INTERNATIONAL RESEARCH JOURNAL OF PHARMACY
www.irjponline.com
ISSN 2230 – 8407

Research Article

SCREENING OF BIOACTIVE COMPONENTS BY GC-MS ANALYSIS AND THE STUDY ON *IN VITRO* ANTICANCER EFFICACY OF *MICROPORUS AFFINIS* (BLUME & T. NEES) KUNT.

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Article Received on: 27/10/20 Approved for publication: 11/12/20

DOI: 10.7897/2230-8407.1112104

ABSTRACT

Natural compounds with biological activity are normally present in plants, mushrooms and their natural sources. Applied mycology is one of the most stimulating and rapidly evolving areas of the biological sciences. Hence the present study focussed on exploring *Microporus affinis* (Blume & T. Nees) Kunt., the least explored and edible bracket fungus. Chemical characterization by GC-MS analysis resulted in the presence of 47 bioactive compounds. 9, 12- Octadecadienoic acid (Z,Z)- methyl ester, Ergosterol, Monolinolein, Thiacremonone, Stellasterol, n- Hexadecanoic acid, Ribitol, Maltol etc., were the leading compounds. Because of the presence of various bioactive compounds which have been already reported to possess antitumor, antioxidant and anticancer activities, *M. affinis* extract has been tested for in vitro anticancer efficacy on DLD1 cell lines (cultured in DMEM medium) using MTT assay. It resulted in the decrease of percentage of viability as the increase in concentration of the extract. Apoptosis was determined by using Acridine orange and Ethidium bromide staining. Thus, the taxa, *M. affinis* can be recommended for further anticancer assays for validation.

Key words: *Microporus affinis*, chemical compounds, sterols, esters, cell line, apoptosis and anticancer

INTRODUCTION

The use of mushrooms with potential therapeutic properties raises global interests from the scientific and clinical community. Fungal biology has great significance in the field of medicinal research since it had led to the discovery of several wonder drugs. Many fungal members are used for culinary and medicinal purposes. Among these, macro fungi (both gilled and bracket fungus) have more implication. Many novel biologically active compounds have been reported as a result of research on medicinal mushrooms[1]. Bioactive compounds found in medicinal mushrooms may provide anticancer action with a minimum of side effects.

Cancer is a dreadful disease, which is characterised by uncontrolled cell growth and local tissue invasion and cause several deaths per year. It is a multistep disease involving various factors. The synthetic drugs which are in use clinically have not completely succeeded in fulfilling expectations, considering the extensive cost of their development. There is a constant need for the development of cost effective, novel drugs. The chemotherapeutic anticancer compounds can trigger a chain of cellular responses that influence cell proliferation and tumorigenesis. A much-studied cellular response is apoptosis or programmed cell death.

Indian traditional system of medicine has been the focus of cancer research recently, as the drugs are natural and safe. The secondary metabolites are the excellent source of new medicinal compounds. Mushrooms possess medicinal properties because of the presence of different types of secondary metabolites[2]. Thus, the present study is focused on exploring *Microporus affinis* (Blume & T. Nees) Kunt., is an edible and least explored bracket fungus. It belongs to the family Polyporaceae and the class Basidiomycetes. It is a widespread polypore that is common in tropical and subtropical regions. Basidiomycetes produce various classes of secondary metabolites with a wide variety of biological activities[3,4]. Some basidiomycetes are clinically used for anticancer treatment and prevention in Asian countries[5,6]. The objectives of the study are focused on revealing various bioactive components using GC-MS analysis, evaluation of *in vitro* anticancer efficacy of *M. affinis* on DLD1 cells by using MTT assay and determination of apoptosis by acridine orange and ethidium bromide staining.

MATERIALS AND METHODS

M. affinis resembles to *M. xanthopus* but it differs by having lateral stem, slightly shorter spores and distinctly dextrinoid hyphae[7]. Fruiting bodies of *M. affinis* were collected from Iringolkavu (Thusharagiri, Ernakulam), neighbouring areas of Pookod Lake (Wayanad) and Calicut University campus (Malappuram), Kerala, India. Collected specimens were dried and powdered. Powdered materials, 10 g of each were extracted with 100 ml of 100% methanol for 6 hours by using Soxhlet apparatus. The extract thus obtained was cooled, filtered and concentrated by removing the solvent in a vacuum evaporator. Further experiments were done using this dried extract.

Chemical characterization using GC-MS

The methanolic extract was subjected to GC-MS analysis for the characterization of chemical compounds using GC-MS (Shimadzu QP-2010 plus). Helium was used as the carrier gas at a flow rate of 1ml/min. Injection volume was 1 µl and the split ratio was 1:20. 70 ev was the electron ionization voltage. The constituents were identified by comparison of their linear retention indices. Identification of the individual components was done by using the NIST- MS Search and quantification was done by using percentage peak area calculation.

