Chapter 2

Chapter 2. Bioactivity-based screening of different species of *Pleurotus* mushroom with their myco-chemical profiling

2.1. Background

Pleurotus mushroom belongs to a distinct macro-fungus group of Basidiomycetes of the order Agaricales, and family Tricholomataceae. These mushrooms are referred to as 'Mushrooms of high adaptability' and, thus, are distributed worldwide from temperate to tropical regions (10℃ - 32℃), comprising around 40 species. Amongs them, *P. osteratus* and *P. eryngii* are cultivated in the temperature range of 10˚C - 20˚C and 15˚C - 20˚C, respectively. Whereas, the optimum temperature for the cultivation of *P. florida*, *P. sajor-caju*, and *P. roseus* is 20˚C - 30˚C. Additionally, basidiomycetes families are known for their diversity in myco-metabolites. The existence of various myco-metabolites accounts for species' varied pharmacology. Their therapeutic potential includes immunomodulatory, anti-cancer, anti-inflammatory, anti-oxidant, and lowering blood cholesterol levels (Shreya et al., 2022).

In recent decades, varied lower molecular weight myco-metabolites from *Pleurotus* mushroom, such as phenolic acid, steroids, fatty acid esters, statin, terpenoids, etc., have been delved for anti-cancer potential. Phenolic compounds have acquired considerable attention due to their markable anti-oxidant property. They include an army of subclasses comprehending flavonoids, phenolic acids including hydroxybenzoic acids and hydroxyl cinnamic acids, stilbenes, lignans, tannins, and

oxidized polyphenols with diversity in their structure (Gąsecka et al., 2016; Palacios et al., 2011; Reis et al., 2012). Besides phenolic, ergosterol, the most abundant sterol found in *Pleurotus* mushrooms are noted for anti-inflammatory, antimicrobial, antioxidant, and anticancer potential. (Bekiaris, G., Tagkouli, D., Koutrotsios, G., Kalogeropoulos, N., & Zervakis, 2020). The researcher explored the cytotoxicity activity of polyphenols and ergosterol from different species of *Pleurotus* mushroom; a few of them are cited below. Hu Q *et al.* reported the anti-proliferative activity of the polyphenol-rich extract of *P. eryngii* against colon cancer cells (Hu et al., 2018). Similarly, Menega and his coworker isolated 3-methoxy 4-hydroxy benzoic acid, a phenolic acid, from *P. osteratus* and studied its cytotoxicity against lung cancer cell lines (Menaga et al., 2021). Besides polyphenol, as per Krishnamoorthy *et al.*, ergosterol, a sterol derivative with a total content of 48 %, showed anti-cancer activity against DMBA-induced breast cancer in rats (Krishnamoorthy & Sankaran, 2016). In the same direction, Finimundy *et al.* studied the cytotoxic activity of *P. sajor-caju* against colon cancer cell lines. As per the GC-MS report, ergosterol was one of the main bioactive molecules (Finimundy et al., 2018b).

The biomarkers for *Pleurotus* mushrooms were chosen for further research based on the above literature outlook, and these include polyphenols and ergosterol. The present research focuses on a comparative study involving five different species of *Pleurotus* mushroom (*P. osteratus*, *P. eryngii*, *P. florida*, *P. sajor-caju*, and *P. roseus*), their preferential extraction of myco-metabolites from the different solvent systems (DCM: Et and hydroalcoholic) resulting in differential *in-vitro* bioactivity (cytotoxic activity, and free radical scavenging activity). High-performance thin layer chromatography (HPTLC) based methods were developed and validated as per the International council for harmonization (ICH) Q2(R1) for the determination of ergosterol.

2.2. Objectives

- Qualitative and quantitative myco-chemical estimation of DCM:Et and hydroalcoholic crude extract of different species of *Pleurotus* mushroom.
- Evaluation of *in-vitro* cytotoxic activity and free-radical scavenging of DCM:Et and hydroalcoholic crude extract of different species of *Pleurotus* mushroom
- HPTLC-based method development, validation, and quantification of ergosterol content in DCM:Et and hydroalcoholic crude extract of different species of *Pleurotus* mushroom.

2.3. Experimental work

2.3.1. Material, Chemicals, and Reagents

Different species of *Pleurotus* mushroom (*P. osteratus* (PO), *P. eryngii* (PE), *P. sajor-caju* (PS), *P. florida* (PF), and *P. roseus* (PD)) were procured from Sigma bioengineering & technologies, Varanasi, India. Various cell lines, human breast cancer cell line (MDA-MB-231), murine skin cancer cell line (B16F10), and human embryonic kidney cell line (HEK-293) were procured from National Centre for Cell Science, Pune, India. Dulbecco's modified eagle medium (DMEM), Fetal bovine serum (FBS), antibiotic (Pen strep: A001), (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (MTT), and all other cell-culture materials were purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, India. Gallic acid, quercetin, d- glucose, bovine serum albumin (BSA), 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and ergosterol were procured from Sigma-Aldrich, Mumbai, India. All other chemicals used were of analytical grade.

2.3.2. Extraction

To get a detailed insight into the influence of different solvents on myco-chemical extracted and their correlation with *in-vitro* cytotoxicity potential and free radical scavenging, two solvent systems: 1:1 v/v dichloromethane: ethanol (DCM: Et) and 1:1 v/v distilled water: ethanol (hydroalcoholic) were used for cold maceration. Dried whole bodies of mushrooms were powdered with a crude drug disintegrator. Approximately 1 kg of dried powder of different species of *Pleurotus* mushroom was soaked with solvents. This mushroom-solvent mixture was kept for cold-maceration for 48 hours at room temperature with occasional manual agitation. After 48 hours, the mushroom-solvent mixture was filtrated through muslin cloth and followed by Whatman No. 4 filter paper. The resultant filtrate was concentrated by a rotary evaporator (IKA RV 10, IKA, India) at 45°C and 100 RPM. The concentrated crude extract was stored in the refrigerator till further analysis. The schematic representation of the extraction procedure is illustrated in Figure 2.1.

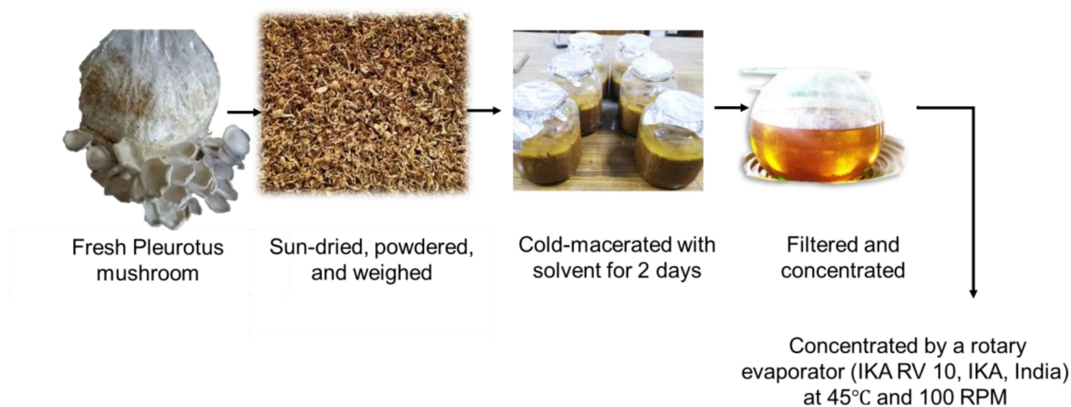


Figure 2.1. Schematic representation of extraction procedure.

