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Pleurotus citrinopileatus Extracts in HepG2 Cancer Cell Study

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ABSTRACT

Introduction: *Pleurotus citrinopileatus* is a mushroom with n-number of biomolecules expressing both nutraceutical and pharmaceutical qualities. In recent years, there are many medicinal properties of the mushroom brought to light, be it the biomolecules which inhibits fat-induced weight gain; cancer treatments for hepatoma, colon or HIV-1 reverse transcriptase inhibitory activity by novel lectins present in it.

Objective: The present study is to find the degree of cell damage in HepG2 cancer cell lines caused by the extracts of *Pleurotus citrinopileatus*.

Method: Herein, we are going to compare the level of cell damage caused by acetone, ethanol and aqueous extracts of *Pleurotus citrinopileatus* using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay.

Result: Acetone extracts of the mushroom showed higher cell damage in HepG2 cell line, indicated by MTT assay with the half maximal inhibitory concentration (IC50) value of 37.03µg/ml, compared to the ethanolic and aqueous extracts of *Pleurotus citrinopileatus*.

Conclusion: The acetone extract appears to possess biologically active compounds that might help reducing the viable cancer cells. If used as an adjuvant drug with cisplatin can reduce the effects caused by it and activate P53 action.

Novelty: Further study on the biomolecules present in oyster mushrooms can give us an insight into the apoptotic behavior of cancer cells.

Key Words: Acetone, Aqueous extract, Cell damage, Cell viability, Ethanol, *Pleurotus citrinopileatus*, Chemo-embolization

INTRODUCTION

Over 50,000 liver cancer cases are reported in India annually. Surgical removal, ablations, chemotherapy, radiotherapy, freezing of cancer cell, embolization, chemo-embolization, drug-targeted therapy, immunotherapy and palliative care are a few first-line treatment plans for cancer suggested by the health professional based on the invasion of cancer.

An anticancer agent called cisplatin is used to treat liver cancer by hepatic arterial infusion chemotherapy method. Based on the extent of malignancy, liver functions and patient choice only can the treatment plan be decided. So, cisplatin can only be used on hepatocellular carcinoma patients without other therapeutic interventions.

The late 20th and early 21st century has embarked the use of non-toxic mushrooms as immunotherapeutic drugs or adjuvant drugs. In this study, we are to investigate the extracts of *Pleurotus citrinopileatus* (*P. citrinopileatus*) and their effect on hepatocarcinoma cell line (HepG2 cell line). In vitro toxicology determinations of unknown compounds were made by counting viable cells after staining with a vital dye. Measurement of radioisotope incorporation as a measure of DNA synthesis, automated counter counting, and other approaches based on dyes and cellular activity are utilized as alternatives. The MTT technique uses mitochondrial dehydrogenases to determine the activity of live cells. The MTT approach is straightforward, precise, and repeatable. The key component is (3-[4, 5-dimethylthiazol-2-yl]-2,

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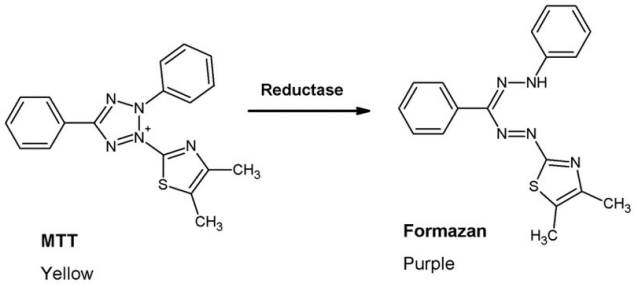
5-diphenyl tetrazolium bromide) or MTT, is a water soluble tetrazolium salt yielding a yellowish solution when prepared in media or salt solutions lacking phenol red.[1] Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by mitochondrial dehydrogenase enzymes of viable cells. DMSO, acidified isopropanol, or other solvents (Pure propanol or ethanol) can be used to dissolve this water insoluble formazan. The purple solution produced is spectrophotometrically measured. A change in the amount of formazan generated in response to a change in cell number indicates the degree of cell damage caused by the test material.