Effect on cell line

For anticancer effect determination using MTT assay, DLD1 (Human Colorectal Adenocarcinoma) cell was used. The effective extract was also tested on L929 cell lines in order to find out the effect of the extract on normal cells. The cell line was cultured in DMEM medium. The culture was maintained up to 70% confluence. From the stock solution, various concentrations (6.25, 12.5, 25, 50 and 100 µg in 500 µl of 5% DMEM) were prepared. Each concentration of 100 µl were added in triplicates and incubated in 37^0C for 24 hours. Non treated control cells were also maintained. These were observed after 24 hours of treatment in an inverted phase contrast microscope. Microscopic observations were recorded as images. Any detectable changes in the morphology of the cells such as rounding, shrinking of cells and granulation in the cytoplasm of cells were considered as indicators of cytotoxicity.

Anticancer assay using MTT method

Fifteen mg of MTT was reconstituted in 3 ml PBS until completely dissolved and sterilized by filter sterilization. After 24 hours of incubation period, the sample content in wells were removed and 30 µl of reconstituted MTT solution was added to all test and control wells and incubated for 4 hours. After the incubation period, supernatant was removed and 100 µl MTT solubilisation solution (DMSO) was added in order to solubilize the formazan crystals. The absorbance was measured by using microplate reader at a wavelength of 540 nm[8]. From this, percentage of cell viability was calculated.

Determination of apoptosis by Acridine orange and Ethidium bromide

After attaining sufficient confluency, LC 50 concentration of sample (171.759µg/mL) was added. After treatment with the samples, the cells were washed by cold PBS and then stained with a mixture of Acridine orange (100 µg/ml) and Ethidium bromide (100 µg/ml) at room temperature for 10 min. The stained cells were washed twice with 1X PBS and observed in blue filter of fluorescent microscope (Olympus CKX41 with Optika Pro5 camera).

RESULTS AND DISCUSSION

GC-MS analysis showed the presence of different active compounds. In the present study, 47 compounds were characterized and were categorized into 11 classes. In which, Fatty acid, sterol, fatty acid ester, ketone, ester, alcohol *etc*. were the leading classes. 9,12- octadecadienoic acid (Z,Z) - methyl ester, ergosterol, monolinolein, thiacremonone, stellasterol, n-hexadecanoic acid, pentadecanoic acid, ribitol, maltol, dihydroxyergosterol *etc*. were the major compounds. Presence of biologically active components such as thiacremonone, maltol, decanoic acid, ergosterol, stellasterol *etc.* is the highlight of *M. affinis* extract. Among the wide spectrum of chemical constituents, some are already reported to possess antioxidant and antitumor activities, which may be exploited for the production of mycopharmaceuticals.

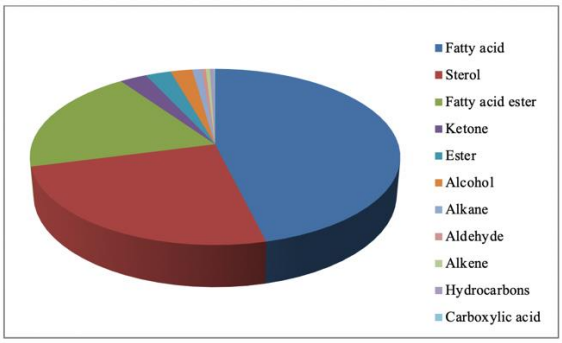


Fig. 1: Class of compounds obtained by GC-MS analysis of *M. affinis*

Earlier report suggests thiacremonone as a novel sulphur containing chemotherapeutic agent[9]. Hence presence of thiacremonone in *M. affinis* extract may boost up its anticancer efficacy. Previous works have shown that, ergosterol exhibit some degree of antitumor activities[10,11], which will be steppingstone for further studies in anticancer assays. Recent study on *M. affinis* has reported a new cadinane type sesquiterpenoid[12].

Cancer is one of the leading causes of death worldwide. The most common treatment of cancer is chemotherapy, which usually has side effects. Therefore, it is necessary to find an alternative method which is most effective and non-toxic. In recent years, mushrooms have gained a lot of alteration as a source of physiological functional food and drug because of their medicinal value[13]. Because of the presence of sterols and esters which possess antitumor and antioxidant properties, *M. affinis* was tested for *in vitro* anticancer efficacy by using MTT assay on DLD1 cell line. Present study reported the presence of rounding and shrinking of DLD1, which forms the clear indicators of cytotoxicity. From the absorbance that was measured by using microplate reader at a wavelength of 540 nm, it is clear that the percentage of viability for lower concentration (6.25 µg/ml) was found to be 89.39 % and for higher concentration (100 µg/ml), it was 66.81 %. Thus, it is clear that as concentration of the extract increases, percentage of viability decrease.

