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Comparative assessment of *In vitro* antiamoebic and cytotoxic activities of *Cyperus rotundus* L. leaf and rhizome extracts

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Abstract
Amoebiasis, caused by *Entamoeba histolytica*, remains one of the most important protozoan infections worldwide and continues to be a major public health problem, particularly in developing countries where poor sanitation and unsafe drinking water facilitate disease transmission. Although metronidazole is the drug of choice for the treatment of amoebiasis, its prolonged use is associated with adverse effects, treatment failures, and concerns regarding the emergence of drug resistant strains, emphasizing the need for safer and more effective alternative therapies. Medicinal plants represent an important source of structurally diverse bioactive compounds with significant antiparasitic potential. The present study aimed to comparatively evaluate the *in vitro* antiamoebic activity and cytotoxicity of leaf and rhizome extracts of *Cyperus rotundus* L. prepared using ethanol, methanol, ethyl acetate, and chloroform. Antiamoebic activity against *Entamoeba histolytica* trophozoites was determined using a 96-well microplate assay at concentrations ranging from 20–100 µg/mL, with metronidazole serving as the reference drug. Cytotoxicity was evaluated on L929 mouse fibroblast cells using the MTT assay. All extracts exhibited concentration dependent antiamoebic activity. Among the leaf extracts, the ethyl acetate extract demonstrated the highest activity, producing 58.98% inhibition at 100 µg/mL with an IC_{50} value of 91.02 µg/mL. Among the rhizome extracts, the methanolic extract exhibited the greatest antiamoebic efficacy, producing 66.11% inhibition with an IC_{50} value of 70.95 µg/mL. Cytotoxicity studies revealed that all extracts maintained more than 60% viability in L929 fibroblast cells at the highest tested concentration, indicating relatively low toxicity toward normal mammalian cells. Notably, the methanolic rhizome extract demonstrated the most favorable balance between antiamoebic activity and cytocompatibility. The findings provide scientific evidence supporting the traditional medicinal use of *Cyperus rotundus* and suggest that its rhizome is a promising source of bioactive compounds for the development of novel plant-derived antiamoebic agents. Further investigations involving bioassay-guided fractionation, isolation and characterization of active constituents, mechanistic studies, molecular docking, pharmacokinetic evaluation, and in *vivo* validation are warranted to establish its therapeutic potential.

Keywords: *Cyperus rotundus*, *Entamoeba histolytica*, amoebiasis, antiamoebic activity, cytotoxicity, l929 fibroblast cells, medicinal plants, natural products

Introduction

Amoebiasis is a major parasitic disease caused by the protozoan *Entamoeba histolytica* and remains a significant public health concern, particularly in low- and middle-income countries where poor sanitation, inadequate hygiene, and contaminated food and water facilitate transmission. Although many infected individuals remain asymptomatic, invasive infections can result in amoebic colitis, severe dysentery, and extraintestinal manifestations such as amoebic liver abscess, contributing substantially to morbidity and mortality worldwide (Stanley, 2003; Shirley *et al*., 2018) [13, 15]. According to the World Health Organization, amoebiasis continues to rank among the leading causes of death due to parasitic protozoan infections, highlighting the need for improved therapeutic strategies. Nitroimidazole drugs, particularly metronidazole, remain the first-line treatment for invasive amoebiasis. However, their use is associated with several limitations, including gastrointestinal adverse effects, neurotoxicity following prolonged therapy, inability to eliminate luminal cysts without combination treatment, poor patient compliance, and growing concerns regarding reduced parasite susceptibility and the emergence of drug resistance. These limitations have intensified the search for safer and more effective antiamoebic agents derived from natural sources (Bansal *et al*., 2006; Haque *et al*., 2022 [3, 7]; Centers for Disease Control and Prevention, 2024) [4].