MATERIALS & METHODS

Mushroom extracts

The fruiting bodies of *P. citrinopileatus* were freshly collected, sun-dried and finely grinded. Aqueous extract, ethanol extract and acetone extracts of the mushroom was obtained from the following solvent-solute mixture, five grams of the pulverized mushroom sample in (i) 100 ml of water; (ii) 80% ethanol solution and (iii) 80% acetone solution, run at 150 rpm for 24 hours maintaining the temperature at 25˚C. Whatman No. 1 filter paper was used to filter the mixture and the residue/extract was collected. The extracts were allowed to evaporate and remaining solvent was removed with a freeze drier. These dry extracts are considered the test solutions.

Cisplatin:

A platinum based chemotherapeutic agent which goes by the trade name Platinol or CDDP. It is classified as an alkylating agent used in treatment of various cancer types. The efficiency of the agent is limited because of the drug resistance and varied side effects.

Preparation of test solutions

For Anticancer studies, serial two-fold dilutions (3.125-100µg/ml) were prepared from this for carrying out anticancer studies.

Cell lines and culture medium

Human liver carcinoma cell lines (HepG2) were procured from NCCS, stock cells was cultured in medium supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) in an humidified atmosphere of 5% CO_2 at 37°C until confluent. The cell was dissociated with TPVG solution (0.2 % trypsin, 0.02 % EDTA, 0.05 % glucose in PBS). The viability of the cells is checked and centrifuged. Further 50,000 cells / well was seeded in a 96 well plate and incubated for 24 hrs at 37°C, 5% CO_2 incubator.

Source of reagents: DMEM, FBS, Pen strip, Trypsin procured from Himedia.

Procedure

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using respective media containing 10% FBS. To each well of the 96 well microtiter plate, 100µl of the diluted cell suspension (50,000cells/well) was added. When a partial monolayer had developed after 24 hours, the supernatant was flicked off, the monolayer was washed once with media, and 100l of different test drug doses were applied to the partial monolayer in microtiter plates. The plates were then incubated at 37°C for 24hrs in a 5% CO2 atmosphere. After incubation the test solutions in the wells were discarded and 100µl of MTT (5 mg/10 ml of MTT in PBS) was added to each well. The plates were incubated for 4h at 37°C in 5% CO_2 atmosphere. The supernatant was removed and 100µl of DMSO was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 570 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (IC_{50}) values is generated from the dose-response curves for each cell line.[2,3,5]

IC_{50} Value:

The half maximum inhibitory concentration (IC50) is a measure of a compound's ability to block biological or metabolic function. This numerical value specifies how much of a medicine or other chemical (inhibitor) is required to block a biological process (or component of a process, such as an enzyme, cell, cell receptor, or microbe) by half.

By building a dose-response curve and studying the effect of different concentrations of antagonist on reversing agonist activity, the IC50 of a medication can be established. The IC50 value for a specific antagonist can be obtained by finding the concentration required to inhibit half of the agonist's maximum biological response. IC_{50} values for cytotoxicity tests were derived from a nonlinear regression analysis (curve fit) based on sigmoid dose response curve.[3,5]

The direct microscopic observations of drug treated images of cell lines by inverted biological microscope was performed and percentage of HepG2 cell viability against of

test samples of serial two-fold dilutions (3.125-100µg/ml) were assessed and compared with cisplatin, a commonly used anticancer agent in chemotherapy for hepatocarcinoma patients. Table 1 shows the comparative study made on the HepG2 cell lines with the sample extracts and cisplatin.

Table 1: Serial dilution results for Acetone extracts, Ethanol extracts, Aqueous extracts and cisplatin over Hepatocarcinoma cell line viability percentage.

