Research article

**In vitro anticancer efficacy of Cyperus rotundus (L.) on breast adenocarcinoma cells via the induction of DNA fragmentation and apoptosis**

Hema Nidugala¹, Ashwini Prabhu², Ramakrishna Avadhani³, Ravishankar B.⁴,⁵

¹Department of Anatomy, Kanachur Institute of Medical Sciences, Deralakatte, Mangalore, 575018, Karnataka, India
²Yenepoya Research Centre, Yenepoya (Deemed to be University), Deralakatte, Mangalore, 575018, Karnataka, India
³Department of Anatomy, Yenepoya Medical College, Yenepoya (Deemed to be University), Deralakatte, Mangalore, 575018, Karnataka, India
⁴BRS Research Adviser, Sri Sri College of Ayurvedic Science and Research, Bengaluru, Karnataka, India
⁵Former Director, SDM Centre for Research in Ayurveda and Allied Sciences, Udupi, Karnataka, India

(Received: November 2022   Revised: July 2023   Accepted: August 2023)

Corresponding author: Ashwini Prabhu. Email: ashwinibiosciences@gmail.com

**ABSTRACT**

**Introduction and Aim:** *Cyperus rotundus* (L.), commonly known as nutgrass, has been in use in Indian medicine traditional system against several ailments. This investigation was performed to assess the *in vitro* antineoplastic activity of extracts prepared from water and ethanol with *C. rotundus* rhizomes.

**Materials and Methods:** Toxic effects induced by the *C. rotundus* extracts on MCF-7 breast adenocarcinoma cells was evaluated using Sulphorhodamine- B (SRB) Assay, Trypan blue assay, Hoechst nuclear staining assay, Acridine Orange-Ethidium bromide (AO-EB) differential fluorescence staining assay and DNA fragmentation assays.

**Results:** The results of SRB assay indicated the IC₅₀ value of aqueous extract as 510.887 µg/mL and that of ethanol extract as 122.98 µg/mL. Trypan blue assay also indicated the similar results, wherein ethanol extract exerted higher cytotoxicity compared to the aqueous extract. MCF-7 cells treated with aqueous and ethanol extracts of *C. rotundus* rhizomes were exposed to Hoechst staining and AO-EB nuclear staining assays, wherein and ethanol extract induced clear apoptotic bodies in the tested cells. Further, the cells were treated with the ethanol extract and DNA fragmentation was confirmed by DNA ladder assay. MCF-7 cells administered with ethanol extract were subjected to DNA fragmentation assay for assessing the apoptosis induction nature of the extract.

**Conclusion:** Results from our study indicate a promising approach into the possibility of developing *C. rotundus* ethanol extract into an effective anticancer moiety after appropriate pre-clinical and clinical validations.

**Keywords:** *C. rotundus*; cytotoxicity; breast cancer; DNA fragmentation; apoptosis.

**INTRODUCTION**

Cancer is the leading cause of death globally and continues to be a significant healthcare challenge. The prevalence and mortality rates indicate the severity in developing and developed countries (1). Breast cancer represents the most common gynecological malignancy among women globally, with an estimate of 3.4 million new cases worldwide annually by the year 2030 (2,3). Most of the breast cancer cases are curable, when they are diagnosed at early stages. However, distant relapse is seen in case of 30% cases, involving metastases (4,5).

Important predisposing factors for the development of breast cancer development include family history, reproductive status, lifestyle habits and hormonal imbalance (6). Intratumoral heterogeneity and intertumoral heterogeneity constitute an integral part of the breast cancer progression (7).

Currently employed treatment approaches for cancer include surgical resection, radiation, chemotherapy, immunotherapy, and angiogenesis inhibition. Although these approaches have shown success rates up to certain extent, their adverse effects limit the applicability. Most of the drugs used as chemotherapeutic agents exert adverse effects including nausea, GI discomfort, fatigue, low blood counts, myelotoxicity, cardiotoxicity, and renal toxicity (8). Hence, there exists a strong urge to find an alternative approach based on economy, ease of accessibility and effective anticancer compounds. Looking at the plant-based compounds looks promising in countering the harmful consequences of commercial chemotherapeutics.