The extraction yield was calculated for DCM: Et, and hydroalcoholic crude extract of the different species of *Pleurotus* mushroom by the formula depicted in equation 2.1.

$$Extraction\ yield = \frac{w_1}{w_2} \times 100 \qquad \textbf{Equation 2.1.}$$

Where, w_1 is the weight of extract residue obtained by solvent removal, and w_2 is the initial weight of the dried mushroom.

2.3.3. Qualitative myco-chemical analysis

DCM: Et, and hydroalcoholic crude extract of different species of *Pleurotus* mushroom were screened for primary and secondary myco-chemicals by preliminary myco-chemical test (Kokate & Gokhale, 2008).

2.3.4. Quantitative myco-chemical analysis

2.3.4.1. Estimation of total phenolics

The total phenolic content estimation was carried out by Folin-coicalteau calorimetric method with some modifications (Kumaran & Karunakaran, 2006). Gallic acid was used to calculate the standard curve (10-50 µg/mL). All measurements were performed in triplicate, and the results were expressed as mg of gallic acid equivalent (GAE) per gram of dried mushroom.

2.3.4.2. Estimation of total flavonoids

The total flavonoid content was determined using the aluminum chloride method (Kumaran & Karunakaran, 2006). Quercetin was used to prepare the standard curve (2-10 µg/mL). The assay was performed in triplicate, and results were expressed in mg of quercetin equivalent (QE) per gram of dried mushroom.

2.3.4.3. Estimation of total protein content

Protein content was estimated by Bradford protein assay with some modifications (He, 2011). BSA was used to construct a standard calibration curve (62.5-1000 µg/mL). The estimation was repeated thrice, and results were expressed in mg of BSA equivalent (BSAE) per gram of dried mushroom.

2.3.4.4. Estimation of total carbohydrates content

Total carbohydrate content was determined by the Anthrone method (Ludwig & Goldberg, 1956). D-glucose was used for the standard calibration curve (20-100 µg/mL). The experiment was conducted in triplicates, and results were expressed in mg of d-glucose equivalent (DGE) per gram of dried mushroom.

2.3.5. *In-vitro* cytotoxic activity

In-vitro cytotoxic activity of the crude extracts was screened by MTT assay using MDA-MB-231, B16F10, and HEK-293 cell lines, following the published procedure (Trivedi et al., 2018). Approximately 5×10^3 cells/well were seeded in 96 microtiter well plates, in DMEM/F-12 media with 10% FBS solution and antibiotics, and incubated with a 5% CO_2 atmosphere at 37 °C for 24 hours. After incubation, the medium was removed, washed twice with phosphate buffer saline (PBS 10 mM, pH 7.4), and further incubated with different concentrations of DCM: Et and hydroalcoholic crude extract of different species of *Pleurotus* mushroom (2000 µg/mL, 1000 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL, 31.25 µg/mL and 15.625 µg/mL) for 24 hours. After washing with PBS, the samples were replaced with 200 µL of fresh media containing 20 µL MTT solution (5 mg/mL), and cells were incubated for 4 hours at 37°C. The insoluble formazan crystals produced by viable cells were dissolved by 150 µL DMSO, and the absorbance was taken by a microplate reader (Bio-Rad Laboratories, Munchen, Germany) at 570 nm. The percentage of cell viability was calculated using equation 2.2.

$$Percentage\ cell\ viability = \frac{A_s}{A_C} \times 100 \qquad \textbf{Equation 2.2.}$$

where, A_s and A_C are the absorbance with sample and absorbance of the control (without any treatment), respectively.

2.3.6. *In-vitro* free radical scavenging activity

The *in-vitro* free radical scavenging activity of the crude extract was evaluated by scavenging free radicals of DPPH and $ABTS^{\cdot+}$.

2.3.6.1. DPPH assay

Accurately 0.1 mM DPPH solution in methanol was prepared and kept in the dark. Precisely 2 mL of DPPH solution was added to 1 mL of the solution of crude extract in methanol at different concentrations (100 µg/mL-1000 µg/mL). The mixtures were shaken vigorously and allowed to stand at room temperature in the dark for 30 mins. The absorbance was measured at 517 nm using a UV-Vis spectrophotometer (Cary 60, UV-Vis, Agilent, USA). DPPH solution with methanol was considered as control, and the absorbance of control was adjusted below 1 a.u. Lower absorbance values indicate higher free radical scavenging potential. The capability of scavenging the DPPH radical was calculated using equation 2.3.

$$Percentage\ inhibition = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100 \qquad \textbf{Equation 2.3.}$$

Where, $Abs_{control}$ is the absorbance of the control reaction and Abs_{sample} is the absorbance of the crude extract. All the tests were repeated in triplicates.

2.3.6.2. $ABTS^{\cdot+}$ assay

The $ABTS^{\cdot+}$ radical was chemically prepared by mixing 7 mM ABTS aqueous solution with 2.4 mM aqueous potassium persulfate in the ratio of 1:1 v/v and left for 16 hours for the completion of the reaction. This primary $ABTS^{\cdot+}$ solution was further diluted with water to yield a secondary $ABTS^{\cdot+}$ solution with an absorbance of less than 1 a.u. Accurately 2 mL of secondary $ABTS^{\cdot+}$ solution was added to 2 mL of crude extract solution in methanol at different concentrations (100 µg/mL-1000 µg/mL). The obtained solution was incubated in a dark chamber for 5 mins, and immediately the absorbance

was observed at 734 nm. The capability of scavenging the $ABTS^{\cdot+}$ radical was calculated similarly as described in the DPPH assay.

2.3.7. HPTLC-based method development, validation, and quantification of ergosterol content

Ergosterol marker-based method development, validation, and quantification were as per ICH Q2(R1) guidelines.

2.3.7.1. Preparation of standard and sample solution

Accurately 1 mg/mL of ergosterol in methanol and 10 mg/mL of crude extract in methanol were used for method development, validation, and quantification.

