SJIF Impact Factor: 6.711

WORLD JOURNAL OF ADVANCE HEALTHCARE RESEARCH

ISSN: 2457-0400
Volume: 9.
Issue: 9
Page N. 179-184
Year: 2025

Original Article www.wjahr.com

IN VITRO STUDY ON NEUROPROTECTIVE EFFECT OF LEAST EXPLORED BRACKET FUNGI, *MICROPORUS AFFINIS* (BLUME & T. NEES) KUNT. AND *MICROPORUS XANTHOPUS* (FR.) KUNT.

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Article Received date: 06 July 2025 **Article Revised date:** 26 July 2025 **Article Accepted date:** 17 August 2025

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ABSTRACT
The species richness of mushrooms and their natural beauty has long fascinated mankind. We have treasured them for their medicinal and culinary properties. *Microporus*, a least explored bracket fungus belongs to the family polyporaceae under the class basidiomycetes. Fungal secondary metabolites have shown promising neuroprotective effects, offering potential therapeutic avenues for neurodegenerative diseases like Alzheimer's and Parkinson's. Neurodegeneration is mainly characterized by the accumulation of amyloid – β-peptide (Aβ). Since it is an important cause of neuronal death, there is a need for neuroprotective studies. Thus, neuroprotective activity of *M. affinis* and *M. xanthopus* was screened using IMR-32, neuroblastoma cell lines using MTT assay and apoptotic determination by using Acridine orange and ethidium bromide double staining method. Even though, *M. xanthopus*, is more potent in antiapoptotic properties, further validations are needed to support its neuroprotective efficacy.

KEYWORDS: Neuroprotection, neurodegeneration, *Microporus*, apoptosis, cell viability.

INTRODUCTION

Many polypore species are however, employed in pharmacology for the treatment of a wide range of ailments including bacterial and viral infections, allergies, neurological problems and cancer (Lindequist et al. 2005). In modern day, its biotechnological potential has been highlighted recently with pharmacological studies indicating significant antimicrobial, anticancer, anti-inflammatory and neuroprotective activities (Pleszczyn´ska et al. 2017). Earlier studies (Lee et al. 2019) reported that, edible-medicinal mushrooms can improve memory and cognitive functions and slow dementia and neurodegeneration in age-related neurodegenerative disorders, including Alzheimer's and Parkinson's. In an earlier study conducted by Kou et al., 2022 suggests that mushrooms for its potential as a dietary supplement for neurodegenerative diseases.

Microporus, a least explored genera of bracket fungi. Among these, *M. affinis* is edible (Arko et al., 2017) and *M. xanthopus* is an inedible (Ao et al. 2016), bracket fungus which belong to the family Polyporaceae under the class Basidiomycetes. The genera *Microporus* has been explored taxonomically and medicinally to some extent. The mature fruiting bodies of *M. xanthopus* have thin, funnel shaped caps that are concentrically zoned in various shades of brown and is commonly called yellow footed polypore (Mohan, 2013). Fruiting bodies of *M. affinis* is characterized by the presence of approximately parallel bands of purple, brown, cream or black of varying widths with the outer margin white. Lower surface is white to fawn and finely pored (3-5 pores per mm). Stipe is up to 2 cm long, lateral, hard and brown to reddish often black coloured. Due to the presence of variety of chemical constituents, the present study is focussed to reveal the neuroprotective potential of *M. affinis* and *M. xanthopus*.

MATERIALS AND METHOD

Fruiting bodies of both the species were collected from Wayanad, Kozhikode, Ernakulam, Palakkad, Kannur and Malappuram districts of Kerala, India. Methanolic extracts of the collected specimens were subjected to neuroprotective screening. Neuroprotective studies were carried out by using neuroblastoma cells (IMR 32 cell line), purchased from NCCS (National Centre for Cell

Sciences), Pune and was maintained in Dulbecco's modified eagles' media (HIMEDIA).

i. *In vitro* neuroprotective effect determination by MTT assay

The cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10 % FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100 µg/mL), Streptomycin (100 µg/mL) and Amphoteracin B (2.5 µg/mL). Cultured cell lines were kept at 37ºC in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany).