The use of plants for various pharmacological applications has attracted attention in the recent decades (9). Most of the effective anticancer compounds are derived from plants and approximately 50% of the therapeutic agents under clinical trials are derived from natural sources (10,11). Medicinal plants serve as the rich source of bioactive molecules that could be used as potential antineoplastic agents (12). Vincristine, vinblastine, taxol, camptothecin, and podophyllotoxin represent some classic examples of anticancer drugs derived from plants (13). Despite several efforts, clinical use of plant-based chemotherapeutic agents is limited due to the lack of studies governing safety and efficacy in the larger

DOI: https://doi.org/10.51248/v43i4.2309
population. Hence, a constant demand for safer and more effective anticancer compounds continues to subsist.

In this investigation, we have assessed the anticancer efficacy of *Cyperus rotundus* root extracts against breast adenocarcinoma cells *in vitro*. *C. rotundus* belongs to the family Cyperaceae, widely distributed in the Asian continent (14). The plant harbours a variety of pharmacological activities and rhizomes are used as astringent, analgesic, vermifuge, antispasmodic, carminative and antibacterial (15). This investigation assesses the anticancer, apoptotic and DNA fragmentation inducing efficacy of *C. rotundus* rhizome extracts on breast cancer cells.

**MATERIALS AND METHODS**

**Preparation of *C. rotundus* rhizome aqueous and ethanol extracts**

Rhizomes of *C. rotundus* were procured from the local vendors of Mangalore, Karnataka, India. Dr. Sunil Kumar, Department of Pharmacognosy, SDM Centre for Research in Ayurveda and Allied Sciences, Udupi performed the authentication of voucher specimen (No. 11110101). The powder was prepared from the rhizomes using a mechanical blender and was stored in sealed covers at 4°C till further analyses. Aqueous and ethanol extracts of the rhizome powder were made.

**Cells and culture conditions**

MCF-7 breast adenocarcinoma cells were procured from National Center for Cell Sciences (NCCS), Pune. Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% antibiotic-antimycotic solution and maintained at 37°C and 5% CO₂ conditions. Cells were passaged once they attain 70% confluence and were utilized for the experiments after they have been passaged more than three times.

**Evaluation of anticancer activity of the extract using sulforhodamine-B (SRB) assay**

MCF-7 cells (5000 cells per well) were plated onto 96 well microtiter plates. After attachment, they were subjected to treatment with aqueous and ethanol extracts at different concentrations, ranging from 25 - 200 µg/mL for 48 h. After the treatment period, media with the drug was aspirated and cells were given a wash with 200 µL of PBS. Cell pellet was fixed by the addition of 30 % TCA (50 µL) and incubated at 4°C for 1 h. Cells were gently washed with Milli-Q water for 5 times to remove the traces of TCA. The plate was air-dried and 50 µL of 0.05% SRB dye was added and kept for incubation in the dark for 30 min. Acetic acid (1% v/v, 200 µL) was used to wash off the unbound SRB. The plate was again subjected to air drying and cell-bound dye was solubilized by adding trizma base buffer (200 µL). The plate was subjected to shaking on an orbital shaker for 20 min and the absorbance was recorded at 540 nm with a reference wavelength of 630 nm using an ELISA plate reader. Cell viability percentage was calculated with respect to untreated control. Results were expressed as Mean ± S.E.

**Trypan blue dye exclusion assay**

Cell suspension (100 µL) prepared from the stock containing 1X10⁶ cells in PBS was dispensed in sterile test tubes. The cells were subjected to treatment with the test drugs (100 µL) at varying concentrations, followed by the addition of PBS (700 µL). The cells were incubated at 37°C for 3 h. Trypan blue (100 µL) was added, and this suspension was mixed. The total numbers of dead and viable cells were counted using a haemocytometer and the percentage viability was calculated as follows:

\[ \% \text{ viable cells} = \frac{\text{Number of unstained cells}}{\text{Total number of cells}} \times 100 \]