2.3.7.2. Chromatographic operative condition

The sample application was executed on a 20×10 cm pre-coated TLC aluminum silica gel 60 F254 plates (Merck KGaA, Darmstadt, Germany) with a band length of 8 mm, syringe turret position (Y) of 8 mm, and the distance between the center of the first band to the left-hand side of the plate (X) as 20 mm. Aliquots of the sample were applied by semiautomatic TLC applicator Linomat-V (CAMAG, Muttenz, Switzerland) with a 100 μL syringe (CAMAG, Muttenz, Switzerland) by Nitrogen gas flow. Before the application of aliquots, the plates were activated at 60°C for 5 mins. Linear ascending chromatogram development was performed in 20×20 cm Twin-Trough-Chamber (CAMAG, Muttenz, Switzerland), saturated with 20 mL of mobile phase comprising of toluene: ethyl acetate in the ratio of 15:5 v/v. The optimized chamber saturation time was 20 mins at room temperature. The migration distance was 80 mm. TLC plates were dried by hair-dryer (HP8100/46, Philips India Ltd, Himachal Pradesh, India). Densitometric scanning by (CAMAG TLC scanner IV) was performed with a slit width of 6.00 mm × 0.45 mm with a scanning speed of 20 mm/s in absorbance-reflectance mode at λmax (283 nm) after scanning between 200-800 nm using deuterium and white lamp. After scanning,

developed plates were visualized under the CAMAG TLC visualizer 2 (CAMAG, Muttenz, Switzerland). During the analysis, the operative and analytical parameters were controlled by the user interface 'WinCATS' software (CAMAG, Version 1.4.7.2018).

2.3.7.3. Method validation

2.3.7.3.1. Calibration curve

To determine the linearity of the developed method, different volumes (n = 5) 1 µL, 2 µL, 3 µL, 4 µL, and 5µL of 1mg/mL of ergosterol with the amount per spot as 1 µg/spot, 2 µg/spot, 3 µg/spot, 4 µg/spot, and 5 µg/spot were used. For the calibration curve, the mean peak area *versus* the amount per spot of ergosterol was plotted with n = 3. The linear regression and correlation coefficient were determined.

2.3.7.3.2. Specificity

The specificity of the developed method was achieved by comparing the spectra, peak area, and retardation factor (Rf) of the crude extract with that of standard ergosterol.

2.3.7.3.3. Sensitivity

The sensitivity of the developed method was estimated in terms of the limit of detection (LOD) and limit of quantification (LOQ), where LOD is $3.3\sigma/S$, and LOQ is $10\sigma/S$. Wherein, σ is the standard deviation of y-intercept and S is the slope of the calibration curve.

2.3.7.3.4. Precision

Precision, defined in terms of percentage relative standard deviation (RSD), was conducted to look over the repeatability and reproducibility of the developed method. Intra-day and inter-day precision were performed on three different concentrations, *i.e.,* low, medium, and high concentrations of ergosterol: 1 µg/spot, 3 µg/spot, and 5 µg/spot were repeated on three different consecutive days and three times on the same days.

2.3.7.3.5. Accuracy

Accuracy, defined in terms of percentage recovery by analyzing 50%, 100%, and 150% spiked concentration to the pre-analyzed standard solution (3 µg/spot was spiked to 4.5 µg/spot, 6.0 µg/spot, and 7.5 µg/spot).

2.3.7.4. Quantification of ergosterol in the crude extract

To quantify the ergosterol in the crude extract, 5 µL of 10 mg/mL of crude extract was spotted with the standard linearity ranges concentration of ergosterol under the above-mentioned chromatographic operative condition. The analysis was repeated in triplets. The quantity of ergosterol in the crude extract was estimated using the linear regression equation of standard ergosterol.

2.4. Results and discussion

2.4.1. Extraction yield

Extraction yield of the DCM: Et and hydroalcoholic crude extracts of the different species of the *Pleurotus* mushroom are tabulated below in Table 2.1.

Table 2.1. Extraction yield of DCM: Et and hydroalcoholic crude extracts of different species of the *Pleurotus* mushroom.

Pleurotus mushroom	***Extraction yield (%)***	
	DCM: Et	**hydroalcoholic**
P. osteratus (PO)	2.73 ± 1.760^{b}	42.28 ± 3.650^{a}
P. eryngii (PE)	3.11 ± 1.458^{b}	43.83 ± 2.097^{a}
P. florida (PF)	2.03 ± 2.983^{b}	37.32 ± 1.393^{a}
P. sajor-caju (PS)	2.12 ± 1.329^{b}	38.76 ± 2.019^{a}
P. djamor var. roseus (PD)	2.00 ± 1.564^{b}	36.91 ± 1.215^{a}

Values are expressed as mean ± standard deviation (SD) of three parallel measurements. One-way ANOVA was performed, followed by a turkey's multiple comparison test using Graph pad prism 5.0. Different letters (a, b) represent a significant difference at ($p < 0.05$), and the same letter indicates the absence of a significant difference between the results.

Findings from tabulated extraction yield demonstrated a significant difference in the extraction yield of DCM: Et and hydroalcoholic crude extracts of different species of

Pleurotus mushroom. Hydroalcoholic crude extracts were found to have a significantly high extraction yield than DCM: Et. crude extracts.

2.4.2. Qualitative myco-chemical analysis

A preliminary myco-chemical screening test is the pioneering step in detecting types of myco-chemical. It provides a blueprint of the class of the myco-chemical present in the complex structure of crude extracts. The presence of carbohydrates, protein, amino acids, flavonoids, phenols, alkaloids, and glycosides was observed in the DCM: Et and hydroalcoholic crude extracts of different species of *Pleurotus* mushroom. While DCM: Et crude extract of different species of *Pleurotus* mushroom showed the presence of steroids and terpenoids, in contrast to hydroalcoholic crude extracts.

2.4.3. Quantitative myco-chemical analysis

The linear regression analysis of the standard calibration curve of gallic acid (10-50 µg/mL), quercetin (2-10 µg/mL), d-glucose (20-100 µg/mL), and BSA (62.5-100 µg/mL) revealed regression equation of $y = 0.011x + 0.0338$, $y = 0.0054x - 0.0016$, $y = 4.6664x - 0.0008$, and $y = 0.519x + 0.0683$ respectively and co-relation coefficient (r^2) was found to be 0.9919, 0.9908, 0.9822, and 0.9681, respectively (Figure 2.2.).

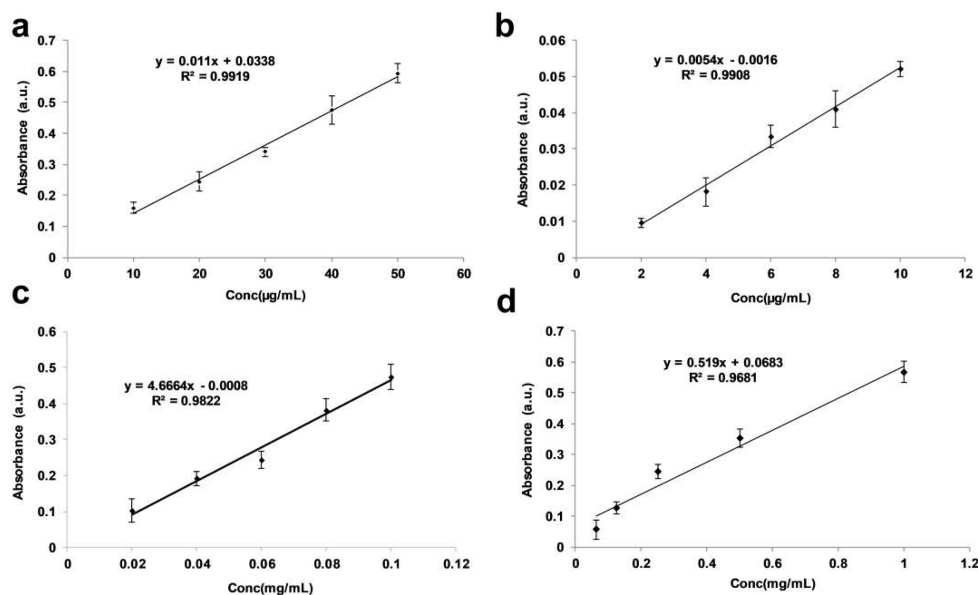


Figure 2.2. Calibration curve of the standard compound (a) gallic acid (10-50 µg/mL), (b) quercetin (2-10 µg/mL), (c) d-glucose (20-100 µg/mL), and (d) BSA (62.5-100 µg/mL).