The viability of cells was evaluated by direct observation of cells by an inverted phase contrast microscope and followed by MTT assay method.

Cells seeding in 96 well plate

Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10 % growth medium, 100 µL of cell suspension (5 x 10^4 cells/well) was seeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5 % CO_2 incubator.

Preparation of compound stock

One mg of the sample was dissolved in 1 mL of DMEM using a cyclomixer. The extract solution was filtered through 0.22 µm Millipore syringe filter to ensure the sterility. Amyloid (10 µM) was added to induce toxicity.

Cytotoxicity evaluation

The prepared extracts in 5% DMEM were five times serially diluted by two-fold dilution (100 µg, 50 µg, 25 µg, 12.5 µg and 6.25 µg in 500 µL of 5% DMEM) and each concentration of 100 µL were added in triplicates to the respective wells and incubated at 37ºC in a humidified 5 % CO_2 incubator.

Cytotoxicity assay by direct microscopic observation

Entire plate was observed at an interval of each 24 hours for 3 days in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observations were recorded as images. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in cytoplasm of the cells were considered as indicators of cytotoxicity.

Cytotoxicity assay by MTT method

Fifteen mg of MTT (Sigma, M-5655) was dissolved in 3 mL PBS and sterilized by filter sterilization.

After 24 hours of incubation period, the sample contents in wells were removed and 30 µL of reconstituted MTT solution was added to all the test and control wells. The plate was gently shaken well and incubated at 37ºC in a humidified 5 % CO_2 incubator for 4 hours. After the incubation period, the supernatant was removed and 100 µL of MTT Solubilization Solution (DMSO) was added and the wells were mixed gently by pipetting up and down in order to solubilize the formazan crystals. The absorbance was measured by using microplate reader at a wavelength of 540 nm (Talarico et al., 2004).

The percentage of growth inhibition was calculated using the formula.

$$\text{Percentage of viability} = \frac{\text{mean OD of samples}}{\text{mean OD of control}} \times 100$$

ii. Apoptosis determination by using acridine orange and ethidium bromide (AO/EtBr) double staining

DNA-binding dyes Acridine Orange (AO) and Ethidium Bromide (EtBr) (Sigma, USA) were used for the morphological detection of apoptotic and necrotic cells (Zhang et al, 1998). AO is taken up by both viable and non-viable cells and emits green fluorescence if intercalated into DNA. EtBr is taken up only by non-viable cells and emits red fluorescence by intercalation into DNA.

After treatment with sample (*M. xanthopus*) for 24 hours, the cells were washed by cold PBS and then stained with a mixture of AO (100 µg/mL) and EtBr (100 µg/mL) at room temperature for 10 min. The stained cells were washed twice with 1X PBS and observed by a fluorescence microscope in blue filter of fluorescent microscope (Olympus CKX41 with Optika Pro5 camera).

The cells were divided into four categories as follows: living cells (normal green nucleus), early apoptotic (bright green nucleus with condensed or fragmented chromatin), late apoptotic (orange stained nuclei with chromatin condensation or fragmentation) and necrotic cells (uniformly orange stained cell nuclei).

RESULTS AND DISCUSSION

Neuroprotective effects refer to the ability of substances to protect the nervous system from neurodegenerative diseases like Alzheimer's, Parkinson's, stroke *etc*. As the case of neurodegenerative diseases are increasing now a days, there is a need for the studies on neuroprotective areas. Alzheimer's disease (AD) is characterized by an increase in amyloid β peptide (Aβ) aggregation. One strategy in the search of new treatments for AD focuses on compounds that decrease Aβ accumulation. Compounds containing a benzofuran ring have been described to play an important role in decreasing Aβ-induced toxicity; however, only synthetic benzofurans have been tested thus far (Gonz´alez-Ram´ıreza et al., 2018). So, as a need for new sources, the neuroprotective effect of two species of *Microporus* was evaluated in IMR 32 neuroblastoma cell lines using MTT assay and apoptosis determination by AO/EtBr double staining.

i. *In vitro* neuroprotective effect determination by MTT assay

The cell viability of the fungal extract was compared with control and the cells treated with amyloid. Cell

viability was found to be declined in cells treated with amyloid (44.84 ± 0.33).