Medicinal plants represent an invaluable reservoir of structurally diverse bioactive compounds with considerable therapeutic potential. Plant-derived secondary metabolites, including flavonoids, phenolic acids, terpenoids, alkaloids, tannins, saponins, and essential oils, have demonstrated broad-spectrum antimicrobial, antiparasitic, antioxidant, anti-inflammatory, and immunomodulatory activities. Advances in phytochemistry and pharmacology have renewed interest in medicinal plants as promising sources for the discovery of novel antiparasitic drugs with improved safety profiles and multiple mechanisms of action (Newman & Cragg, 2020; Atanasov *et al*., 2021). *Cyperus rotundus* L. (Cyperaceae), commonly known as nut grass or purple nutsedge, is a perennial medicinal herb widely distributed throughout tropical and subtropical regions. The plant occupies an important place in traditional systems of medicine, including Ayurveda, Traditional Chinese Medicine, and Unani medicine, where it is used for the management of gastrointestinal disorders, fever, inflammation, pain, menstrual abnormalities, microbial infections, and metabolic diseases (Sivapalan, 2013;

Peerzada *et al*., 2015) [11, 14]. Phytochemical investigations have revealed that *C. rotundus* contains a wide spectrum of bioactive constituents such as flavonoids, phenolic compounds, sesquiterpenes, monoterpenes, alkaloids, glycosides, tannins, and volatile essential oils.

These phytochemicals are believed to contribute to its diverse pharmacological activities through antioxidant, antimicrobial, anti-inflammatory, and antiproliferative mechanisms (Peerzada *et al*., 2015 [11]; Atanasov *et al*., 2021). Numerous experimental studies have demonstrated that extracts of *C. rotundus* possess antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory, antidiabetic, hepatoprotective, gastroprotective, and anticancer activities. Despite these promising pharmacological properties, relatively few investigations have explored its efficacy against *Entamoeba histolytica*, and comparative studies evaluating the antiamoebic activities of different plant parts remain scarce. Furthermore, assessment of cytotoxicity using normal mammalian cell lines is essential for determining the therapeutic safety and selectivity of plant-derived antiparasitic agents before their further development.

Therefore, the present study aimed to comparatively evaluate the *in vitro* antiamoebic activity of leaf and rhizome extracts of *Cyperus rotundus* prepared using different solvents against *Entamoeba histolytica.* In addition, the cytotoxic effects of these extracts were assessed using L929 mouse fibroblast cells to determine their safety and to identify extracts with favorable therapeutic potential for future antiparasitic drug development.

Materials and Methods

1. Plant Material Collection and Authentication

Fresh leaves and rhizomes of *Cyperus rotundus* L. were collected from different locations in the Marathwada region of Maharashtra, India, during the appropriate growing season. The collected plant material was taxonomically authenticated by a qualified plant taxonomist based on standard floristic characteristics. A voucher specimen was prepared, assigned an accession number, and deposited in the departmental herbarium for future reference. Plant nomenclature was verified using the Plants of the World Online (POWO) database maintained by the Royal Botanic Gardens, Kew (POWO, 2024) [12].

2. Preparation of Plant Extracts

The collected leaves and rhizomes were thoroughly washed with distilled water to remove adhering soil and debris, shade-dried at room temperature (25–30°C) until constant weight was achieved and pulverized into a fine powder using a mechanical grinder. Approximately 20 g of each powdered sample was extracted separately using a Soxhlet apparatus with ethanol, methanol, ethyl acetate, and chloroform as extraction solvents. Extraction continued until the siphon tube became colorless. The extracts were concentrated under reduced pressure using a rotary vacuum evaporator at temperatures below 45°C to avoid degradation of thermolabile phytochemicals. The concentrated extracts were weighed to determine extraction yield and stored in airtight, ambercolored containers at 4°C until further analysis (Azwanida, 2015; Uddin *et al*., 2023) [2, 16].