**Hoechst staining**

Cell suspension (100 µL) containing 1000 cells/mL cells were added into each well of a 96-well plate and the cells were allowed to adhere for 24 h. 100 µL of the extracts was administered to the wells, followed by incubation for 24 h. After the treatment period, the media containing drugs was removed, 100 µL of chilled methanol was added and incubated for 30 min at room temperature. Methanol was aspirated, the wells were added with 50 µL of Hoechst reagent, followed by incubation at 37°C under dark condition for 15 min. Staining solution was removed from the plate, excess stain was washed-off with PBS, and plate was observed under an inverted microscope (Nikon eclipse TS100), with excitation filter 460/490 nm and emission filter 535/540 nm.

**Acridine orange- ethidium bromide staining for apoptosis detection**

MCF-7 cells were seeded onto 96-well plates and incubated at 37°C and 5% CO₂. After adherence, extracts were added to the wells (100 µg/mL & 200 µg/mL) and the plate was incubated for 24 h. After the treatment with the extract, the media containing the drug was removed, PBS wash was given to the cells and cells were stained using AO and EB (2 µg/mL; 1:1) for 10 min under dark at 37°C. Excess stain was removed with PBS wash, and cells were imaged using an inverted microscope (Nikon Eclipse TS100) with excitation filter 460/490 nm and emission filter 535/540 nm.

**Apoptosis-DNA ladder assay**

Detection of apoptosis induced by the *C. rotundus* extract in MCF-7 breast cancer cells was evaluated as per the method outlined by Mizuho Aoki et al., 2000. MCF-7 breast adenocarcinoma cells (1X10⁶) were seeded in 2 mL of complete DMEM in a 6 well plate

**DOI:** https://doi.org/10.51248/v43i4.2309

**Biomedicine- Vol. 43 No. 4: 2023**

1199
and incubated for 24 h. The cells were added with different concentrations of ethanol extracts. The plate containing cells were incubated at 37°C for 48 h. Cell pellets were collected from the culture supernatants containing floating dead cells by centrifugation at 1000 rpm for 5 min. Cells attached to the wells were lysed using 300 µL of lysis buffer. The DNA from the cells was added to the cell pellet and incubated for 1 h at 37°C. RNAse (150 µL) was added to the above solution and kept at 50°C for 1 h to destroy the RNA. A brief exposure at 65°C for 2 min was followed to inactivate the unreacted RNAse. The processed cell lysates were then cooled to room temperature and added with 30 % glycerol: bromophenol blue (1:1) and loaded into the wells of agarose gel (1.5%) prepared in the TBE buffer. Electrophoresis was carried out at 400 mA, 60 V for 90 min in the TBE buffer.

**Statistical analysis**

Data are presented in terms of Mean ± SEM. The data were subjected to one-way ANOVA followed by Dunnett’s test, to find out the differences between the test groups. Analysis was performed using GraphPad Prism version 5.0. Levels of significance were considered at p < 0.05.

**RESULTS**

The cytotoxic activity of both aqueous and ethanolic extracts of *C. rotundus* was assessed by SRB assay on MCF-7 human breast adenocarcinoma cells. The results of SRB assay revealed that IC50 value of aqueous extract is 510.89 µg/mL and that of ethanol extract is 122.98 µg/mL (Fig. 1). In trypan blue assay, IC50 value of aqueous extract is 4187.12 µg/mL and ethanol extract is 65.08 µg/mL (Fig. 2). Standard drug Doxorubicin showed IC50 values of 11.22 µg/mL and 2.49 µg/mL respectively, in case of SRB assay and Trypan blue dye exclusion assay. The aqueous extract was found to be less cytotoxic on breast cancer cells in both assays.