In case of cells treated with the extract of *M. affinis*, percentage of cell viability increased from 51.70 ± 0.32 (for the concentration of 6.25 µg/mL) to 57.87 ± 0.08 (for the concentration of 100 µg/mL). Even though, there is an increase in percentage of viability, it is not concentration dependent as viability percentage greater in 25 µg/mL and 50 µg/mL than 100 µg/mL of the extract.

There is a gradual increase in the percentage of cell viability for cells treated with the extract of *M. xanthopus* (**Fig. 1**). For the lowest concentration (6.25 µg/mL) it was found to be 46.20 ± 0.63 and for the highest concentration (100 µg/mL) viability percentage is increased to 84.51 ± 0.34. This clearly indicates dose dependent efficiency of *M. xanthopus* in the recovery of degenerated neuronal cells. Microphotographs of the morphology of treated cells also affected similarly and is recorded in **Fig. 2** and **3.**

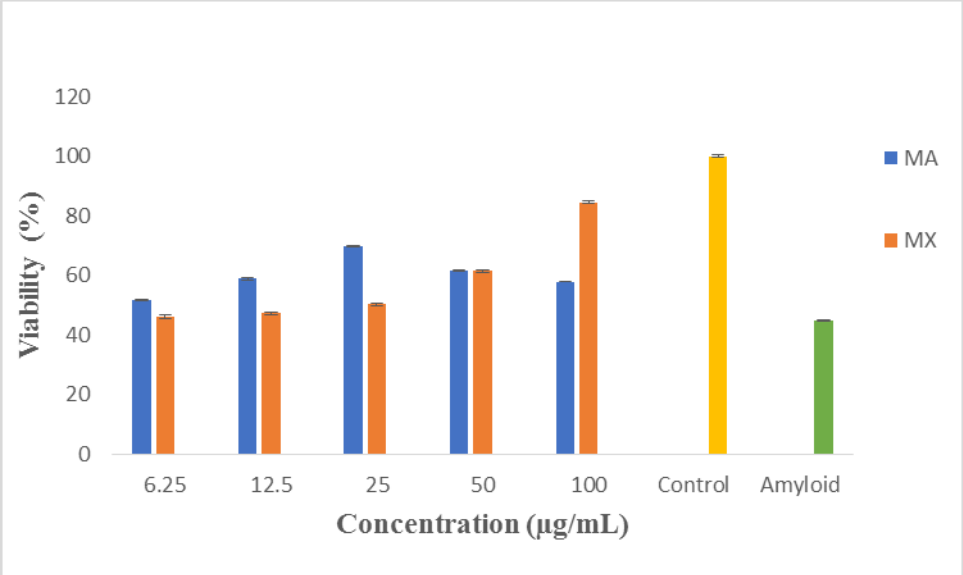
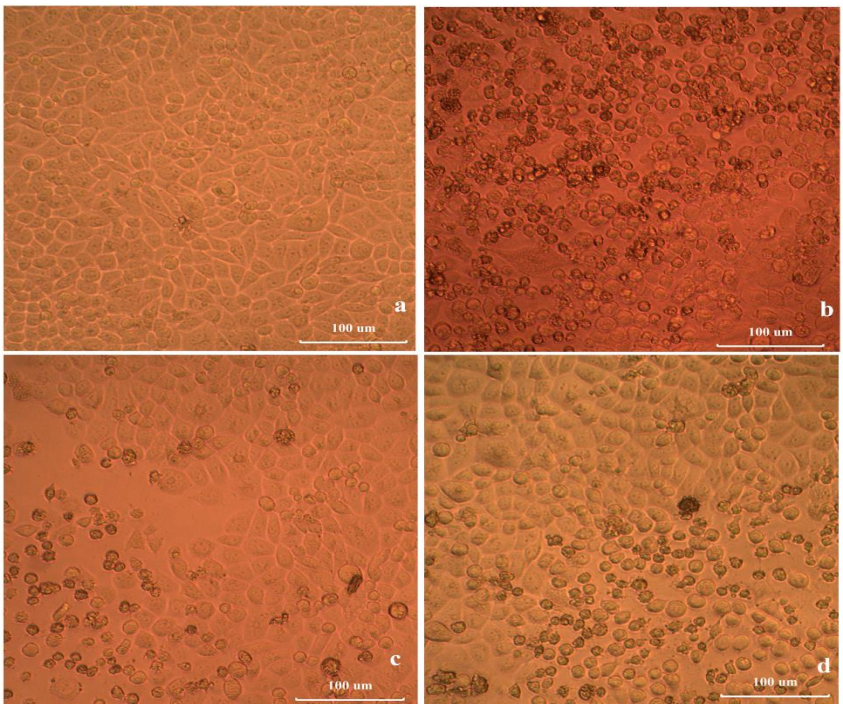