			Concentration Unit: µG					
Acetone	**BLANK**	**UNTREATED**	**3.12**	**6.25**	**12.5**	**25**	**50**	**100**
Reading 1	0.002	1.059	0.988	0.922	0.854	0.74	0.484	0.104
Reading 2	0.002	1.038	0.984	0.934	0.846	0.764	0.475	0.19
Reading 3	0.002	1.043	0.998	0.928	0.863	0.757	0.489	0.185
Mean	0.002	1.0466667	0.99	0.928	0.8543333	0.7536667	0.4826667	0.15967
Mean OD-Mean B		1.0446667	0.988	0.926	0.8523333	0.7516667	0.4806667	0.15767
STANDARD DEVIATION		0.0109697	0.0072111	0.006	0.0085049	0.0123423	0.0070946	0.04827
Viability %		100	94.575622	88.640715	81.589024	71.952776	46.011487	15.0925
							IC 50 VALUE= 37.03	
Ethanol	**BLANK**	**UNTREATED**	**3.12**	**6.25**	**12.5**	**25**	**50**	**100**
Reading 1	0.002	1.059	1.017	0.964	0.9	0.786	0.528	0.143
Reading 2	0.002	1.038	1.011	0.976	0.91	0.76	0.498	0.132
Reading 3	0.002	1.043	1.023	0.97	0.916	0.777	0.513	0.141
Mean	0.002	1.0466667	1.017	0.97	0.9086667	0.7743333	0.513	0.13867
Mean OD-Mean B		1.0446667	1.015	0.968	0.9066667	0.7723333	0.511	0.13667
STANDARD DEVIATION		0.0109697	0.006	0.006	0.0080829	0.0132035	0.015	0.00586
Viability %		100	97.160179	92.661136	86.790045	73.931078	48.915124	13.0823
							IC 50 VALUE= 39.57	
Aqueous	**BLANK**	**UNTREATED**	**3.12**	**6.25**	**12.5**	**25**	**50**	**100**
Reading 1	0.002	1.059	1.04	1.003	0.965	0.949	0.895	0.574
Reading 2	0.002	1.038	1.028	0.99	0.941	0.945	0.878	0.555
Reading 3	0.002	1.043	1.032	1.011	0.953	0.958	0.884	0.567
Mean	0.002	1.0466667	1.0333333	1.0013333	0.953	0.9506667	0.8856667	0.56533
Mean OD-Mean B		1.0446667	1.0313333	0.9993333	0.951	0.9486667	0.8836667	0.56333
STANDARD DEVIATION		0.0109697	0.0061101	0.0105987	0.012	0.0066583	0.0086217	0.00961
Viability %		100	98.723676	95.660498	91.033823	90.810466	84.588385	53.9247
							IC 50 VALUE= >100	
Cisplatin	**BLANK**	**UNTREATED**	**3.12**	**6.25**	**12.5**	**25**	**50**	**100**
Reading 1	0.002	1.059	0.62	0.457	0.316	0.189	0.111	0.049
Reading 2	0.002	1.038	0.634	0.462	0.324	0.177	0.12	0.063
Reading 3	0.002	1.043	0.613	0.455	0.302	0.206	0.105	0.055
Mean	0.002	1.0466667	0.6223333	0.458	0.314	0.1906667	0.112	0.05567
Mean OD-Mean B		1.0446667	0.6203333	0.456	0.312	0.1886667	0.11	0.05367
STANDARD DEVIATION		0.0109697	0.0106927	0.0036056	0.0111355	0.0145717	0.0075498	0.00702
Viability %		100	59.380983	43.650287	29.865986	18.059987	10.529675	5.1372
							IC 50 VALUE= 4.31	

RESULTS

Overall number of live cells after drug treatment, along with changes in cell morphology, were examined using inverted biological microscopic images. The control HepG2 cell line with MTT solution was seen in Figure 1. Compared to ethanol [Fig. 3] or water extracts [Fig. 4], HepG2 cell lines treated with acetone extract [Fig 2] and cisplatin [Fig 5] showed enormous morphological changes and apoptosis-related characteristics such as shrinkage, nuclear collapse, blebbing cell membrane, and apoptotic bodies. Figure 6 depicted a dose-response curve created from three distinct extract concentrations against the HepG2 cell line, which revealed a substantial degree of cytotoxicity in the acetone extract-treated cell line.

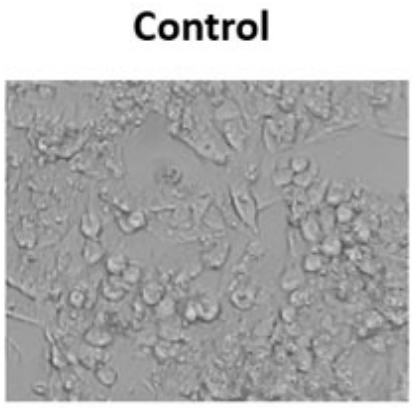


Figure 1: The control HepG2 cell line with MTT alone.