3. Maintenance of Entamoeba histolytica Culture

Trophozoites of *Entamoeba histolytica* were maintained in RPMI-1640 complete medium supplemented with fetal bovine serum and appropriate antibiotics under sterile conditions. Cultures were incubated at 37°C and subcultured periodically to maintain logarithmic growth. Only actively proliferating trophozoites exhibiting >95% viability was used for antiamoebic assays to ensure reproducibility and experimental consistency (Shirley *et al*., 2018; Haque *et al*., 2022) [7, 13].

4. *In vitro* Antiamoebic Assay

Stock solutions of each plant extract were prepared by dissolving 5 mg of dried extract in 50 µL dimethyl sulfoxide (DMSO), followed by dilution with sterile distilled water to obtain a final concentration of 5 mg/mL. The final DMSO concentration in assay wells did not exceed 1%, thereby avoiding solvent-induced cytotoxicity. Working solutions of 20, 40, 60, 80, and 100 µg/mL were freshly prepared before each experiment. The antiamoebic assay was performed in sterile 96-well flat-bottom microplates. Briefly, 80 µL of trophozoite suspension prepared in complete RPMI-1640 medium was added to each well, followed by 20 µL of the respective extract solution. Parasite cultures without extract served as the negative control, while metronidazole was used as the positive control. Plates were incubated at 37°C for 48 h under aseptic conditions. Following incubation, trophozoite viability was determined using the trypan blue exclusion assay, and absorbance was recorded at 510 nm using a microplate reader. Percentage inhibition of parasite growth was calculated according to the method described by Kumarasamy *et al*. (2002) [8], using the following equation:

$$Percentage\ inhibition\ (\%) = [Control\ OD - Sample\ OD)/\ Control\ OD] \times 100$$

5. Cell Line and Culture Conditions

The mouse fibroblast cell line (L929) was used to evaluate the cytotoxic potential of the plant extracts. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 1% antibiotic-antimycotic solution (100 U/mL penicillin and 100 µg/mL streptomycin), and maintained at 37°C in a humidified incubator containing 5% CO_2. Culture medium was replaced every 2–3 days, and cells were subcultured after reaching approximately 80–90% confluence according to standard mammalian cell culture procedures (Freshney, 2021) [5].

6. MTT Cytotoxicity Assay

The cytotoxic effects of the extracts were evaluated using the MTT colorimetric assay based on the reduction of tetrazolium salt to insoluble purple formazan crystals by metabolically active cells (Mosmann, 1983) [9].

L929 cells were seeded into sterile 96-well culture plates at a density of 1×10^4 cells per well and allowed to attach for 24 h. Cells were then treated with various concentrations (20, 40, 60, 80, and 100 µg/mL) of each extract and incubated for an additional 24 h. Untreated cells served as the negative control, while DMSO-treated cells were included as the vehicle control. After treatment, 20 µL of MTT solution (5 mg/mL) was added to each well, followed by incubation for 4 h at 37°C. The culture medium was carefully removed, and the resulting formazan crystals were

dissolved in DMSO. Absorbance was measured at 550 nm using a microplate reader. Cell viability was calculated using the following equation:

$$Cell\ viability\ (\%) = (OD\ of\ treated\ cells\ /\ OD\ of\ control\ cells) \times 100$$

7. Statistical Analysis

All experiments were performed in triplicate, and data were expressed as mean ± standard error of the mean (SEM). Statistical analyses were carried out using GraphPad Prism (Version 10.0) and SPSS (Version 29.0). Differences among treatment groups were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison post hoc test. Values of $p < 0.05$ were considered statistically significant. IC_{50} values were calculated using nonlinear regression analysis, and graphical representations were prepared using GraphPad Prism software (GraphPad Software, 2024) [6].