Fig. 1: Determination of cell viability percentage using MTT assay in the cells treated with amyloid, different concentrations of *Microporus* and the control. (MX= *M. xanthopus* and MA= *M. affinis*).



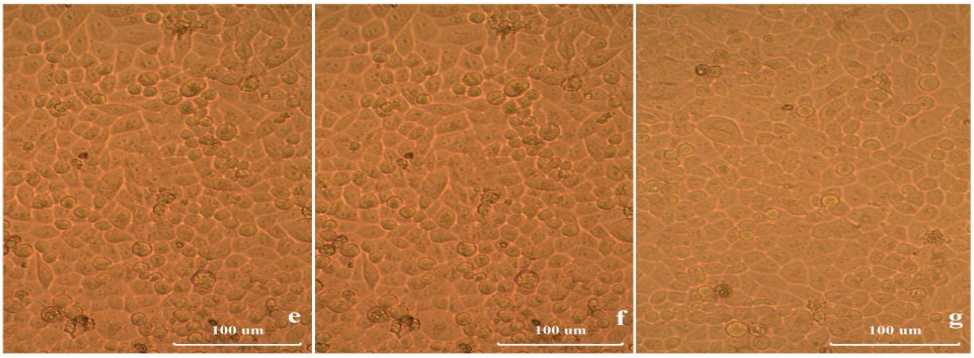


Figure 2: *In vitro* neuroprotective effect determination by MTT assay on methanolic extracts of *M. xanthopus* on IMR32 cell line **a.** Control; **b.** amyloid treated; **c-f:** cells treated with extracts of M. affinis; **c.** 6.25 µg/mL; **d.** 12.5 µg/mL; **e.** 25 µg/mL; **f.** 50 µg/mL; g. 100 µg/mL Scale bar 100 µm

