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IN VITRO EVALUATION OF ANTICANCER ACTIVITY OF *HYPSIZYGOUS ULMARIUS* (BULL.) AGAINST MCF-7 CANCER CELL LINE

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ABSTRACT

This study aimed to investigate *in vitro* anticancer activity of *Hypsizygous ulmarius* (Bull.) fruiting bodies extracts against MCF-7 breast cancer cell line. *Hypsizygous ulmarius* have been extracted using methanol and fractionated by petroleum ether and ethyl acetate, respectively. Phytochemical analysis using standard methods were applied. MTT assay was used to evaluated anticancer activity of methanol extract, ether and ethyl acetate fractions against MCF-7 cancer cell line. Phytochemical analysis showed the presence of alkaloids, tannins and phenolic compounds, flavonoids, anthraquinone, saponin, coumarin

steroids in methanol extract. Petroleum ether fraction contain tannins and phenolic compounds which are absent in ethyl acetate fraction. Anticancer activity using MTT assay showed that ethyl acetate fraction the maximum activity against MCF-7 cancer cell line with IC_{50} value of 10.78 µg/ml followed by extract with IC_{50} value of 24.92 µg/ml. The lowest activity was showed by petroleum ether fraction with IC_{50} of 39.98 µg/ml.

Keywords: *H. ulmarius*, MCF-7, MTT assay, Methanol extract, Petroleum ether fraction, Ethyl acetate fraction

INTRODUCTION

Cancer is a disease that characterized by irregular proliferation of cells due to genetic mutations in DNA. It's one of the most important problems and the second killer disease around the world with more than 100 types **[1-7]**. There are many methods used for cancer treatment such as chemotherapy, but the major problem of this method is non-selectivity in which tumor as well as natural cells will be damaged. Therefore, availability of natural products with higher effectiveness and lower side effects is desired **[8]**. There are many sources for natural extracts such as microorganisms, plants, fungi, vertebrates and invertebrates **[2, 9]**. Mushrooms produce a wide range of medical compounds that can be used for cancer treatment. These compounds are called secondary metabolite. Alkaloids, polyphenols, flavonoids, saponin, and tannins are important constituents of these compounds. Secondary metabolites have biological effects such as antimicrobial, anti-inflammatory, anticancer, antidiabetic etc. **[10-13]**. Evaluation of natural extracts is an essential aspect for drug discovery. In present study we evaluate anticancer activity of methanol extract, petroleum ether and ethyl acetate fractions from fruiting bodies of *Hypsizygous ulmarius* (Bull.) against MFC-7 breast cancer cell line.

MATERIALS AND METHODS

Mushroom material

Dried *H. ulmarius* fruiting bodies were

collected from "S" Mushroom Agritech, Hyderabad, Telangana state, India. Mushroom had been grown at 25°C and dried by solar method.

Mushroom extraction

(650 g) of dried fruiting bodies of *H. ulmarius* were powdered and extracted with methanol (5 L) using a Soxhlet apparatus (14 cycles). The solvent was completely evaporated using rotary evaporator, yielding a viscous extract (133g) **[14]**.

Fractionation of methanolic extract

Part of methanolic extract (only 90 g), was fractionated using petroleum ether and ethyl acetate, respectively. Both solvent extracts were concentrated using rotary evaporator.

Preliminary phytochemical screening

Methanolic extract and its fractions were subjected to phytochemical tests to investigate the presence of phytochemicals using standard methods **[15, 16]**.

Thin layer chromatography (TLC) analysis

Phytochemical screening by TLC analysis using different solvent systems and spray reagents for methanolic extract and its fractions was also characterized **[17, 18]**.

***IN-VITRO* EVALUATION OF ANTICANCER ACTIVITY BY MTT ASSAY**

Cancer cell lines

MCF-7 human breast cancer cell line was provided by National Centre for Cell Science (NCCS), Savitribai Phule Pune University, Pune. Cancer cell line was grown in Eagles Minimum Essential Medium (EMEM) which, contained 10% fetal bovine serum (FBS). Cell line was maintained at 37°C, 100% relative humidity, 5% CO_2, 95% air and subculture twice weekly.