Table 2.2. Total phenolic, flavonoids, carbohydrates, and protein contents in the DCM: Et and hydroalcoholic crude extracts of different species of *Pleurotus* mushroom.

***Pleurotus* mushroom**	**Total phenolic content (mg GAE/g)**	**Total flavonoid content (mg QE/g)**	**Total carbohydrate content (mg DGE/ g)**	**Total protein content (mg equivalent to BSAE/g)**
PO1	12.468 ±1.409[a]	7.540 ±2.341[a]	4.132±0.758[a]	56.306±0.360[a]
PE1	8.693±1.521[bc]	5.828±0.476[a]	12.202±0.317[b]	61.041±1.091[b]
PF1	12.673±1.173[ad]	6.264±1.023[a]	2.147±0.353[ad]	53.829±0.251[ad]
PS1	10.825±1.220[acde]	5.938±1.170[a]	8.261±2.708[c]	56.735±1.279[ae]
PD1	11.645±2.035[ade]	6.550±0.561[a]	6.570±0.582[ac]	59.836±0.464[cb]
PO2	20.048±2.879[f]	9.355±0.452[a]	24.164±3.665[e]	75.028±0.969[f]
PE2	17.130±2.661[g]	6.691±0.723[a]	44.538±1.980[f]	88.973±2.341[g]
PF2	22.073±3.352[f]	7.731±0.978[a]	20.933±0.525[e]	73.459±0.704[fh]
PS2	16.231±2.415[g]	5.937±0.342[a]	27.961±0.752[g]	77.772±0.538[fi]
PD2	17.258±1.210[g]	6.804±2.456[a]	28.459±0.946[g]	73.264±2.601[fhj]

Values are expressed as mean ± SD of three parallel measurements. One-way ANOVA was performed, followed by a turkey's multiple comparison test using Graph pad prism 5.0. Different letters (a, b, c, d, e, f, g, h, i, and j) represent a significant difference at $p < 0.05$, and the same letter indicates the absence of a significant difference between the results.

From the data tabulated above (Table 2.2.), it can be revealed that there was a significant difference in the yield of total phenolic, flavonoid, carbohydrates, and protein content between DCM: Et, and hydroalcoholic crude extract of different species of *Pleurotus*

mushroom. The hydroalcoholic crude extract of different species of *Pleurotus* mushroom (PO2, PE2, PS2, PF2, and PD2) yield comparatively higher total phenolic, carbohydrates, and protein content as compared to DCM: Et extract of different species of *Pleurotus* mushroom (PO1, PE1, PS1, PF1, and PD1). There were non-significant differences observed in the total flavonoid content of DCM: Et and hydroalcoholic crude extract of different species of *Pleurotus* mushroom.

2.4.4. *In-vitro* cytotoxic activity

MTT assay, an enzyme-based colorimetric assay method, was used to screen crude extract for their *in-vitro* cytotoxicity activity. Based on IC_{50} data, DCM: Et crude extract showed a significantly higher cytotoxic potential against MDA-MB-231, and B16F10, as compared to hydroalcoholic crude extract (Figure 2.3.). The IC_{50} value of DCM: Et crude extract (PO1, PE1, PF1, PS1, and PD1) against MDA-MB-231 cancer cell line were in the range < 200 µg/mL (with the exception PE1 with IC_{50} 201.8 µg/mL) (Figure 2.3.a). In contrast, the IC_{50} value of hydroalcoholic crude extract against MDA-MB-231 cancer cell line in the range of 250-350 µg/mL (except PF2 with IC_{50} 196.7 µg/mL) (Figure 2.3.a). Against the B16F10 cancer cell line, DCM: Et and hydroalcoholic crude extract showed IC_{50} value in the range of 300-400 µg/mL, with a significant difference in the IC_{50} value among both the crude extract (except PF1 and PF2) (Figure 2.3.b). Both the crude extract showed minimal cytotoxicity against the HEK-293 cell line, as reflected in Figure 2.3.c.

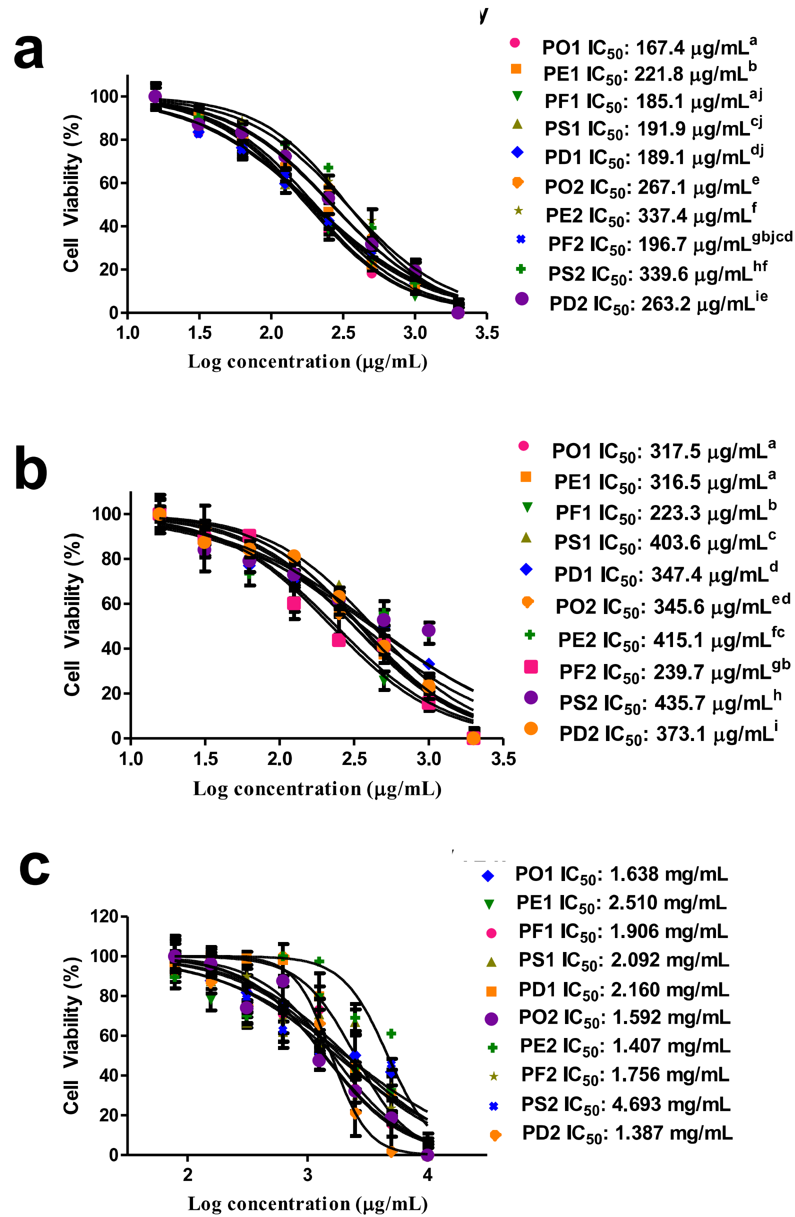
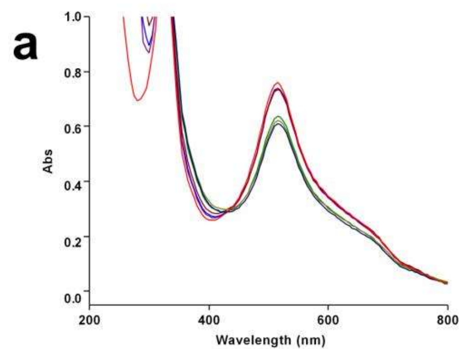