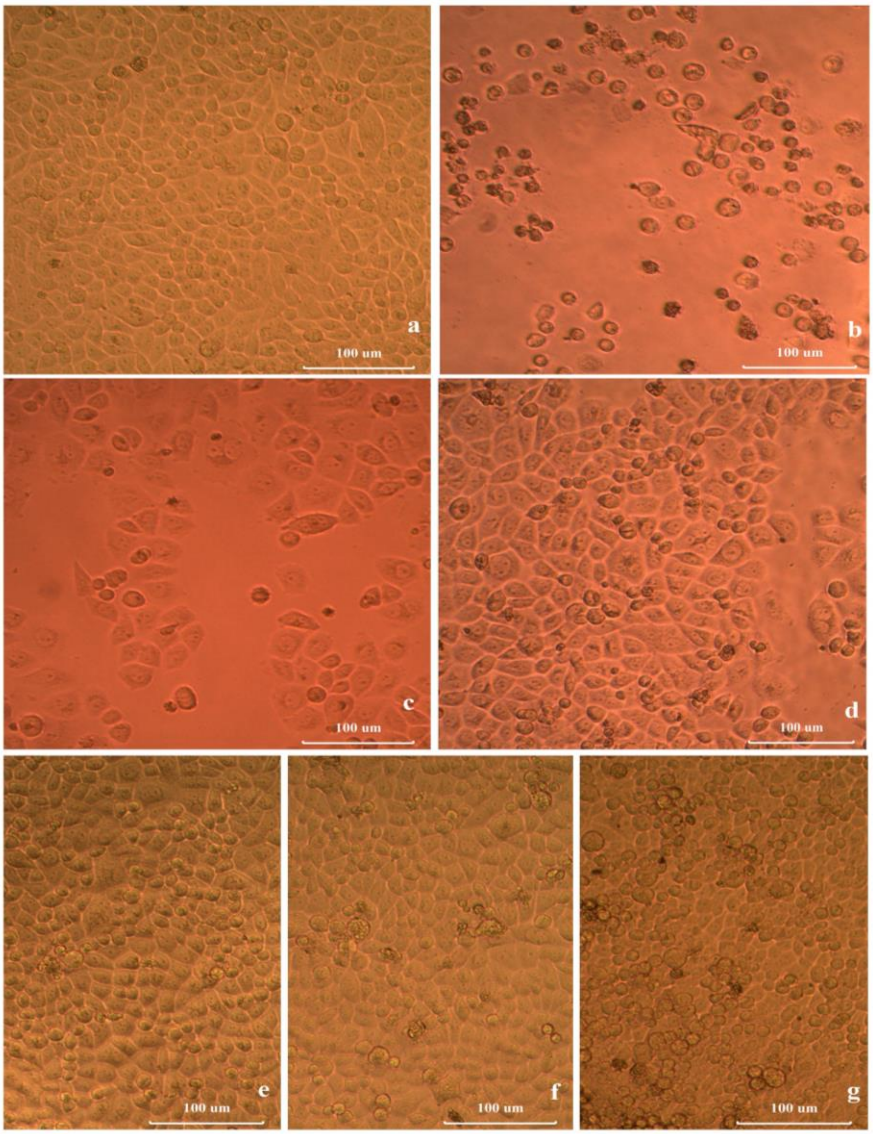


Fig. 3: *In vitro* neuroprotective effect determination by MTT assay on methanolic extracts of *M. affinis* on IMR32 cell line **a.** Control; **b.** amyloid treated; **c-f:** cells treated with extracts of *M. affinis* **c.** 6.25 µg/mL; **d.** 12.5 µg/mL; **e.** 25 µg/mL; **f.** 50 µg/mL; g. 100 µg/mL Scale bar 100 µm

ii. Apoptosis determination by using acridine orange and ethidium bromide (AO/EtBr) double staining

Apoptosis or programmed cell death, plays an important role in the development and progression of neurodegenerative diseases which are characterized by progressive loss of neurons and apoptosis is a key mechanism diving this neuronal death.

For the morphological detection of apoptotic and necrotic cells, DNA-binding dyes Acridine Orange (AO) and Ethidium Bromide (EtBr) are used. AO is taken up by both viable and non -viable cells and emits green fluorescence. EtBr is taken up by only non -viable cells and emits red fluorescence. By observing the stained cells under fluorescence microscope, four categories of cells are obtained: living cells with normal green nuclei, early apoptotic cells with bright green nuclei, late apoptotic cells having orange stained nuclei with chromatin condensation or fragmentation and finally apoptotic cells with uniformly orange stained cell nuclei. In comparison with the extract of *M. affinis*, the extract of *M. xanthopus* is found to be more effective in neuroprotective aspects. The result was compared with the control and cells treated with amyloid. Thus, further studies are carried out in the extract of *M. xanthopus* only. Photographs obtained are given in **Fig. 4**.

