Materials
and
Methods

3.1. Materials

In this study all consumables (chemicals, reagents, glass wares, plastic wares, culture medium ingredients, biological kits and raw materials) were procured from Hi-Media (India), SRL (India), Merck (India), TCI (India), Sigma-Aldrich (India), Tarson (India) and Borosil (India). The chemicals used in this study are given in the Table 3.1.

Table 3.1. List of chemical components used in the experimentation and analysis of present study

Sr. No.	Chemical component	Make
1	Acetonitrile	Merck
2	HPLC water	Merck
3	Methanol	Merck
4	MTT	Merck
5	Etoposide	TCI
6	Paclitaxel	TCI
7	Cyclophosphamide	Hi-media
8	Urea	Hi-media
9	Ascorbic acid	Hi-media
10	Sodium bicarbonate	Hi-media
11	KCl	Hi-media
12	H_2O_2	Hi-media
13	Na_2HPO_4	Hi-media
14	Creatinine	Hi-media
15	KH_2PO_4	Hi-media
16	NaOH	Hi-media

17	Citric acid	Hi-media
18	DMEM	Hi-media
19	Foetal bovine serum	Hi-media
20	Penicillin-Streptomycin	Hi-media
21	Trypan blue	Hi-media
22	DMSO	Hi-media
23	Formic acid	Hi-media
24	Glucose	Hi-media
25	Ammonium tartrate	Hi-media
26	Yeast extract	Hi-media
27	Peptone	Hi-media
28	$CaCl_2 . 2H_2O$	Hi-media
29	$MgSO_4.7H_2O$	Hi-media
30	Thiamine-HCl	Hi-media
31	Nitriloacetate	Hi-media
32	$MnSO_4.2H_2O$	Hi-media
33	$ZnSO_4.7H_2O$	Hi-media
34	$CuSO_4.7H_2O$	Hi-media
35	$FeSO_4.7H_2O$	Hi-media
36	$Na_2MoO_4.2H_2O$	Hi-media
37	ABTS	Hi-media
38	Veratryl alcohol	Hi-media
39	Phenol red	Hi-media
40	Bovine serum albumin	Hi-media
41	Sodium lactate	Hi-media
42	Sodium tartrate	Hi-media

43	Potato dextrose broth	Hi-media
44	Sodium acetate	Hi-media
45	Potato dextrose agar	Hi-media
46	Agar-Type I	Hi-media
47	H_2SO_4	Hi-media
48	NaCl	Hi-media
49	Trypsin- EDTA	Hi-media
50	DNSA	Hi-media

The selected antineoplastic compounds are reported highly toxic in nature, so their handling during the experimentation require proper safety precaution such as use of nitril gloves, wear mask and lab coat. All the stocks of these antineoplastic compounds were prepared in fume hood containing disposal paper on the work surface to protect the surface from their exposure. After the preparation of stock, discard all the tubes and surface paper of hood that were in contact with these compounds in a separate biohazard container.

3.2. Microorganisms and Cell line

Ganoderma lucidum (MTCC-1039), *Phanerochaete chrysosporium* (MTCC-787) were collected from Microbial Type Culture Collection (MTCC), CSIR-IMTECH Chandigarh (India) in lyophilized form and *Trametes versicolor* (AH05) was obtained from Dr. Rajeev Kapoor Laboratory, Maharishi Dayanand University, Rohtak (Haryana) on agar petri plate form. These fungi are white rot fungi considered as microorganisms of Biosafety Level 1 (BSL-1). These are not reported for causing disease in individuals during handling them, and not able cause any hazardous effect to environment and laboratory staff. It is recommended that standard microbiological precautions must be employed while doing experiments with these microorganisms (Buchan et al., 2019).

The cell line Raw 264.7 was obtained from National Centre for Cell Science (NCCS), Pune (India) and Prof. Gobardhan Das, Special Centre for Molecular Medicine (SCMM), Jawaharlal Nehru University, New Delhi, India. This cell line is mouse monocyte macrophage or immune cell line. This is not reported for causing any disease in individual handling but to prevent contamination during their growth and media exchange it require proper handling and precautions.