Results

1. *In vitro* Antiamoebic Activity of *Cyperus rotundus* Extracts

The *in vitro* antiamoebic activity of leaf and rhizome extracts of *Cyperus rotundus* L. was evaluated against *Entamoeba histolytica* trophozoites at concentrations ranging from 20–100 µg/mL. All extracts exhibited concentration-dependent inhibitory activity, indicating the presence of phytochemicals capable of suppressing trophozoite growth and viability (Table 1 and figure 1). The positive control, metronidazole, produced the highest inhibition (88.14%) at 100 µg/mL, with an IC_{50} value of 50.48 µg/mL, confirming the reliability and sensitivity of the assay. Among the leaf extracts, the ethyl acetate extract demonstrated the greatest antiamoebic activity, producing 58.98% inhibition at 100 µg/mL with an IC_{50} value of 91.02 µg/mL. Methanolic and ethanolic leaf extracts exhibited moderate activity (52.86% inhibition), whereas the chloroform extract showed comparatively lower inhibition (46.87%), and an IC_{50} value could not be determined within the tested concentration range.

Rhizome extracts generally exhibited greater antiamoebic activity than the corresponding leaf extracts. The methanolic rhizome extract was the most active plant extract, producing 66.11% inhibition with an IC_{50} value of 70.95 µg/mL. Chloroform and ethyl acetate rhizome extract also showed appreciable inhibitory effects (59.32% and 52.46%, respectively), while the ethanolic rhizome extract demonstrated the lowest activity (38.88%). The superior activity of the methanolic rhizome extract suggests that methanol efficiently extracted polar bioactive constituents, including flavonoids, phenolic acids, tannins, and glycosides, which have previously been associated with antiparasitic activity. Rhizomes of *C. rotundus* are known to contain higher concentrations of sesquiterpenes and phenolic compounds than aerial plant parts, which may explain their enhanced biological activity (Peerzada *et al*., 2015 [11]; Atanasov *et al*., 2021).

Table 1: Comparative *in vitro* antiamoebic activity of *Cyperus rotundus* extracts against *Entamoeba histolytica.*

Extract	% Inhibition at 100 µg/mL	IC_{50} (µg/mL)
Metronidazole	88.14	50.48
Leaf Ethanol	52.86	97.14
Leaf Methanol	52.86	97.14
Leaf Ethyl acetate	58.98	91.02
Leaf Chloroform	46.87	NE
Rhizome Ethanol	38.88	NE
Rhizome Methanol	66.11	70.95
Rhizome Ethyl acetate	52.46	97.54
Rhizome Chloroform	59.32	90.68

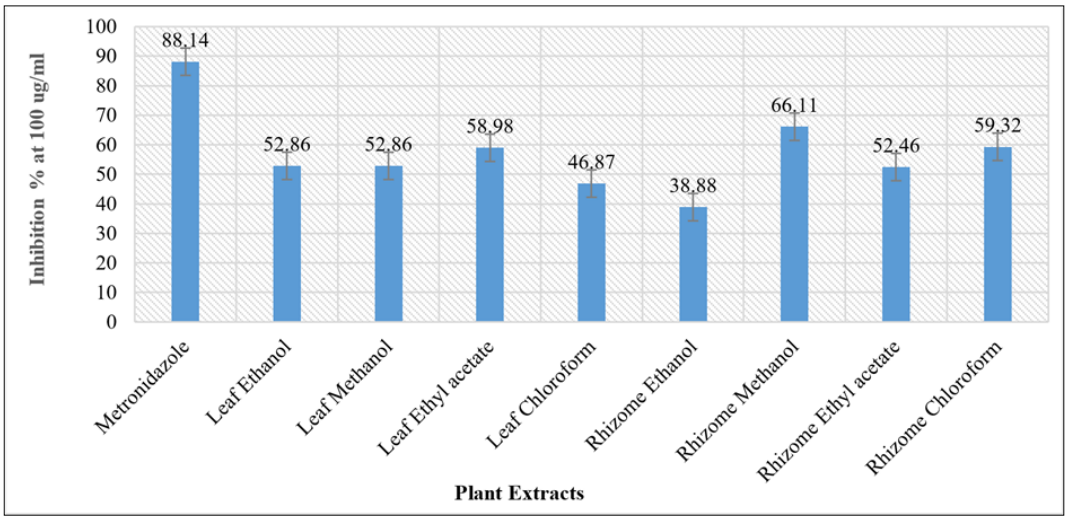

Fig 1: Comparative antiamoebic activity of metronidazole and different leaf and rhizome extracts of *C. rotundus* at 100 µg/mL.