Figure 2.3. *In-vitro* cytotoxic activity of DCM: Et and hydroalcoholic crude extract against (a) MDA-MB-231, (b) B16F10, and (c) HEK-293 cell line. Non-linear regression analysis was performed for the determination of IC_{50}. Values are expressed as mean ± SD of three parallel measurements. One-way ANOVA was performed, followed by a turkey's multiple comparison test using Graph pad prism 5.0. Different letters (a, b, c, d, e, f, g, h, i, and j) represent a significant difference ($p < 0.05$), and the same letter indicates the absence of a significant difference between the results.

2.4.5. *In-vitro* free radical scavenging activity

The *in-vitro* free radical scavenging activities were studied using DPPH and $ABTS^{\cdot+}$ assay. The absorption maxima of DPPH and $ABTS^{\cdot+}$ were detected at 517 nm and 734 nm. The scavenging activities of DCM: Et and hydroalcoholic crude extract against DPPH and $ABTS^{\cdot+}$, represented as anti-oxidant potential, were evaluated by decreased absorption of their respective absorption maxima. Presented methods are easy, cheap, inexpensive, and widely used for assessing *in-vitro* free radical scavenging potential.

As the concentration of the crude extract increased from 100 μg/mL to 1000 μg/mL, there were gradual decreases in the absorption maximum of DPPH and $ABTS^{\cdot+}$, owing to the free radical scavenging potential of the crude extract (Figure 2.4. and Figure 2.5.). The scavenging activities of the hydroalcoholic crude extract against DPPH and $ABTS^{\cdot+}$ were comparatively higher than DCM: Et crude extract (Figure 2.6.). This could be due to a higher yield of the total phenolic content in hydroalcoholic crude extract compared to DCM: Et crude extract.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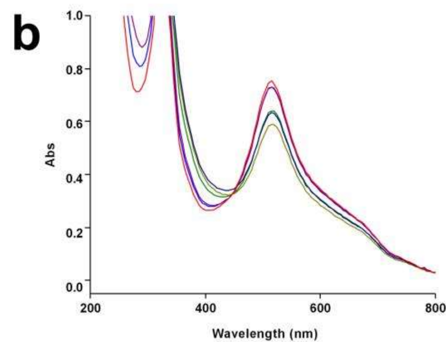
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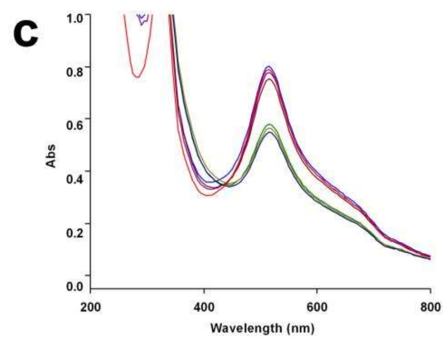
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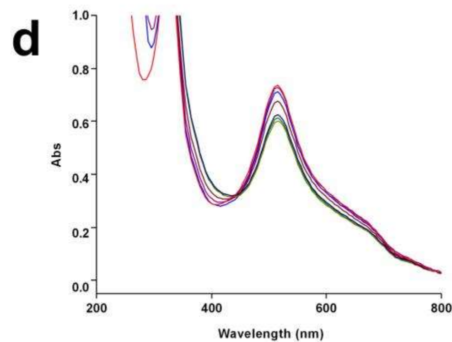
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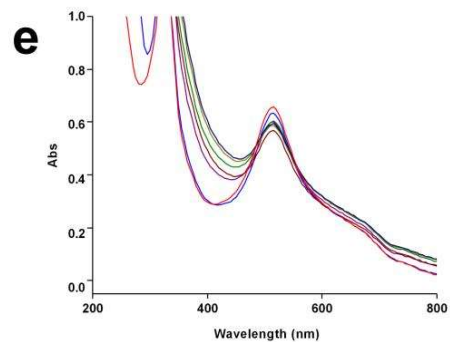
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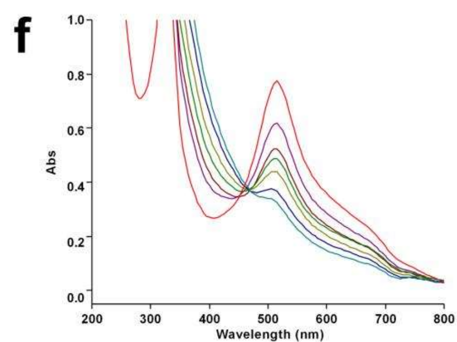
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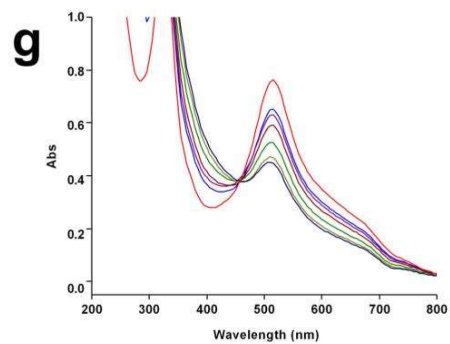
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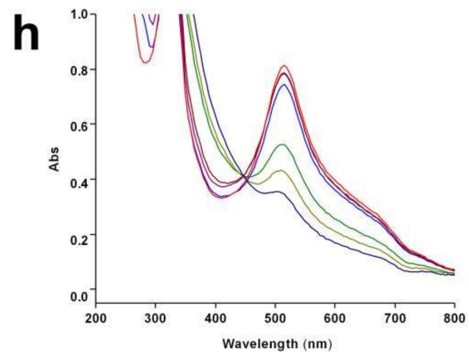
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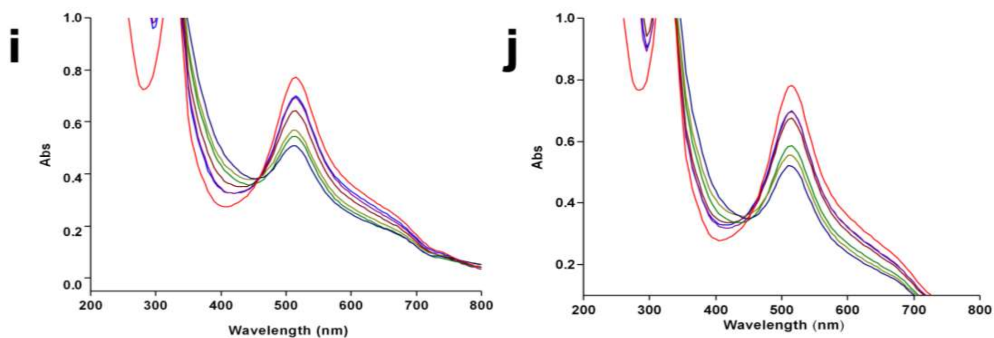
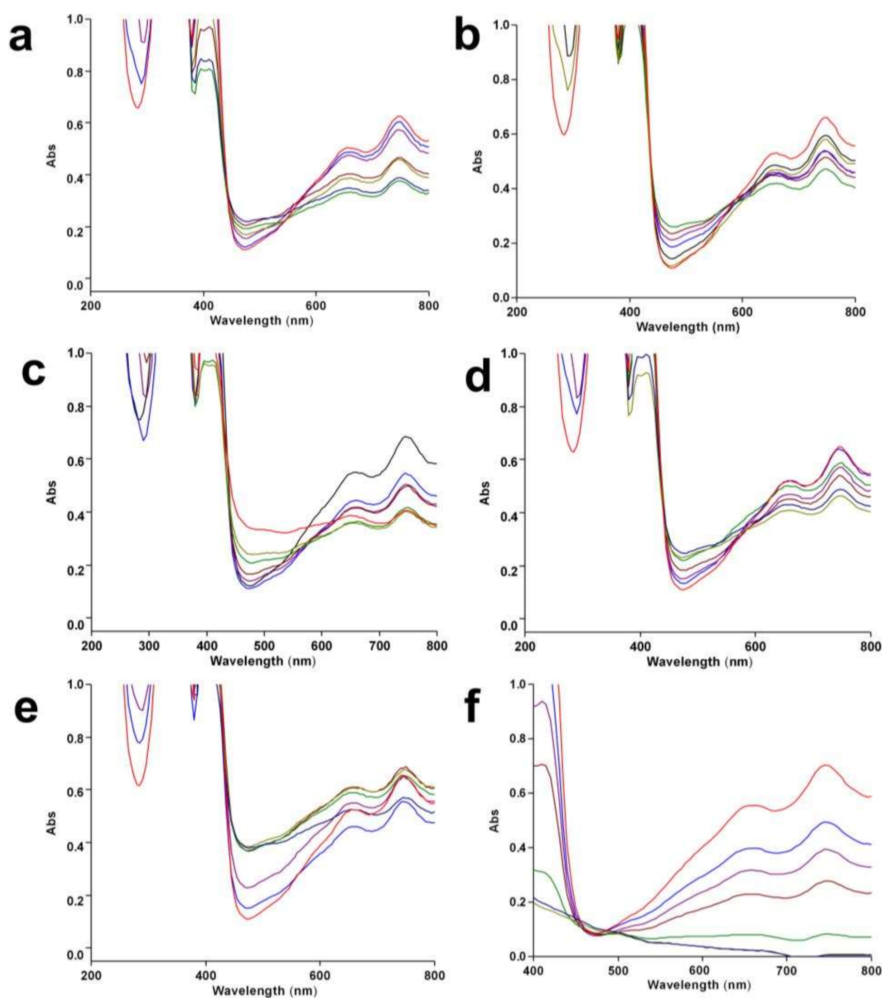


