Tracing the Anti-Cancer Mechanism of *Pleurotus osteratus* Fractionate *via* Integrated Strategy of Network Pharmacology and Experimental Studies

Thesis submitted in partial fulfilment for the Award of Degree

Doctor of Philosophy

By

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2022

Chapter 6

Chapter 6.Summary and Conclusion

Pleurotus osteratus, a species from the *Pleurotus* genus, has been intensively studied for its anti-cancer activity, attributed to higher molecular weight myco-metabolites. Conversely, little is known about the anti-cancer bioactivities associated with lower molecular weight myco-metabolites, to list a few ergosterol, and polyphenolic compounds claimed as lower molecular weight biomarkers responsible for anti-cancer activities of *P. osteratus*. Besides this, the comprehensive myco-metabolite profiling, a correlation between myco-metabolites and their bioactivities, and underlying mechanisms are still elusive.

The presented research work was designed to explore differential bioactivity-based screening of preferential extracted higher molecular weight and lower molecular weight myco-metabolites, detailed myco-metabolite profiling and correlating with bioactivity, and tracing mechanistic pathway involved in the anti-cancer intervention of potential one.

For the execution of the study design, a few objectives were aimed:

1. To screen **different species of *Pleurotus* mushroom** based on *in-vitro* cytotoxic activity.
2. To carry out bioassay-guided fractionation of **screened species**, with comparative myco-metabolite profiling of and correlating with *in-vitro* cytotoxic activity.
3. To study the anti-cancer mechanism of **potential fractionate** by integrative approach of network pharmacology and experimental studies.

Each objectives of the study are framed in the form of a chapter of the study.

In **Chapter 2**, comparative studies were conducted on different species of *Pleurotus* mushroom, extracted in two different solvent systems: 1:1 v/v dichloromethane: ethanol (DCM: Et) and 1:1 v/v distilled water: ethanol (hydroalcoholic). Comparably, hydroalcoholic crude extract had a considerably high extraction yield and total phenolic, carbohydrate, and protein content. Owing to high total phenolic yield, hydroalcoholic crude extract had a better ability to DPPH and $ABTS^{\cdot+}$ radical. Contrarily, DCM: Et crude extract showed strong cytotoxic potential against MD-MBA-231, and B16F10. HPTLC-based methods were developed and validated as per ICH Q2(R1) for the determination of ergosterol. Ergosterol content in hydroalcoholic extract crude extract was undetermined, owing to the absence of the ergosterol band. Whereas, DCM: Et crude extract showed appreciable ergosterol content. Among the studied species of *Pleurotus* mushroom, *P. osteratus* and *Pleurotus florida* had remarkably higher *in-vitro* cytotoxicity and free radical scavenging activity.

In continuation in **Chapter 3**, bio-assay guided fractionation of 1:1 v/v dichloromethane: ethanol crude extract of *P. osteratus* (PO1) against MDA-MB-231, and A549 was carried out. Later, PO1 and its potent hexane (HFPO1) and ethyl acetate (EFPO1) fraction were screened against a panel of human cancer cell lines. HFPO1 fraction possesses higher cytotoxicity followed by EFPO1 and PO1. Literature review revealed that polyphenol and ergosterol are the biomarkers found in *P. osteratus*, and are responsible for its cytotoxic potential. Accordingly, hyphenated liquid chromatography with tandem mass spectrometry (LC-MS/MS) based polyphenol and ergosterol targeted myco-metabolite profiling of PO1, HFPO1 and EFPO1 fractions was carried out. Despite being significantly rich in polyphenol and ergosterol content, EFPO1 fraction showed moderate cytotoxicity. Considering this, LC-QTOF/MS-based untargeted myco-metabolite

profiling of PO1 with HFPO1 and EFPO1 fractions was further conducted to identify a new biomarker. Tentatively, 20 myco-metabolites were identified, belonging to the class of steroids, alkaloids, terpenoids, fatty alcohol, and polyketide. The myco-metabolite variabilities amongst potent samples in correlation to their *in-vitro* anti-cancer activity were explored using the different chemometric tools: principal component analysis (PCA), hierarchical clustering analysis (HCA), and partial least square (PLS). Collectively, PO1 was an amalgamation of myco-metabolites such as alkaloids, steroids, terpenoids, fatty alcohol, and carotenoids. From the PO1 crude extract, HFPO1 fraction extracts preferentially steroids, terpenoid, and a fatty alcohol class of myco-metabolite. While, polyphenol, ergosterol, and alkaloids were primarily extracted in EFPO1 fraction. This differential extraction of myco-metabolites was responsible for the observed variable bioactivities of the studied extract and fraction.

Further in **Chapter 4**, the probable mechanism behind the anti-cancer activity of the HFPO1 were explored using network pharmacology and experimental validation. HFPO1 myco-metabolites targets and targets related to the cancer were mined from the online webserver, and overlapping targets were screened. Out of the 74 overlapping targets, 33 targets were identified in the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway of cancer. Further, the main active myco-metabolites and hub targets were identified by network analysis of the compound-targets network and protein-protein interaction, respectively. Molecular docking results showed good binding affinity of the hub targets with their respective myco-metabolites. Before *in-vitro* experimental validation, the mRNA expression of selected hub targets (PI3KCA, and AKT1), and its prognostic potential were explored from the online webserver. The results showed up-regulation of PI3KCA, and AKT1 targets genes in tumor samples, and the less expressed patients showed a good prognosis. HFPO1 induced *in-vitro* anti-cancer activity by down-

expressing the PI3K-AKT-mTOR proteins, besides apoptotic cell bodies formation. Additionally, a significant tumor volume reduction was observed in HFPO1-treated Ehrlich ascites carcinoma (EAC) bearing Swiss albino mice. Overall, HFPO1 induces anti-cancer potential by modulating the PI3K-AKT-mTOR signalling pathway.

In parallel to **Chapter 5**, the probable mechanism behind the anti-cancer activity of the EFPO1 were explored using network pharmacology and experimental validation. EFPO1 myco-metabolites targets and targets related to the cancer were mined from the online webserver, and overlapping targets were screened. Out of the 90 overlapping targets, 44 targets were identified in the KEGG pathway of cancer. Further, the main active myco-metabolites and hub targets were identified by network analysis of the compound-targets network and protein-protein interaction respectively. Molecular docking results showed good binding affinity of the hub targets with their respective myco-metabolites. Before *in-vitro* experimental validation, the mRNA expression of selected hub targets (BCL2L1), the proteomic expression and its prognostic potential were explored from online webserver. The results showed up-regulation of BCL2L1 targets genes, along with high proteomic expression in tumor samples, and the less expressed patients showed a good prognosis. EFPO1 induced *in-vitro* anti-cancer activity by down-expressing the BCL2L1 protein, besides apoptotic cell bodies formation. Additionally, a significant tumor volume reduction was observed in EFPO1-treated EAC bearing Swiss albino mice.

Collectively, the presented research work scientifically explored the differential bioactivity, owing to preferential extraction of myco-metabolites, a detailed myco-metabolite profiling, correlating with bioactivity, and tracing the bioactivity *via* integrating *in-silico*, *in-vitro*, and *in-vivo* approach.