Cell treatment

The monolayer cells were detached and single cell suspensions were made using trypsin-ethylenediaminetetraacetic acid (EDTA). A hemocytometer was used to count the viable cells and the cell suspension was diluted with a medium containing 5% FBS in order to obtain final density of $5x10^4$

cells/ml.

MTT assay

MTT colorimetric assay was applied to evaluate anticancer activity of methanolic extract, petroleum ether and ethyl acetate fractions on MCF-7 cancer cell line **[19]**. Briefly, cancer cells were seeded in 96-well plate at a density of 5×10^3 cells/well and then incubated at 37°C, 5% CO_2, 95% air and 100% relative humidity to allow cell attachment. After 24 hours, the medium was discarded and various concentrations (2.5, 5, 10, 15 and 25 µg/ml) of methanolic extract, petroleum ether and ethyl acetate fractions were added and incubated for 48 hours at 37 °C, 5% CO_2, 95% air and 100% relative humidity. After 48 hours incubation period, the medium was removed and fresh medium with 100 µL MTT (5 mg/mL) was added to each well and the plate was further incubated for four hours. The medium with MTT was flicked off and the formed formazan crystals were solubilized in 100µl of DMSO. Absorbance was read in a micro plate reader spectrophotometer at 570 nm. Cell viability was calculated by the formula:

Cell viability (%) = Test OD /control OD ×100.

Where OD is the optical density. Studies were performed in triplicate.

STATISTICAL ANALYSIS

Experimental data are expressed as means ± standard error. Statistical analyses were performed by one-way ANOVA using SPSS ver. 20.0 software. IC_{50} values were calculated using Excel linear regression by plotting growth inhibition (%) versus concentrations. IC_{50} which is half the maximal inhibitory concentration of extract which results in the reduction of biological activity by 50%.

RESULTS AND DISCUSSION

Phytochemical and TLC analysis

The major phytochemical compounds in methanolic extract (M) are alkaloids, tannins and phenolic compounds, flavonoids, anthraquinone, saponin, coumarin and steroid. In Petroleum ether fraction (P)

tannins and phenolic compounds, flavonoids, anthraquinone, saponins and steroids were present while in ethyl acetate fraction (E) flavonoids, anthraquinone, coumarins and steroids were present.

Similar result was reported, in which alkaloids, saponin, phenolics, tannins and glycosides were found in methanolic extract of *H. ulmarius* fruiting bodies **[20]**. Also, presence of phenols and saponins in aqueous extract of *H. ulmarius* have been indicated **[21]**. Since *H. ulmarius* contain a good number of phytochemical compounds, which have been reported to possess many pharmacological properties **[10-13]**, extracts of this mushroom may will be a good alternative for the treatment of many diseases.

TLC analysis showed similar chromatographic results using different solvent systems and different spray reagents **(Figures 1, 2, 3 and 4)**. Bright blue fluorescence zones in UV-365nm may be due to coumarins (e.g., scopoletin, umbelliferone) **(Figures 1 and 4(A))**. Saponins from colored (vis.) zones with Anisaldehyde sulfuric acid (ASA) and Vanillin sulfuric acid (VSA) reagents **(Figures 1, 4 (B), 2, 3 (A) and 4(C)**. Colored fluorescent zones may be due to all anthraglycosides, coumarins, and flavonoids **(Figures 1 (C), 2 (B)**. Green-blue, yellow, yellow-brown in UV-365 nm is due to Furano- and pyranocoumarins **(Figure 2(C))**. Most constituents react with VSA and H_2SO_4 reagents with colored zones in vis. Both reagents are sufficient to detect bitter principles and saponins (**Figure** 3(B, C)) **[11, 18]**.

TLC results of methanolic extract and its fractions confirmed results of preliminary phytochemical tests in which coumarins, flavonoids and saponins was detected. Glycosides detected only by TLC and this may be due to its low concentrations in methanolic extract and its fractions. In a previous study, HPTLC analysis of methanolic extracts of *Pleurotus ostreatus* and *Lentinus edodes* fruiting bodies showed the presence of ferulic acid, coumarins aglycones, polyphenolic and ergosterol in *P.*

ostreatus extract and coumarins and ergosterol in *Lentinus edodes* extract **[22]**. Also, the presence of coumarin and oleanolic acid in 95% aqueous ethanol extracts of *Russula medullata, Russula virescens, Russula helios* and *Russula alboareolata* have been reported **[23]**. In another study, 95% ethyl alcohol extracts of 16 mushrooms were analyzed using TLC and steroid and terpene were detected in all extracts **[24]**.