Figure 2.4. DPPH scavenging potential of DCM:Et crude extract and hydroalcoholic crude extract (a) PO1 (b) PE1 (c) PF1 (d) PS1 (e) PD1, (f) PO2 (g) PE2 (h) PF2 (i) PS2 and (j) PD2.



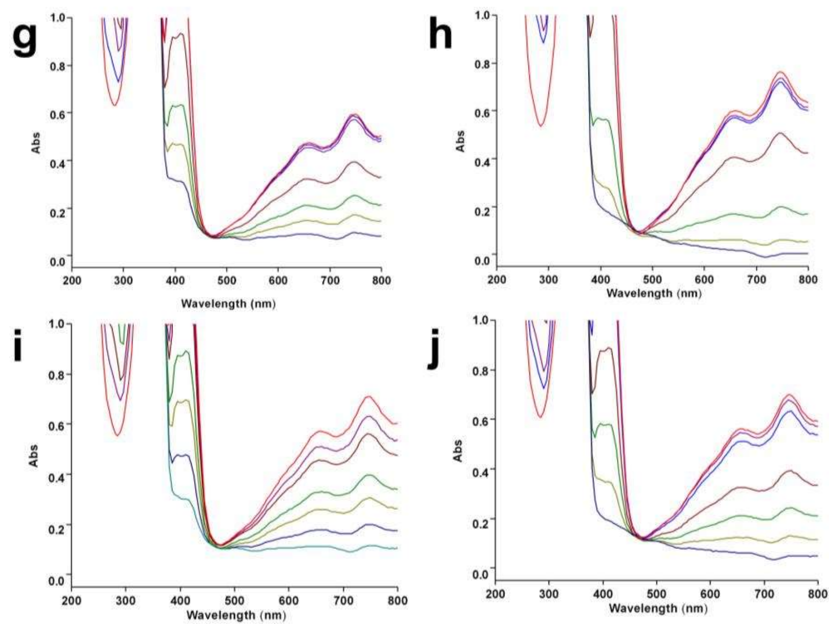


Figure 2.5. ABTS$^{\cdot+}$ Scavenging potential of DCM:Et crude extract and hydroalcoholic crude extract (a) PO1 (b) PE1 (c) PF1 (d) PS1 and (e) PD1, (f) PO2 (g) PE2 (h) PF2 (i) PS2 and (j) PD2.