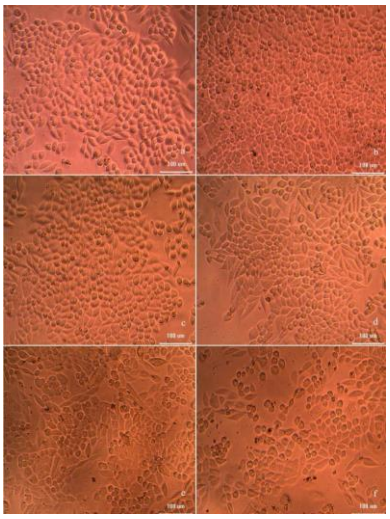
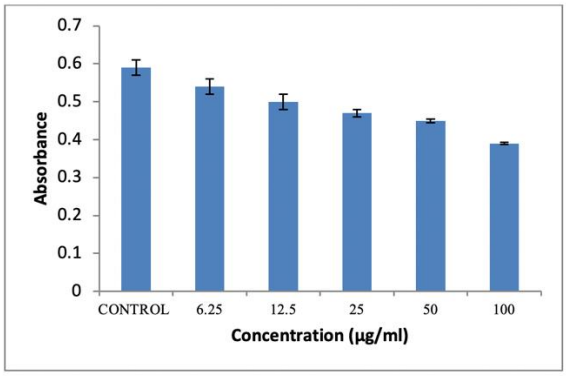


Fig. 2: Anticancer effect of methanolic extract of *M. affinis* on DLD1 cells. a. Control, b. 6.25 µg/ml, c. 12.5 µg/ml, d. 25 µg/ml, e. 50 µg/ml, f. 100 µg/ml



DNA-binding dyes Acridine Orange and Ethidium Bromide (Sigma, USA) were used for the morphological detection of apoptotic and necrotic cells[14]. Acridine orange is taken up by both viable and non-viable cells and emits green fluorescence if intercalated into double stranded nucleic acid (DNA). Ethidium bromide is taken up only by non-viable cells and emits red fluorescence by intercalation into DNA.

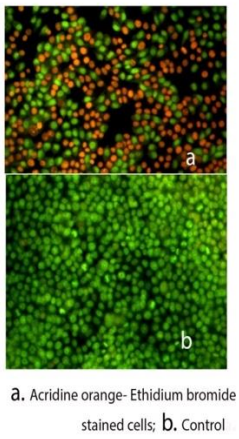


Fig. 3: Apoptotic effect of *M. affinis* on DLD1 cells using acridine orange and ethidium bromide

In the apoptotic assay the living cells were observed with normal green nucleus, early apoptotic cells with bright green nucleus showing condensed or fragmented chromatin, late apoptotic cells with orange-stained nuclei showing chromatin condensation or fragmentation and necrotic cells with uniformly orange stained cell nuclei.

In earlier works there is a report on the medicinal and bioactive potential of a related species like *M. xanthopus*[15].Thus the genus Microporus is medicinally significant.

CONCLUSION

This magnificent cytotoxic impetus of *M. affinis* can be exploited in future for developing the taxa as a drug candidate. It is unambiguous that the genus *Microporus* is a therapeutically important one. Thus, it can form a key material for further studies and can be extended to animal assays for drug validation.

ACKNOWLEDGEMENTS

The first author gratefully acknowledges Prof. P. Manimohan (Department of Botany, University of Calicut, Kerala, India) and Dr T. K. Arun Kumar (Assistant Prof., Department of Botany, Zamoris Guruvayurapan College, Kozhikode, Kerala, India) for authenticating the study material.

ABBREVIATIONS

- **GC-MS:** Gas Chromatography and Mass Spectrometry
- **MTT :** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
- **DMEM:** Dulbecco's Modified Eagle Medium
- **DLD1:** Human colorectal adenocarcinoma cell line
- **NIST MS:** National Institute of Standards and Technology for Mass Spectrometry
- **DMSO:** Dimethyl sulfoxide
- **PBS :** Phosphate-buffered saline

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Cite this article as:

S. Aneesh and J. E. Thoppil. Screening of bioactive components by GC-MS analysis and the study on In vitro anticancer efficacy of *Microporus affinis* (Blume & T. Nees) Kunt. Int. Res. J. Pharm. 2020;11(12):31-34.
http://dx.doi.org/10.7897/2230-8407.1112104

Source of support: Nil, Conflict of interest: None Declared