***In vitro* anticancer activity**

Anticancer activity of methanolic extract, petroleum ether and ethyl acetate fractions on MCF-7 cancer cell line are represented in **Table 1 and Figure 5 (A, B and C)**.

Results obtained from **Table 1** indicated that at concentrations of 10, 15 and 25 µg/ml, ethyl acetate fraction showed an inhibition activity against MCF-7 cells growth with 55.56, 66.26 and 74.72 %, respectively. IC_{50} of ethyl acetate fraction was 10.78 µg/ml. This is followed by methanolic extract with growth inhibition at 25 µg/ml (50.89 %) and IC_{50} of 24.92 µg/ml. Petroleum ether fraction is found to be less active against MCF-7 cancer cell line with only 38.24% growth inhibition at 25µg/ml and IC_{50} of 39.98 µg/ml in compare to other two extracts **(Figure 5)**. Anticancer activity increases gradually as dose is increased (dose dependent manner).

Mushrooms contain a variety of medical compounds that can be used in cancer treatment **[25]**. These compounds are called secondary metabolite which include flavonoids, alkaloids, saponin, polyphenols and tannins. Secondary metabolites have biological effects such as anticancer, antimicrobial, antidiabetic, anti-inflammatory, etc. **[22-28]**. Evaluation of natural extracts is an essential aspect for drug discovery.

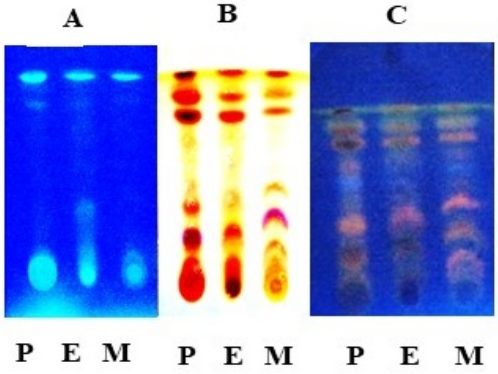

Figure 1: TLC analysis of methanol extract and its fractions. The TLC plate solvent system was chloroform: ethyl acetate (6:4). (A) TLC plate under UV light (365nm), (B) TLC plate sprayed with VSA and (C) seen under UV light (365nm)

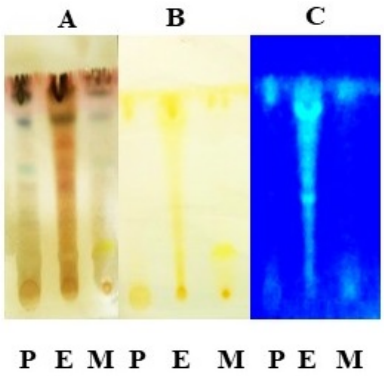

Figure 2: TLC analysis of methanol extract and its fractions. TLC plate solvent system was ethyl acetate: methanol: water (10:1.3:1). (A) TLC plate sprayed with VSA and seen in visible light, (B) TLC plate sprayed with KOH and (C) seen under UV light (365nm)

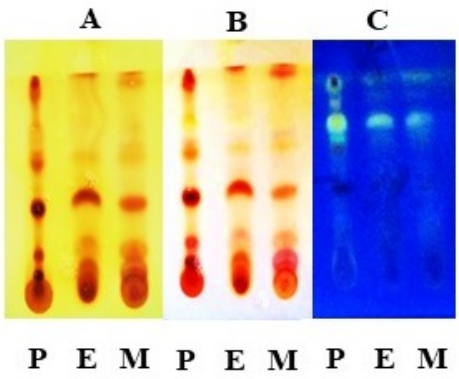

Figure 3: TLC analysis of methanol extract and its fractions. TLC plate solvent system was toluene: ethyl acetate (7:3). (A) TLC plate sprayed with VSA and seen in visible light, (B) TLC plate sprayed with Conc. sulfuric acid and (C) seen under UV light (365nm)