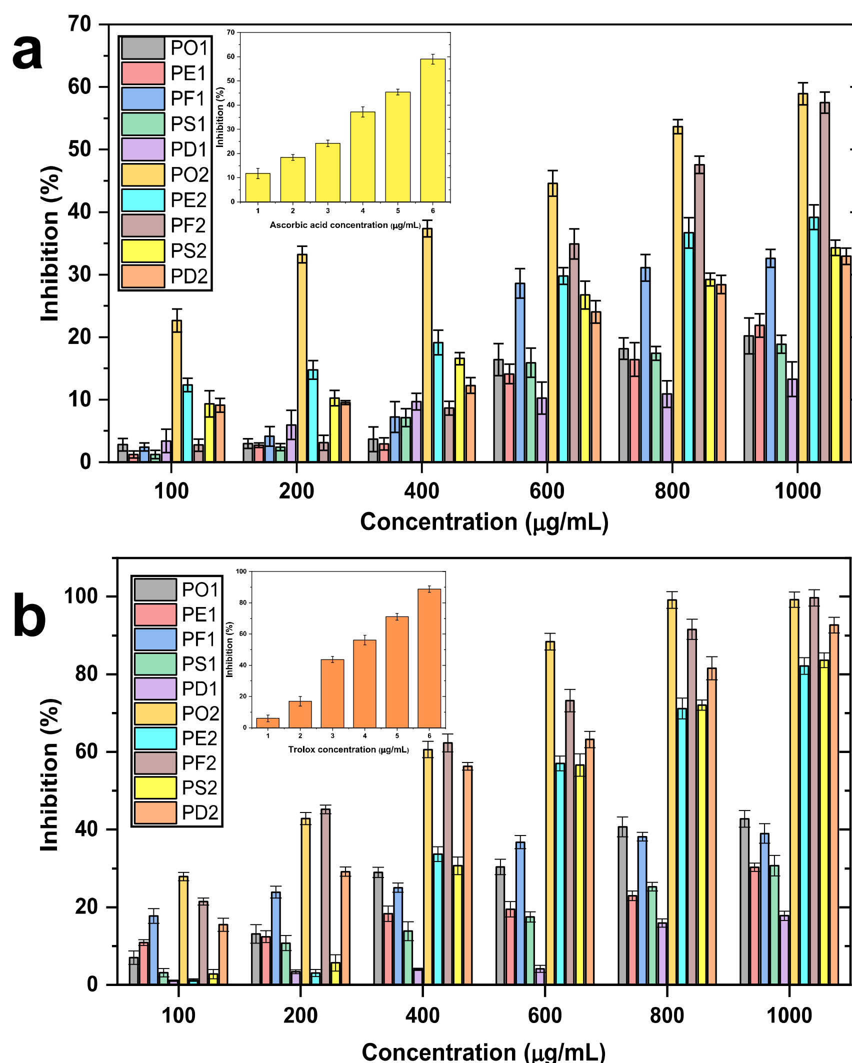


Figure 2.6. *In-vitro* free radical scavenging activity of DCM: Et and hydroalcoholic crude extract against (a) DPPH radical with inset figure of ascorbic acid, and (b) ABTS$^{\cdot+}$ radical with inset figure of trolox.

2.4.6. HPTLC-based method development, validation, and quantification of ergosterol content

HPTLC-based method for quantification of ergosterol in the DCM: Et and hydroalcoholic crude extract of different species of *Pleurotus* mushroom were developed and validated for linearity, specificity, sensitivity, accuracy, and precision as per ICH guidelines. The Rf values of standard ergosterol were approximately 0.58±0.2 with λmax

around 283 nm. The detection of ergosterol bands in the crude extract was confirmed by comparing the λmax spectra, peak area, and Rf of the crude extract with that of standard ergosterol (Figure 2.7. and Figure 2.8.).

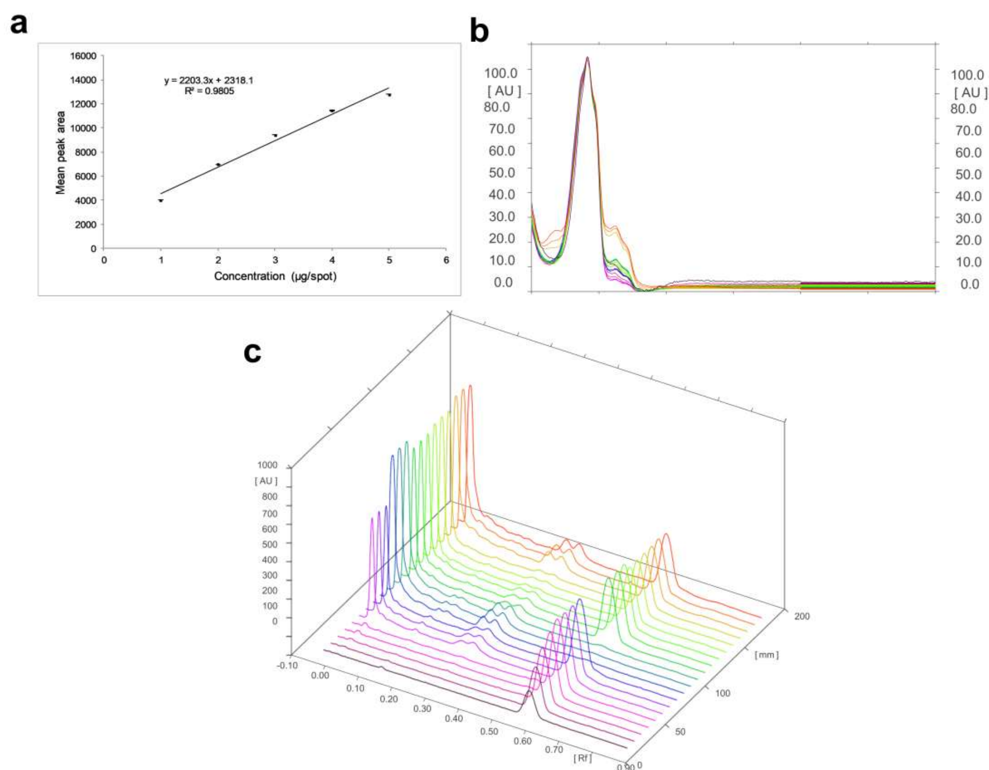


Figure 2.7. Calibration curve and densitometric chromatogram of standard ergosterol with DCM: Et crude extract (a) Calibration curve of standard ergosterol mean peak area *versus* concentration (µg/spot) (n = 3) (b) 3-D Overlay λmax spectra of standard ergosterol with DCM: Et crude extract at 283 nm, and (c) 3-D Densitometric chromatogram of standard ergosterol with DCM: Et crude extract at 283 nm.

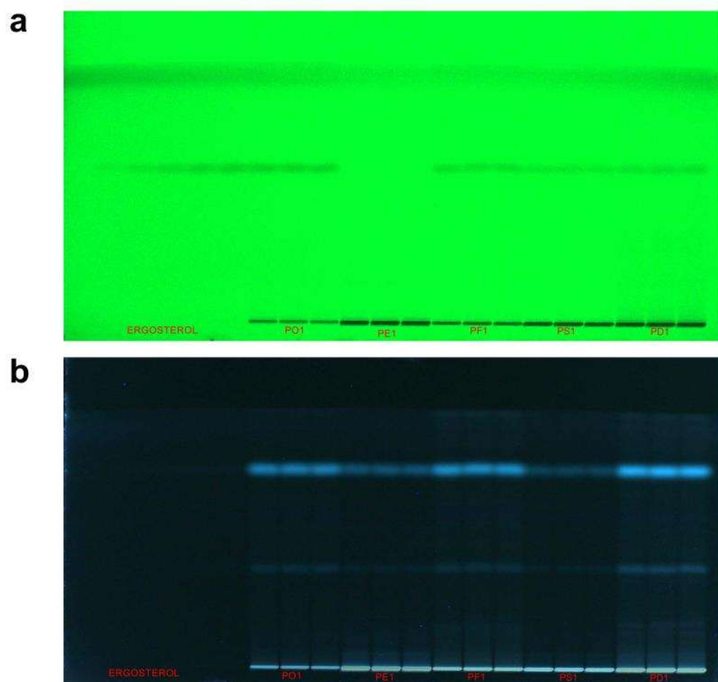


Figure 2.8. HPTLC fingerprinting of standard ergosterol with DCM: Et crude extract (a) captured at 256 nm, and (b) captured at 366 nm.