![Graph 1: Cytotoxic activity of *C. rotundus* extract on breast cancer cells, as evaluated using SRB assay](image1)

![Graph 2: Cytotoxic activity of *C. rotundus* extract on breast adenocarcinoma cells, as assessed using Trypan blue dye exclusion assay](image2)

![Fig. 3: Nuclear integrity assessment of breast cancer cells after treatment with *C. rotundus* extract](image3)
MCF-7 cells were subjected to treatment with both aqueous and ethanol extracts of *C. rotundus* for 48 h and subjected to AO-EB dual fluorescence staining assay to visualize the nuclear changes including apoptosis and necrosis. Nuclei with green fluorescence with uniformly distributed chromatin were recorded in DMSO (vehicle control) treated cells inferring their viability. Doxorubicin (positive control) treated MCF-7 cells displayed nuclei fluorescing green and orange-red color, indicating the chromatin condensation and fragmentation, which are the hallmarks of apoptosis. Treatment with ethanol extracts of *C. rotundus* in two doses resulted in early apoptotic cells indicated by yellowish green nuclei as well as orange-red fluorescing nuclei indicating late apoptotic cells, characterized by the presence of fragmented chromatin and formation of apoptotic bodies (Fig. 4A). There were no such cells in aqueous extract treated groups. Thus, these results indicated that ethanol extracts showed potential anticancer property against breast adenocarcinoma cells by inducing apoptosis. Fig. 4B displays the apoptosis percentage induced by *C. rotundus* ethanol extracts on MCF-7 cell line using AO/EB staining. Cytotoxicity of ethanol extract was almost comparable to that of standard drug doxorubicin. DNA fragmentation assay results indicated that treatment with *C. rotundus* ethanol extracts induced DNA ladder formation like that of doxorubicin (Fig. 5).

**DISCUSSION**

Several previous reports have indicated that the plant extracts possess various phytochemicals, which exert anticancer activity (16,17). The breast cancer incidence has been elevating in a constant manner due to the lifestyle habits and exposure to harmful environmental chemical substances (18). Although several efforts have been put forward for the early diagnosis and routine screening programs, breast cancer claims higher mortality rates worldwide. Hence, there is an immediate need to identify the novel therapeutic candidates with specificity on cancer cells and limited toxicity on the normal cells (19). Our results marked the potential cytotoxic effects induced by *C. rotundus* root extract on breast cancer cells. Ethanol extract was found to be highly cytotoxic compared to the aqueous extract. This may be due to its higher solubility, higher stability, and higher antioxidant activity.

Our study indicated that cytotoxicity of ethanol extract was almost comparable to that of the standard drug doxorubicin. The hallmark event of apoptosis which does not occur in necrosis is the depagination of the nuclei and their deposition near the nuclear membrane, presence of intact nuclear membrane and chromatin condensation (20). Acridine orange-ethidium bromide staining is a widely utilized method in assessing the nuclear structure *in vitro* upon treatment with various drugs.
(21). Acridine orange stains both healthy and dead cells, while ethidium bromide stains apoptotic cells only. Upon merging the green and red channels, early apoptotic cells appear yellowish green, while late apoptotic nuclei appear orange-red in fluorescence. Results of our study indicated the clear apoptosis initiating effect by C. rotundus ethanolic extract on breast cancer cells.

DNA fragmentation assay could be used effectively for evaluating the hallmarks of apoptosis. A peculiar motif of low and high molecular weight bands is produced in case of apoptotic DNA cleavage (22).

CONCLUSION

From the above study, it was confirmed that ethanol extract of C. rotundus could be utilized as a potent natural anticancer drug after confirmation with preclinical and clinical studies. Since breast cancer is the primary cause of cancer-related mortality in many countries, the study’s findings are highly pertinent given that this herb is readily available locally and may be a practical and affordable treatment option. The active components of C. rotundus rhizome extracts implicated in the antiproliferative or cytotoxic actions require further investigation. To determine the precise molecular process by which the components in ethanol extract could achieve therapeutic efficacy, a thorough study could be done.

ACKNOWLEDGEMENT

Authors acknowledge the support from Yenepoya (Deemed to be University) and Manipal University for providing the facilities to carry out this study. Authors acknowledge the kind support of Dr. Nitesh Kumar and Dr. Mallikarjuna Rao, Manipal College of Pharmaceutical Sciences.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

REFERENCES


DOI: https://doi.org/10.51248/v43i4.2309