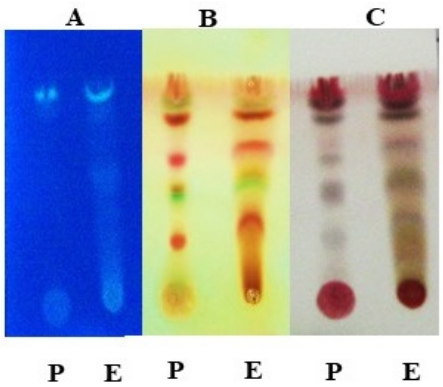

Figure 4: TLC analysis of petroleum ether and ethyl acetate fractions. TLC plate solvent system was petroleum ether: ethyl acetate (2:8). (A) TLC plate under UV light (365nm), (B) TLC plate sprayed with ASA and seen in visible light and (C) TLC plate sprayed with VSA and seen in visible light

Table 1: Percentage of growth inhibition and IC_{50} of methanolic extract and its fractions against MCF-7

Extract and its fraction	(IC_{50} ± SEM)
Methanol	24.92 ± 0.02
Petroleum ether	39.98 ± 0.03
Ethyl acetate	10.78 ± 0.02

Note: All IC_{50} values are expressed as Mean ± SEM (n = 3)

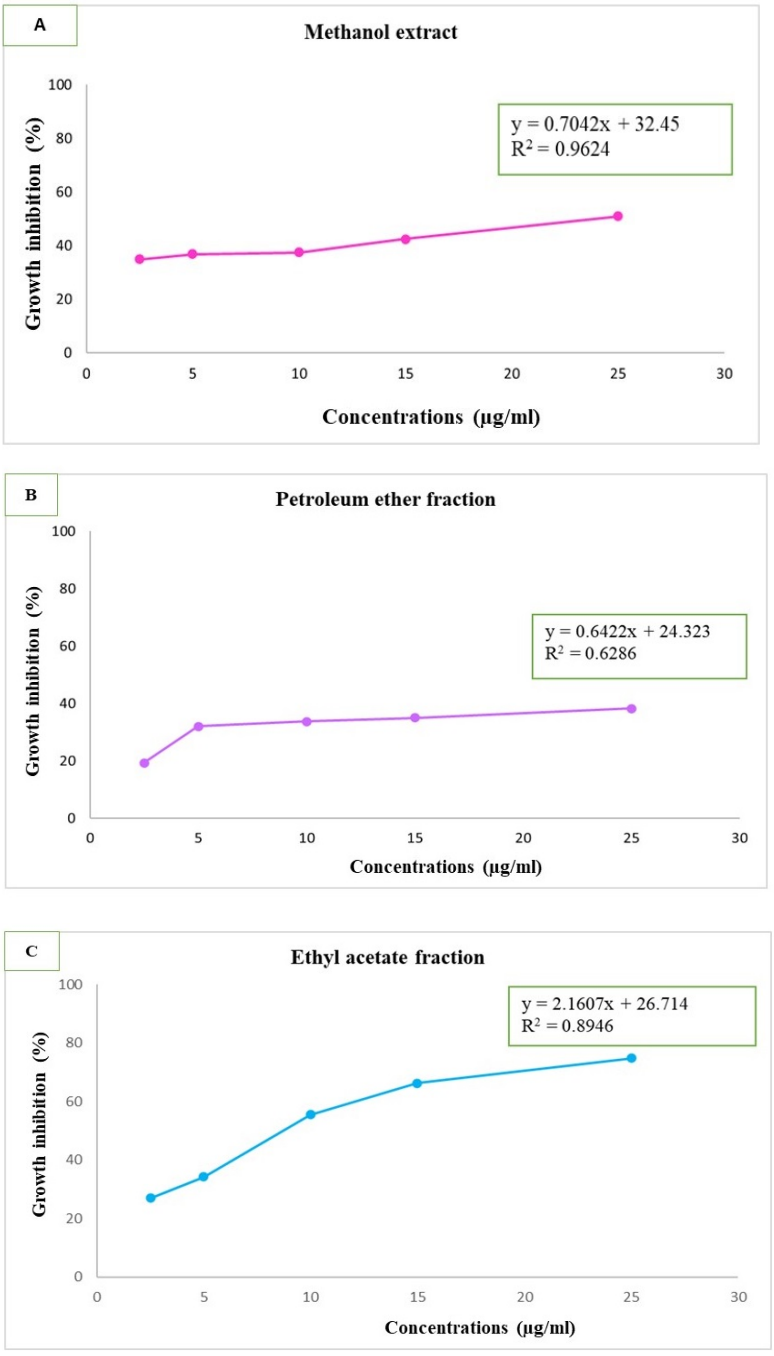

Figure 5: Anticancer activity of (A) methanol, (B) petroleum ether and (C) ethyl acetate fractions against MCF-7 cancer cell line

Anticancer activity of ethanol extracts of *Hypsizygus ulmarius, Pleurotus djamor, Ganoderma lucidum* and *Pleurotus florida* fruiting bodies against HeLa (cervical cancer) cell line was evaluated **[29]**. Their result was similar to our finding in which ethanol extract of *H. ulmarius* showed activity on HeLa cancer cell line in dose dependent manner. At concentrations of 12.5, 25, 50,100 and 200 µg/ml the percentage of HeLa cancer cell viability are 79.842%, 76.151%, 73.421%, 67.942% and 65.891%, respectively.

In a recent study, cytotoxic activity against DLA cancer cell line using aqueous ethanolic extracts of fruiting bodies and cultured mycelia of *H. ulmarius* was evaluates **[30]**. The IC_{50} of fruiting bodies and mycelia extracts were found to be 30µg/ml and 35µg/ml, respectively. Also, in another study, purified exopolysaccharides isolated from *H. ulmarius* and *P. florida* exhibited anticancer activity against MCF-7 breast cancer cell line with cell viability of 47.63% and 66.48%, respectively. Exopolysaccharides of *H. ulmarius* demonstrated encouraging results in terms of anticancer potential **[31]**.