3.3. Development of high-performance liquid chromatography methods

Here, three different revere phase HPLC methods for the detection and quantification of cyclophosphamide, etoposide and paclitaxel were developed (Fig. 3.1). Initially, three different stock solution of cyclophosphamide and etoposide were prepared according to their solubility. Cyclophosphamide and etoposide were prepared in acetonitrile, while paclitaxel was prepared in methanol and finally diluted in HPLC grade water. The final concentration of stock solution was 1000 $\mu g.ml^{-1}$ for each compound. After the preparation of stock of antineoplastic compounds, synthetic urine was prepared. The chemical compositions of synthetic urine are given in Table 3.2. Further, the antineoplastic compounds from the prepared stocks were spiked with synthetic urine and serially diluted in various concentrations *viz.* 10, 50, 100, 250, 500, 750 $\mu g.ml^{-1}$ for the determination of the LOD and LOQ. All the prepared solutions were filtered through 0.22 µm syringe filter prior to use in HPLC. The prepared stocks solutions were kept in dark condition at -20 ºC temperature for further analysis or use.

3.3.1. Optimization of prepared method

The chromatographic analysis was carried out on HPLC (CECIL Instrument Service Ltd., U.K) system with C_{18} column (250mm×4.6mm×5µm) using acetonitrile and water as mobile phase in different ratio at both gradient and isocratic mode for cyclophosphamide, etoposide and paclitaxel. Prior going to HPLC run, we scanned the prepared solution for each compound

using spectrophotometer to check the absorbance wavelength (nm) at which the compounds were giving maximum absorbance. For gradient as well as isocratic mode, different parameters were optimized. On the basis of best efficiency, the mode of run *i.e.* isocratic mode was selected for further selection and optimization of conditions. Finally, in isocratic mode the total run time, flow rate and temperature were optimized (Table 3.3).

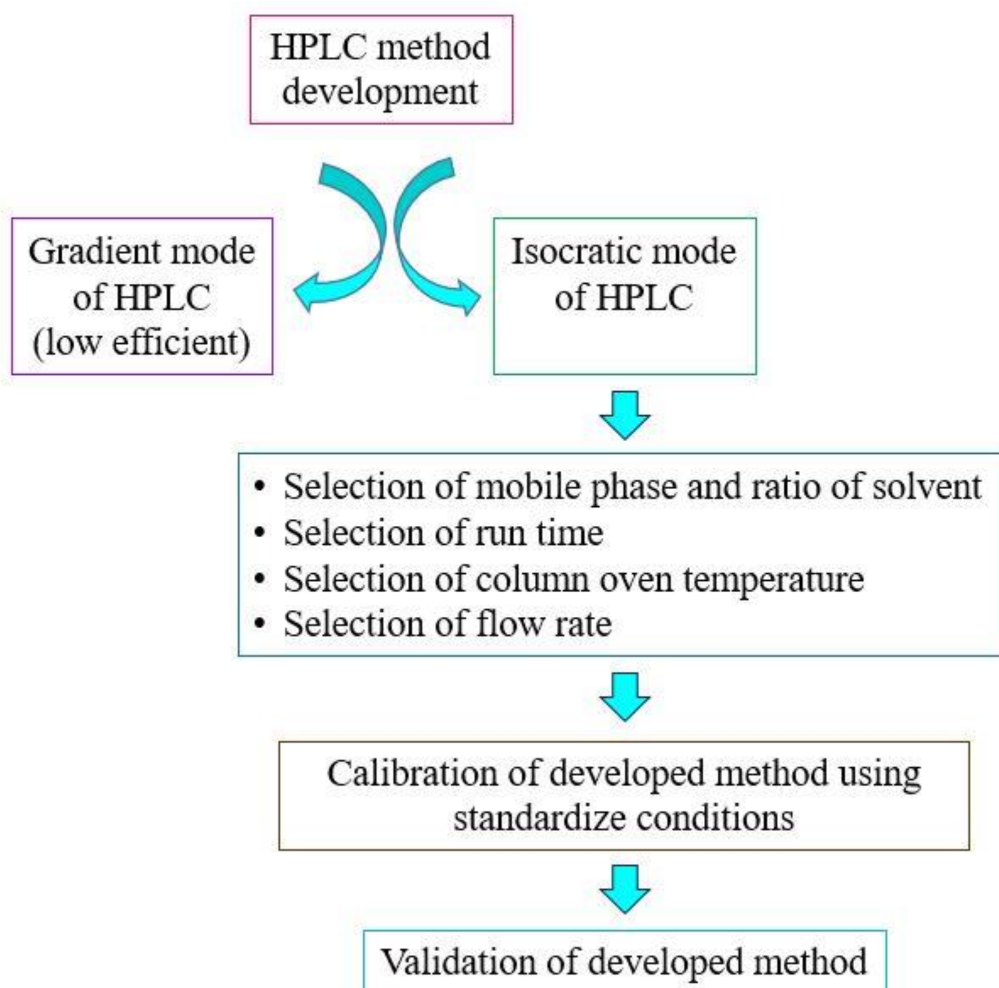

Fig. 3.1. Workflow of HPLC method development for selected antineoplastic compounds