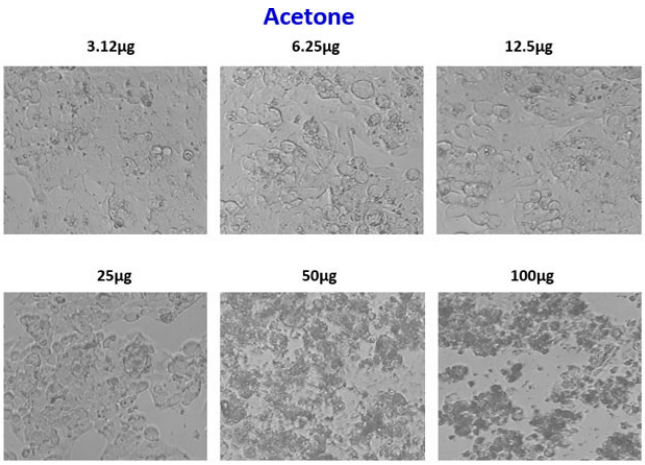


Figure 2: The effect of Acetone extracts on the HepG2 cell line.

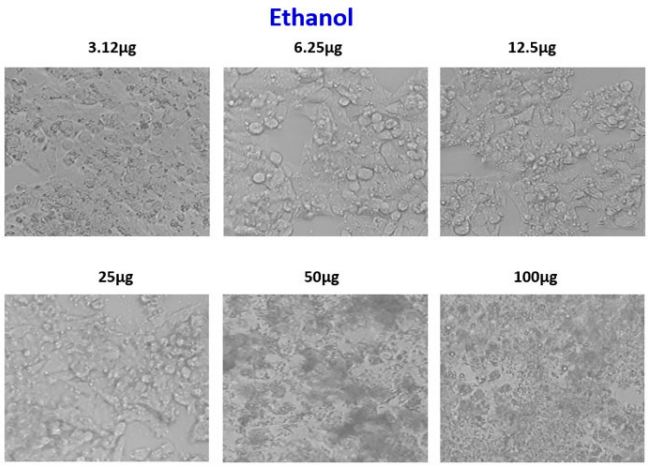


Figure 3: The effect of Ethanol extracts on HepG2 cell line.

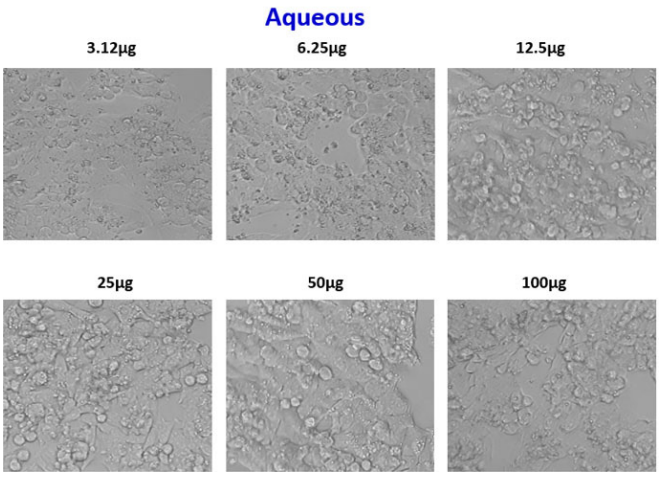


Figure 4: The effect of aqueous extracts on HepG2 cell line.

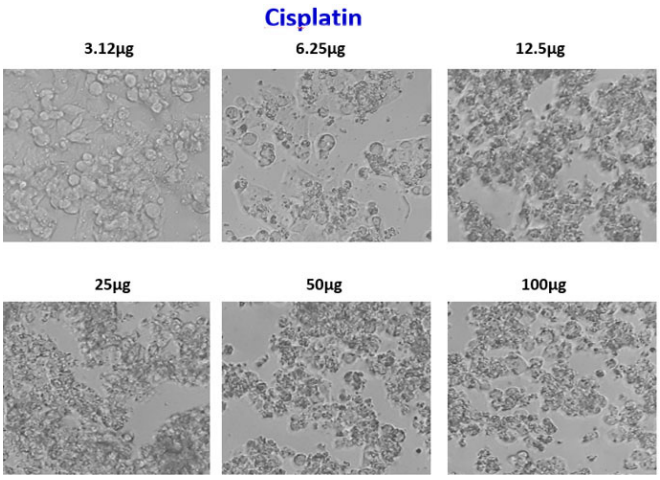


Figure 5: The effect of Cisplatin on HepG2 cell line.