The linearity ranges for standard ergosterol at five different levels of concentration were in the range 1µg/spot-5 µg/spot, as reflected in (Table 2.3.) (Table 2.4.) (Figure 2.7.a). The mean peak area was calculated for three parallel sets of calibration curve data and plotted against the amount per spot (µg/spot). Regression equation (Y) and correlation coefficient (r^2) for standard ergosterol were $y = 2203.3x + 2318.1$ and 0.980, respectively, as presented in (Table 2.4.) (Figure 2.7.a), thus proving the linearity of the developed method. The LOD and LOQ for standard ergosterol were found to be 0.347 µg/spot and 1.04 µg/spot, respectively, as shown in (Table 2.4.).

Table 2.3. Calibration curve of standard ergosterol amount per spot *versus* mean peak area.

Amount per spot (µg/spot)	Mean Peak Area ± SE (a.u.)	%CV
1	4011.240 ± 4.096	0.919
2	6972.043 ± 8.048	1.039
3	9415.833 ± 15.794	1.509
4	11454.570 ± 13.006	1.022
5	12786.637 ± 21.127	1.487

SE: Standard error CV: Coefficient variance (Calibration carried out 5 different level of concentration, repeated in triplets).

Table 2.4. Linear regression analysis of calibration curve and LOD and LOQ of standard ergosterol.

Parameters	Ergosterol
Linearity-range	1µg/spot - 5µg/spot
Regression equation (Y)	2203.3x + 2318.100
Correlation coefficient	0.980
Slope standard error	171.900
Intercept standard error	570.129
95% slope- confidence interval	1654.371-2748.499
95% intercept- confidence interval	467.343 - 4096.155
LOD	0.347
LOQ	1.04

The precision of the developed method was calculated in terms of % RSD for intra-day and inter-day repeatability, as revealed in (Table 2.5.). The %RSD for intra-day and interday repeatability was below 2%, which signifies the reproducibility of the concerned developed method.

Table 2.5. Intra-day and inter-day precision of standard ergosterol (n = 3).

Amount per spot (µg/spot)	Intra-day precision		Inter-day precision	
	Mean peak area ± SD (a.u.)	**%RSD**	**Mean peak area ± SD (a.u.)**	**%RSD**
1	4057.883 ± 45.117	1.111	4061.376 ± 68.026	1.69
3	9367.370 ± 134.494	1.438	9421.717 ± 113.283	1.202
5	12451.470 ± 191.370	1.537	12513.740 ± 143.107	1.144

SD: Standard deviation RSD: Relative standard deviation.

The accuracy of the developed method was estimated in terms of % Recovery, as manifested in (Table 2.6.). % Recovery of the developed method for ergosterol was

within an acceptable range (96.75% - 99.21%), thus indicating no interference in the developed method by spiked concentration.

Table 2.6. Accuracy data of standard ergosterol (n = 3).

Pre-analyzed concentration	Spiked concentration (µg/spot)	Total concentration (µg/spot)	Mean peak area ± SD (a.u.)	%Recovery
3µg/spot	1.5	4.5	11948.180 ± 138.161	97.604
	3.0	6.0	15295.860 ± 292.667	98.553
	4.5	7.5	18399.840 ± 214.529	97.646

SD: Standard deviation.

Based on the above-validated HPTLC-densitometric method development, the ergosterol contents in PO1, PF1, PS1, and PD1 were found to be 9.675 ± 0.012%w/w, 8.536 ± 0.037%w/w, 6.431 ± 0.033%w/w, and 6.613 ± 0.155%w/w respectively. Contrarily, owing to the absence of a band of ergosterol in PE1, the ergosterol content in PE1 was undetermined (Figure 2.8.). Moreover, the hydroalcoholic crude extract showed a lack of ergosterol band; hence the ergosterol content in the PO2, PE2, PF2, PS2, and PD2 was found to be undetectable (Figure 2.9.).

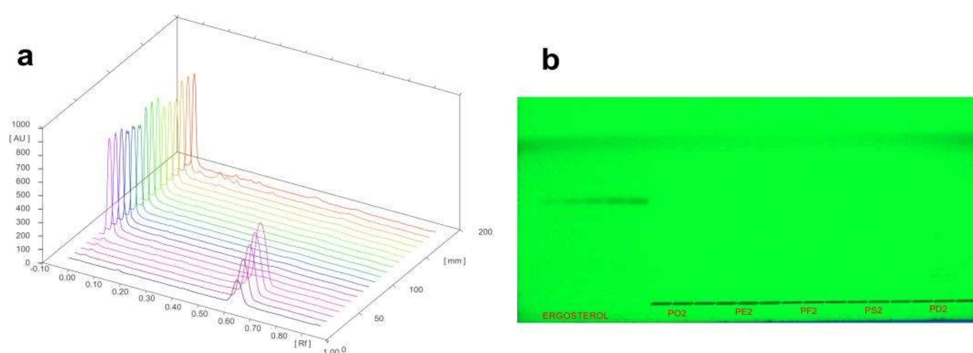


Figure 2.9. Densitometric chromatogram and HPTLC fingerprinting of standard ergosterol with hydroalcoholic crude extract (a) 3-D Densitometric chromatogram of standard ergosterol with hydroalcoholic crude extract at 283 nm, and (b) Developed

chromatoplate image of standard ergosterol with hydroalcoholic crude extract captured at 256 nm.

2.5. Summary

The myco-chemical of DCM: Et and hydroalcoholic crude extract of *P. osteratus*, *P.eryngii*, *P. florida*, *P. sajor-caju*, and *P. roseus* species of *Pleurotus* mushroom were screened for qualitative myco-chemical test and further quantified for total phenolic, flavonoid, carbohydrates and protein content. *In-vitro* free-radical scavenging and *in-vitro* cytotoxicity activity were evaluated for bioactivity-based screening of different species of *Pleurotus* mushroom. Furthermore, ergosterol, a biomarker, was quantified in different species of *Pleurotus* mushrooms by HPTLC. Collectively and comparatively, the hydroalcoholic crude extract had a significantly higher yield of total phenolic, carbohydrates, and protein content. The higher total phenolic content of hydroalcoholic crude extract leads to comparatively higher free radical scavenging activity against DPPH and $ABTS^{\cdot+}$ radicals. Contrarily, DCM: Et crude extract had significantly higher cytotoxicity potential against MDA-MB-231, and B16F10 (compared to hydroalcoholic crude extract), owing to preferential extraction of ergosterol content, quantified by HPTLC. Among the studied species of *Pleurotus* mushroom, *P. osteratus* and *P. florida* had remarkably higher *in-vitro* free radical scavenging and cytotoxicity activity.