2. Cytotoxicity Evaluation on L929 Fibroblast Cells

The cytotoxic potential of *C. rotundus* extracts was assessed using the MTT assay on L929 mouse fibroblast cells. All extracts exhibited concentration-dependent reductions in cell viability; however, none reduced cell viability below 50% within the tested concentration range, indicating relatively low toxicity toward normal mammalian cells (Table 2 and figure 2). Among the leaf extracts, the methanolic extract exhibited the highest cytotoxicity, with cell viability decreasing to 67.04% at 100 µg/mL. The ethanolic, ethyl acetate, and chloroform leaf extracts-maintained viability values between 68% and 73%, suggesting comparatively lower toxicity.

Among rhizome extracts, the ethyl acetate and chloroform fractions exhibited relatively greater cytotoxicity, maintaining cell viabilities of 62.34% and 62.47%,

respectively. Importantly, the methanolic rhizome extract, which demonstrated the strongest antiamoebic activity, retained 66.06% viability in L929 fibroblast cells.

This finding suggests a favorable balance between antiparasitic efficacy and mammalian cell safety. The selective inhibitory effect against E. histolytica compared with normal fibroblast cells indicates that the observed antiparasitic activity is unlikely to result from nonspecific cytotoxicity. Such selectivity is an important criterion in the preliminary screening of natural products for drug development.

Table 2: Cytotoxicity of *Cyperus rotundus* L. extracts on L929 fibroblast cells.

Extract	Cell Viability (%) at 100 µg/mL
5-Fluorouracil	88.12
Leaf Ethanol	72.66
Leaf Methanol	67.04
Leaf Ethyl acetate	68.80
Leaf Chloroform	69.33
Rhizome Ethanol	68.80
Rhizome Methanol	66.06
Rhizome Ethyl acetate	62.34
Rhizome Chloroform	62.47

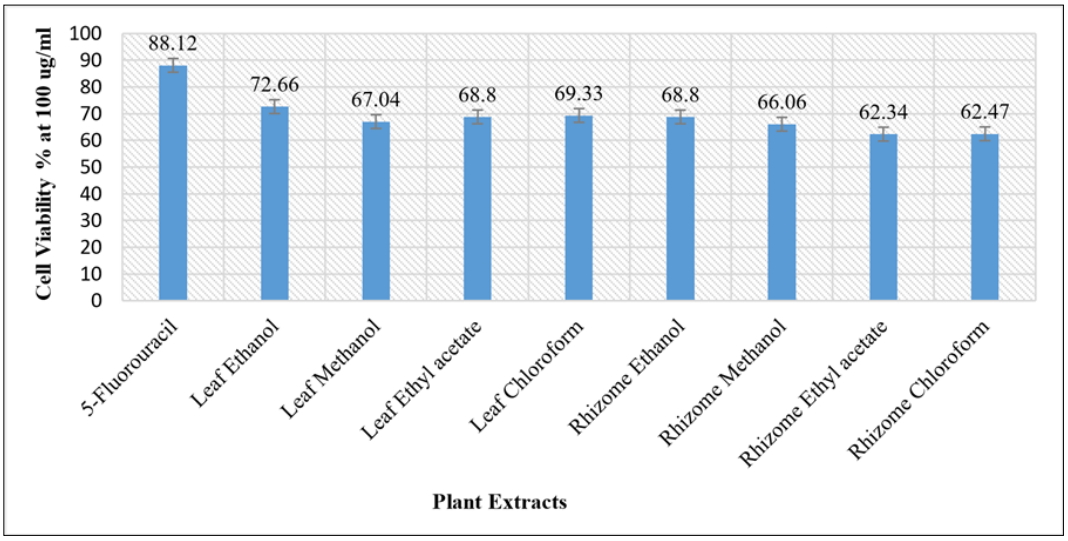

Fig 2: Percentage viability of L929 fibroblast cells following treatment with different *C. rotundus* extracts.

