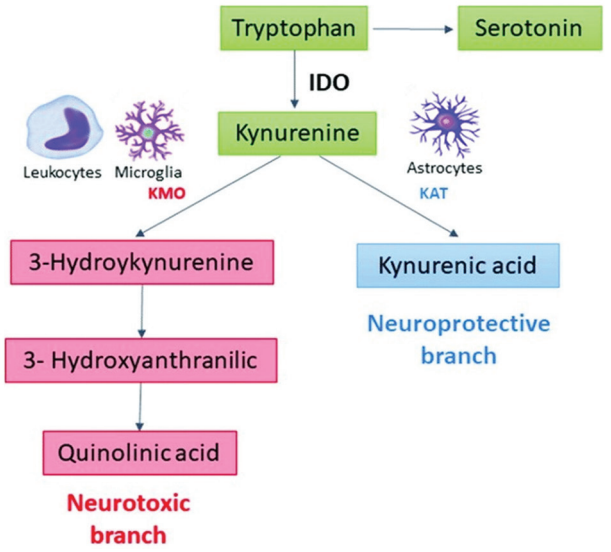

Figure 1. Tryptophan metabolism pathway.

availability may have rate-limiting effects on 5-HT synthesis[7]. Tryptophan is hydrolyzed by the action of tryptophan hydroxylase into 5-hydroxytryptophan, which is rapidly metabolized into 5-HT. 5-HT is stored in synaptic vesicles, in serotonergic neurons, until it is used and/or metabolized to 5-hydroxyindoleacetic acid[8].

5-HT produced from tryptophan, which belongs to the class of small-molecule and fast-acting neurotransmitters, is responsible for acute responses of the CNS and one of its characteristics is to intensify satiety[9]. In addition, low levels of 5-HT can lead to psychopathologies, such as depression, suicide, aggression, anxiety[10], anorexia, and bulimia. Elevated levels of tryptophan result in inhibition of gluconeogenesis, increase in blood glucose level and glucose release to the brain, thereby reducing appetite[11]. Variation in brain serotonergic activity (tryptophan and 5-HT) has been implicated in the regulation of appetite, anxiety and impulse/binge control. 5-HT inhibits neuropeptide Y, resulting in the suppression of hunger and food intake[11].

Eventually, tryptophan can still be converted into melatonin, which is a hormone synthesized by the pineal gland, and is a hormone related to sleep regulation in humans[9]. It is also important to mention that, although it is known to be found in lower concentrations in patients with schizophrenia, the importance of melatonin for some specific symptoms of schizophrenia and in inhibiting some side effects of antipsychotics is still underestimated[9]. Interestingly, the melatonin pathway, which is recognized as a genetic susceptibility factor for bipolar disorder, contributes to the increased risk of cardiovascular complications and decreased life expectancy, and is also frequently reported in schizophrenia[9].

When there is a deficiency of niacin (vitamin B3) in the body, tryptophan can be used for the synthesis of the essential cofactor nicotinamide adenine dinucleotide (NAD^+), which is an important carrier of electrons and fundamental in the production of energy for the cell[12].

Kynurenine pathway is the main route of tryptophan metabolism (Figure 2), responsible for metabolizing more than 95% of this acid amine in the human body, and the catabolic action of some important enzymes in this pathway is dependent on the presence of vitamins B2 and B6, which act as cofactors[10].

However, it is already known that the degradation of tryptophan and the activation of the kynurenine pathway generates several neuroactive compounds, such as KYNA, which is an antagonist of the N-methyl-D-aspartate (NMDA) receptor and the alpha-7-nicotinic cholinergic receptor[13]. It is also known to have a neuroregulatory role in contrast to other kynurenine products[14]. The effect of KYNA on schizophrenia can be explained by its blocking action on glutamate receptors, which are found at elevated levels in patients with schizophrenia. Recent findings have specifically supported the neuroregulatory role of KYNA[15]. KYNA impairs glutamatergic and dopaminergic neurotransmission, and its elevation in the brain is related to psychotic symptoms and cognitive impairments[16].