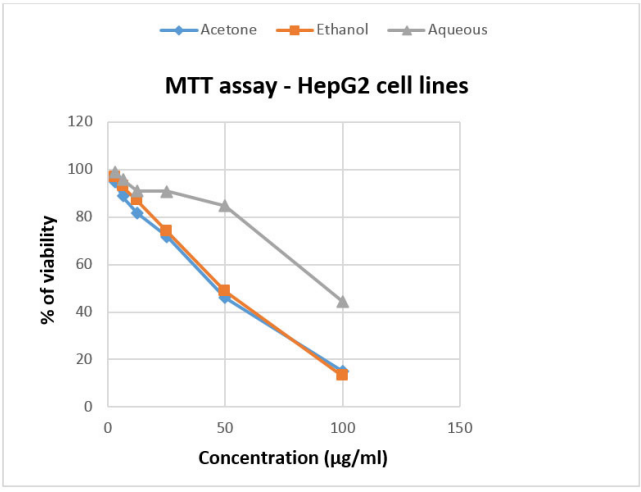


Figure 6: Dose-Response Curve.

The IC_{50} value of the given samples (Acetone, Ethanol, Aqueous) is 37.03, 39.57, >100 µg/ml, which proves that the

100 µg/ml of acetone extract has greater degree of cytotoxicity compared to ethanol and aqueous extracts.

DISCUSSION

In our study the finding that the acetone extracts of *P. citrinopileatus* fruiting bodies exhibited strong inhibitory activity against the HepG2 cells compared to ethanol or aqueous extracts which completely contradicts the study by Younis and his colleagues in which the water extract of *Pleurotus* species shows highest cytotoxic effect against HepG2 and two other cancer cell lines.[8]

More water extracts of *Pleurotus* species have exhibited higher cytotoxicity so far, followed by methanolic extracts rather than acetone or ethanol extracts of the oyster mushroom.

In our study, the use of the acetone extract as an adjuvant enhanced the drug sensitivity to the chemotherapeutic drug, cisplatin, bringing the IC50 value to 4.31 µg/ml. The results obtained were very similar to that conducted by Xu and his colleagues where exposure of liver cancer cells to polysaccharide-protein complex isolated from oyster mushrooms reduced the *in vitro* cancer cell proliferation and invasion but also enhanced the drug-sensitivity to Cisplatin.[9]

Cisplatin has a few limitations in its usage, likely increased drug resistance with multiple side effects. Nausea, vomiting, low blood counts, kidney toxicity, ototoxicity, blood test abnormalities, peripheral neuropathy, loss of appetite, taste change, liver problems are common side effects. As the platinum-based agent wears off early, mechanical stress promotes cisplatin-induced hepatocellular carcinoma cell death. Peripheral blood mononuclear cells are found in elevated numbers upon induced mechanical stress either with or without Cisplatin.[10] Oyster mushrooms have the ability to protect and restore the antioxidant defense mechanism in cisplatin-induced nephrotoxicity in mice.[4]

In Hepatocellular carcinoma patients, mostly with the underlying cancer cell line HePG2, gene mutation in Telomerase reverse transcriptase (TERT) or TP53 is observed.[7] To stop the malignancy, it is important to use an adjuvant drug which would enhance the treatment. Pleurotus species (oyster mushroom species) stimulate NK cell, macrophage, and T cell proliferation, maturation of lymphocytes, natural killer cells, and macrophages results in an increase in the weight and size of the spleen. The above stated immune response activates caspase-dependent apoptosis, thus causing the activation of P53 gene.[6]

CONCLUSION

Through the study, the use of oyster mushroom extract as an adjuvant drug is reiterated.

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Conflict of Interest

Authors have no conflict of interest to declare.

Author Contributions

Author 1: R. Saroja Preethy

Contributed in collection of data and designing, performed laboratory analysis, and wrote the paper.

Author 2: Dr. Anbuselvi

The Author has contributed towards proof-reading the data interpreted after analysis.

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