Discussion

The present study demonstrated that extracts of *Cyperus rotundus* L. possess promising *in vitro* antiamoebic activity against *Entamoeba histolytica*, accompanied by relatively low cytotoxicity toward normal mammalian fibroblast cells. The concentration-dependent inhibition observed for all extracts indicates that the plant contains multiple bioactive metabolites capable of impairing parasite growth. Among all extracts evaluated, the methanolic rhizome extract exhibited the highest antiamoebic activity, achieving 66.11% inhibition with an IC_{50} value of 70.95 µg/mL. This enhanced activity is likely attributable to the efficient extraction of polar phytochemicals such as flavonoids, phenolic acids, tannins, glycosides, and other oxygenated secondary metabolites. Previous phytochemical investigations have reported that *C. rotundus* rhizomes are particularly rich in sesquiterpenoids (cyperene, cyperotundone, cyperol, α-cyperone), flavonoids, and phenolic constituents, many of which possess broad-spectrum antimicrobial and antiparasitic activities (Peerzada *et al*., 2015 [11]; Atanasov *et al*., 2021).

The ethyl acetate leaf extract exhibited the greatest activity among leaf extracts, suggesting that semi-polar constituents also contribute significantly to the antiamoebic effect. Ethyl acetates commonly extract flavonoid aglycones, phenolic acids, and moderately polar terpenoids, compounds that have been reported to exhibit membrane-disruptive and enzyme-inhibitory effects against protozoan parasites. Similar observations have been reported for several medicinal plants in which ethyl acetate fractions displayed superior antiprotozoal activity due to enrichment of phenolic metabolites (Newman & Cragg, 2020; Atanasov *et al*., 2021). The antiamoebic activity observed in this study may result from multiple complementary mechanisms. Polyphenols and flavonoids are known to induce oxidative stress within protozoan cells, alter plasma membrane permeability, inhibit essential metabolic enzymes, interfere with mitochondrial function, and suppress nucleic acid synthesis. Terpenoids and essential oils may additionally disrupt membrane integrity and cellular signaling pathways, ultimately leading to trophozoite death. Because plant extracts contain numerous chemically diverse metabolites, their biological activity is likely mediated through synergistic interactions rather than a single active compound (Atanasov *et al*., 2021; Newman & Cragg, 2020).

Assessment of cytotoxicity is an essential component of antiparasitic drug discovery because potent antiparasitic activity must be accompanied by acceptable safety toward host cells. In the present study, all extracts maintained more than 60% viability in L929 fibroblast cells at the highest concentration tested. Although the methanolic rhizome extract exhibited the strongest antiamoebic activity, it retained 66.06% cell viability, indicating only moderate cytotoxicity. These findings suggest a degree of selectivity toward *E. histolytica*, which is highly desirable during early-stage screening of plant-derived therapeutic agents. The comparatively low cytotoxicity observed is consistent with previous reports demonstrating the safety of *C. rotundus* extracts in mammalian cell systems and experimental animal models. Numerous investigations have shown that C. rotundus exhibits antioxidant, anti-inflammatory, hepatoprotective, gastroprotective, and antimicrobial

activities with relatively low toxicity at pharmacologically relevant doses, supporting its extensive traditional medicinal use (Peerzada *et al*., 2015; Sivapalan, 2013 [11, 14]; Atanasov *et al*., 2021).