QA is a selective agonist of NMDA-sensitive ionotropic glutamate receptors[17]. The known neurotoxic role of QA is executed by triggering excitotoxicity and neurodegeneration. Besides, an increase of QA production, which is detrimental to other pathways, elevates the risk for neurological and psychiatric disorders, including depression and schizophrenia[17]. The balance between QA and KYNA, which act as agonist and antagonist, respectively, can be controlled by the relative expression and activity of kynurenine aminotransferases, kynureninase, and kynurenine-3-mono-oxygenase, perhaps indicating a significant physiological or pathological relevance of the quinolinate/Kynurenate relationship. Likewise, disturbances in cognition are associated with high levels of kynurenate and attributed to the slowing down or suppression of excitatory neurotransmission by this compound[13].

There is evidence that dysregulation of the tryptophan-kynurenine pathway can culminate in certain psychiatric disorders such as schizophrenia with increased tryptophan degradation that can induce serotonin depletion and depressed mood[18]. The downstream metabolites from this pathway, such as 3-HK, QA, and KYNA[18], are neuroactive components that can modulate several neurotransmissions, such as glutamatergic, GABAergic, dopaminergic, and noradrenergic neurotransmissions.

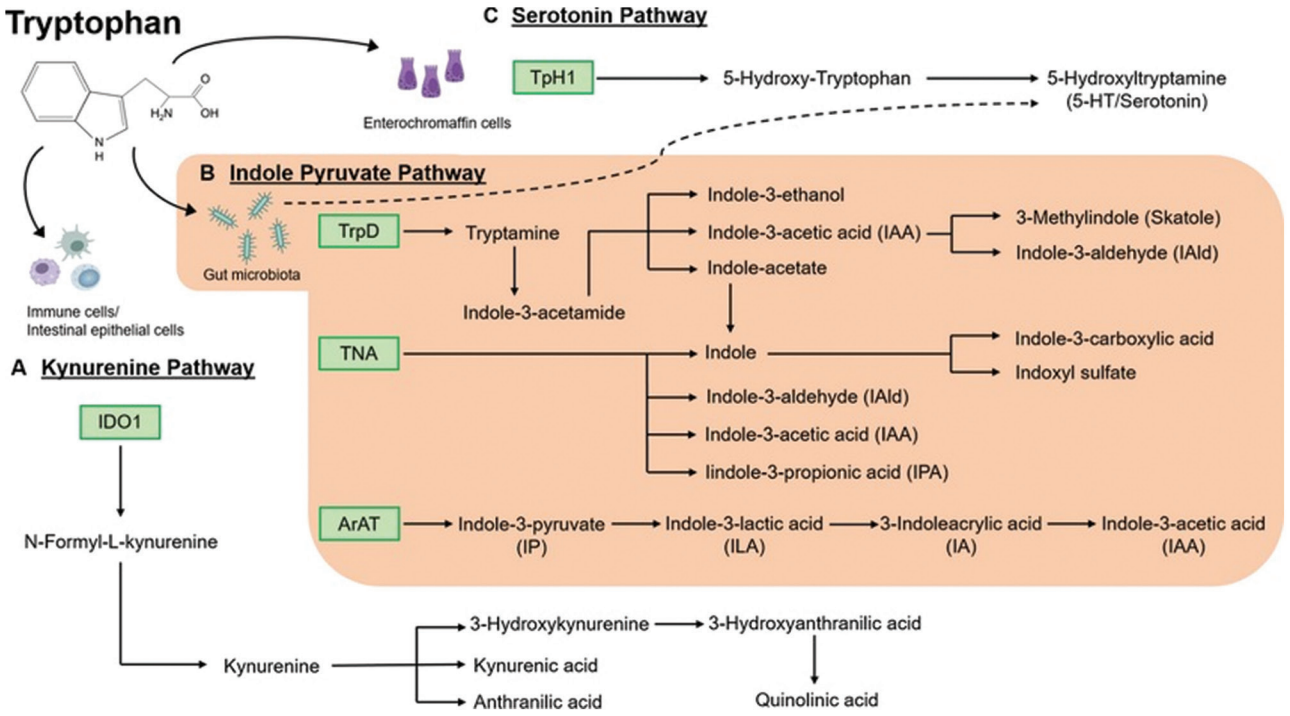

Figure 2. Tryptophan metabolism. Kynurenine (A), indole pyruvate (B), and serotonin pathways leading to different secondary metabolites.

In turn, these neurotransmissions can induce changes in the neuronal-glial network and result in neuropsychiatric consequences[19]. Myint and Kim[19] have shown that upregulation of tryptophan pyrrolase results in increased kynurenine/tryptophan ratio and decreased serotonin/tryptophan ratio in the bilateral hippocampus of the brain. Either tryptophan pyrrolase gene knockout or pharmacological inhibition of hippocampal tryptophan pyrrolase activity attenuates both nociceptive and depressive behavior[20].