The human neuronal apoptosis inhibitory protein (NAIP) gene has been discovered as candidate gene for spinal muscular atrophy, a genetic disorder characterized by motor neuron loss in the spinal cord (Gotz et al., 2000). In this study, *M. xanthopus* is found to be more effective antiapoptotic activities, which are crucial in the neuronal disease recovery. It offers potential for preventing excessive neuronal death. However, consideration of potential challenges with utmost care and the development of targeted and effective therapies are essential for successful implementation. Despite the promising medicinal and therapeutic potential of the mushrooms, the pharmacological applications are not commercialized sufficiently. More efforts are needed on the exploration of the therapeutic potential of medicinal mushrooms and in the field of drug development. Essential tasks include successful human-based clinical studies using high-quality mushroom-derived products for the treatment of diseases and establishing economical ways to produce these products under controlled conditions (Ito et al., 2020).

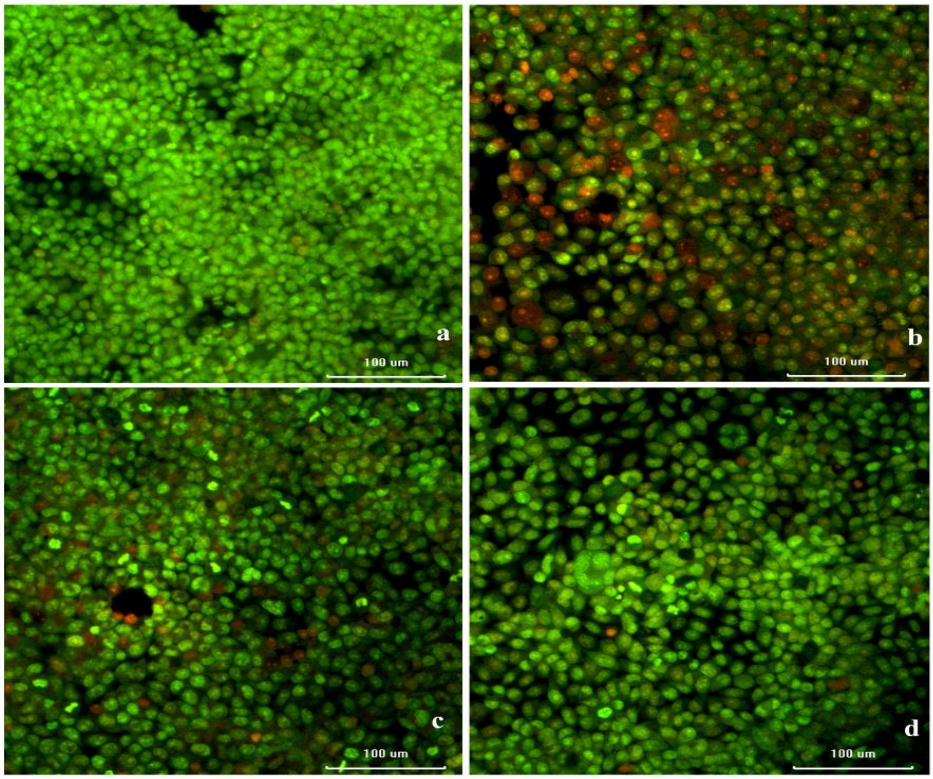


Fig. 4: Determination of apoptosis by using AO/EtBr double staining on IMR32 cell line on methanolic extracts of *M. xanthopus* on **a.** Control; **b.** amyloid treated cells; **c.** Cells treated with the extracts of *M. affinis*; **d.** Cells treated with the extracts of *M. xanthopus*. Scale bar 100 µm

CONCLUSION

M. xanthopus is found to possess better neuroprotective activity than that of *M. affinis.*

Even though, the neuroprotective potential of *Microporus* is promising, further research is needed to obtain an in-depth understanding of their mechanisms and to optimize their therapeutic applications.

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