Although the antiamoebic activity observed was lower than that of metronidazole, the results are encouraging because crude plant extracts generally contain complex mixtures of compounds at relatively low concentrations. Bioassay-guided fractionation and purification may substantially enhance biological activity by concentrating the active constituents. Furthermore, combinations of purified phytochemicals with conventional antiamoebic drugs could potentially improve therapeutic efficacy while reducing drug dosage and minimizing adverse effects, an approach that has attracted increasing attention in antiparasitic drug research. The present findings identify the methanolic rhizome extract of *C. rotundus* as the most promising candidate for further investigation. Future studies should focus on bioassay-guided isolation and structural characterization of the active compounds using chromatographic and spectroscopic techniques, elucidation of their molecular mechanisms of action, evaluation of selectivity indices against additional mammalian cell lines, and validation of antiamoebic efficacy in suitable *in vivo* models before considering clinical applications.

Conclusion

The present study demonstrates that *Cyperus rotundus* L. possesses promising *in vitro* antiamoebic activity against *Entamoeba histolytica*, while exhibiting relatively low cytotoxicity toward normal mammalian cells. All leaf and rhizome extracts produced concentration-dependent inhibition of trophozoite growth, confirming the presence of bioactive phytochemicals with antiparasitic potential. Among the leaf extracts, the ethyl acetate fraction exhibited the highest antiamoebic activity, producing 58.98% inhibition at 100 µg/mL with an IC_{50} value of 91.02 µg/mL. However, the methanolic rhizome extract demonstrated the greatest overall efficacy, achieving 66.11% inhibition with an IC_{50} value of 70.95 µg/mL, suggesting that the rhizome is a richer source of bioactive constituents than the aerial parts of the plant. The cytotoxicity assessment using L929 fibroblast cells further indicated that all extracts possessed acceptable safety profiles under the experimental conditions. Cell viability remained above 60% for all extracts at the highest concentration tested, demonstrating relatively low toxicity toward normal mammalian cells. Notably, the methanolic rhizome extract, despite exhibiting the highest antiamoebic activity, maintained 66.06% cell viability, indicating a favorable balance between antiparasitic efficacy and cytocompatibility. Such selectivity is an important prerequisite for the development of plantderived antiparasitic agents with improved therapeutic indices (Mosmann, 1983 [9]; Atanasov *et al*., 2021).

The superior biological activity of the methanolic rhizome extract is likely attributable to its abundance of phenolic compounds, flavonoids, terpenoids, and other secondary metabolites that have previously been reported to exhibit antimicrobial and antiparasitic activities through multiple mechanisms, including disruption of parasite membrane integrity, inhibition of essential metabolic enzymes, induction of oxidative stress, and interference with nucleic acid synthesis (Peerzada *et al*., 2015 [11]; Newman & Cragg, 2020). The greater activity observed in rhizome extracts compared with leaf extracts further supports previous phytochemical reports indicating that the underground parts of *C. rotundus* contain higher concentrations of pharmacologically active constituents. The findings of the present investigation provide scientific evidence supporting the traditional medicinal use of *Cyperus rotundus* and identify the methanolic rhizome extract as the most promising candidate for the development of novel antiamoebic agents. Although the activity of the crude extracts was lower than that of metronidazole, the observed efficacy, combined with relatively low cytotoxicity, suggests considerable potential for further pharmaceutical development. Bioassay-guided fractionation and purification of the active constituents may further enhance antiparasitic potency and improve selectivity.

Future investigations should focus on the isolation and structural characterization of the active compounds using advanced chromatographic and spectroscopic techniques, elucidation of their molecular mechanisms of action, molecular docking and molecular dynamics studies against validated *E. histolytica* drug targets, determination of selectivity indices, pharmacokinetic and toxicological evaluations, and validation of therapeutic efficacy in appropriate animal models of amoebiasis. Such studies may facilitate the development of safe, effective, and affordable plantderived antiamoebic therapeutics to address the growing need for alternative treatment options in the face of emerging drug resistance and the limitations associated with current chemotherapy (Shirley *et al*., 2018; Haque *et al*., 2022) [7, 13].

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Conflict of Interest: The author declares that there is no conflict of interest.

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