Although the data from the studies are not sufficient to identify the mechanisms involved, it is possible to suggest that the low level of tryptophan is related to the decrease in the supply and the increase in the catabolism of this amino acid, which is, therefore, able to promote greater vulnerability to white matter lesions in patients, by activation of neuroinflammation, neurovascular, and neuroendocrine pathways[2].

From an etiological point of view, it is difficult to establish a single potential pathway involved in the pathophysiology of schizophrenia. Nevertheless, there is a general consensus that the development of schizophrenia is dependent on both predisposing factors (genetic, constitutional, and biochemical) and environmental factors. The dopaminergic hypothesis is widely accepted as it associates dopaminergic stimuli with the symptoms of schizophrenia. Dopamine is a central neurotransmitter released in vesicles at synapses in the CNS; in schizophrenia, the mesolimbic and mesocortical systems are involved. The dopaminergic hypothesis is based on the fact that mediation antipsychotic blocks postsynaptic dopamine receptors of the D2 subtype, resulting in an improvement in symptoms. On the other hand, the administration of dopamine agonists worsens the symptoms of schizophrenia. The emerging "second-generation" atypical antipsychotics, which have a broader profile in addition to drug blockade dopamine receptors, block type 2 serotonin receptors, suggesting a role for serotonin in the pathophysiology of schizophrenia[21].

Another neurotransmitter, glutamate, is also linked to the development of schizophrenia. According to glutamatergic hypothesis, excessive amounts of this neurotransmitter are released and exert a neurotoxic effect that triggers the symptoms of schizophrenia. Therefore, in recent years, it remains controversial whether the dopaminergic hypothesis is the only dysfunction related to schizophrenia. Current studies on the neurobiology of schizophrenia evaluate the multiplicity of factors, including genetics, anatomy (mainly through structural neuroimaging examinations), functional circuits (by functional neuroimaging examinations), neuropathology, electrophysiology, neurochemistry, neuropharmacology, and neurodevelopment[22].

In summary, schizophrenia is one of the most intriguing and well-studied psychiatric conditions. The

psychopathological richness and the clinical characteristics, such as its onset in adolescence and the deteriorating course without major neurological alterations, arouse curiosity and generate a considerable number of studies on the neurophysiological processes involved in the disease. Furthermore, increased KYNA can have several important adverse consequences in schizophrenia. Dysfunctions of these receptors have been associated with cognitive impairments and symptom manifestations seen in people with schizophrenia[3].

Many advances have made it possible to better understand the pathophysiology of schizophrenia. However, much is yet to come. Several research groups around the world, using the wide array of methodologies, are working hard to deepen our understanding of the processes involved in the disease. It is expected that the in-depth knowledge of the pathophysiological mechanisms involved will allow the development of even more effective treatments for the control and eventually the prevention of schizophrenia.

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Conflict of interest

The authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the commentary.

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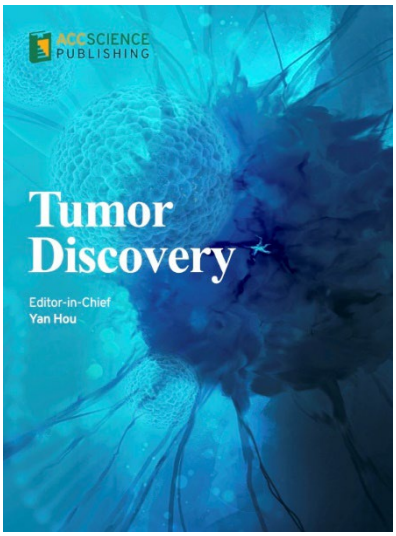

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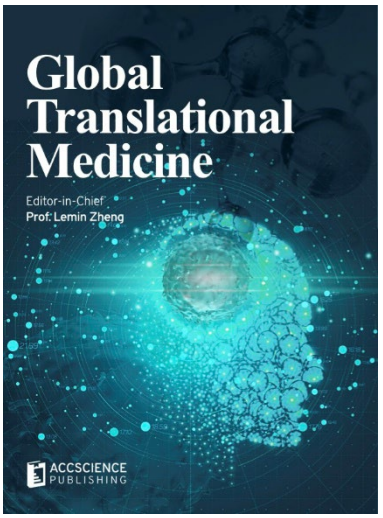

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