Also, water extracts of *Pleurotus ostreatus, Lentinus edodes, Ganoderma lucidum, Hericium erinaceus* and *Volvariella volvacea* were investigated for their anticancer activity against HT-29 colon and H-1299 lungs carcinoma cell lines **[32]**. Among these mushrooms, water extract of *G. lucidum* showed 24% and 29% viability of cells against H-1299 cell line and HT-29 cell line, respectively. The activity was also in dose-dependent manner.

Also, Anjana *et al* **[33]**, indicated that methanol and ethyl acetate extracts from *Pleurotus ostreatus* showed anticancer activity against melanoma cancer cell lines A-37. The higher anticancer activity was achieved by ethyl acetate extract with IC_{50} of 150µg/ml approximately.

Antitumor activity of *Ganoderma lucidum* extract on HL60, K562 and SGC 7901 was recorded **[34]**. Effect of extract is a dose

dependent inhibitory effect on cancer cells. IC_{50} of K562, HL60 and SGC 7901 cells were 0.39, 0.44 and 0.90 mg/ml, respectively. Furthermore, anticancer activity of water, methanol, ether and ethyl acetate extracts from mycelia, broth, fresh and freeze-dried fruiting bodies of *Agaricus bisporus, Lentinula edodes* and *Pleurotus ostreatus* against human liver carcinoma (Hep G2), human colonic epithelial carcinoma (HCT 116) and human cervical cancer cells (HeLa) were reported **[35]**. The highest anticancer activity was showed by water extract of *L. edodes* lyophilized fruiting bodies with IC_{50} values of 12.1, 15.3 and 15.8 µg/ml for Hep G2, HeLa and HCT 116, respectively. Also, water extracts of *P. ostreatus* lyophilized fruiting bodies showed high anticancer activity with IC_{50} of 12.1, 15.1 and 16.7 µg/ml against HeLa, Hep G2 and HCT 116 cells, respectively.

CONCLUSION

Hypsizygous ulmarius fruiting bodies is a good source for phytochemical compounds. Our study indicated its anticancer activity. Further research required to investigate its activity against different cancer cell lines *in vitro* and *in vivo*.

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