Table 3.2. Chemical composition of synthetic urine (Calafat and Sampson, 2012)

Sr. no.	Chemical component	Quantity used ($g.L^{-1}$)
1	KCl	3.8
2	NaCl	8.5
3	Urea	24.5
4	Ascorbic acid	0.34
5	KH_2PO_4	1.18
6	NaOH	0.64
7	Citric acid	1.03
8	Sodium bicarbonate	0.47
9	Creatinine	1.4
10	H_2SO_4	28 ml

Table 3.3. Selection of conditions under isocratic mode for detection and quantification of cyclophosphamide, etoposide and paclitaxel in HPLC

Time (min.)	Mobile phase (Acetonitrile:Water)	Flow Rate ($ml.min.^{-1}$)	Temperature (°C)	Wavelength (nm)
		Cyclophosphamide		
10	50:50, 60:40, 70:30, 80:20, 90:10	0.8, 1.0, 1.2	20, 25, 30	199
		Etoposide		
10	50:50, 60:40, 70:30, 80:20, 90:10	0.8, 1.0, 1.2	20, 25, 30	229
		Paclitaxel		
10	50:50, 60:40, 70:30, 80:20, 90:10	0.8, 1.0, 1.2	20, 25, 30	229 nm

3.3.2. Validation of developed methods

The developed methods were validated by the measurement of different characteristics such as accuracy, sensitivity range, specificity, linearity and robustness.

3.3.2.1. *Linearity, Accuracy and Precision*

To check the linearity of the method developed, we had serially diluted the cyclophosphamide, etoposide and paclitaxel stock solution in synthetic urine to achieve different concentration *i.e.* 10, 50, 100, 250, 500 and 750 $\mu g.ml^{-1}$. Each diluent with respective standard concentration injected into triplicate under optimized condition. The linearity was evaluated by calculation of linear regression (r^2) from the plot between peak area and concentration. The accuracy of the developed method was calculated by the recovery of sample at their different concentration level for cyclophosphamide, etoposide and paclitaxel. The precision of the developed methods was assessed by repeatability testing using standard solution of 500 $\mu g.ml^{-1}$ of cyclophosphamide, etoposide and paclitaxel analysed ten times.

3.3.2.2. *Sensitivity, Specificity and Robustness*

The sensitivity was determined by measuring the signal to noise ratio of LOD and LOQ of test sample in HPLC. The robustness of the method developed was evaluated by changing different parameters such as column temperature, flow rate and variation ratio of mobile phase. The optimized column temperature range was 20, 25 and 30 ºC, flow rate was 0.8, 1.0 and 1.2 $ml.min^{-1}$ and mobile phase ratio of acetonitrile and water was 90:10, 80:20, 70:30, 60:40 and 50:50. Under these optimized parameters, the peak area, peak height and retention were calculated. The specificity was evaluated by the comparing the chromatogram of standard sample and blank.

3.4. Growth of selected fungal culture

Two fungi *G. lucidum* and *P. chrysosporium* culture were present in the form of lyophilized powder in glass vial. Glass vials were broken in biosafety cabinet (Labtech Laminar Air Flow Systems, New Delhi, India). The *G. lucidum* cultured was inoculated on MTCC growth medium (65) containing malt extract 10 $g.L^{-1}$ and agar 20 $g.L^{-1}$ in double distilled water. The pH of culture medium was adjusted at 6.5 ±0.1 before sterilization. *P. chrysosporium* was inoculated on MTCC growth medium (43) containing malt extract 20 $g.L^{-1}$, glucose 10 $g.L^{-1}$, peptone 01 $g.L^{-1}$ and agar 20 $g.L^{-1}$ in double distilled water. *T. versicolor* was sub-cultured on malt extract agar 20 $g.L^{-1}$. Sterilization was conducted in autoclave machine. After inoculation, these fungi were incubated at 25 ^{o}C temperature in incubator under static mode for 5 days.

3.4.1. Mycelium pellet formation

After the growth of fungi on petri plates, a part or slice of fungal colonies from the growing petri plates were transferred into broth medium containing 2 % malt extract, 10 % glucose and 4.5 pH. All fungi were grown at 25 ^{o}C and 150 rpm for 7 days. After 7 days of incubation, mycelium pellets of fungi were formed. Then, the pellet of each fungus was filtered through autoclaved muslin cloth containing jar under aseptic environment and washed with autoclaved deionized water. After this, the pellet was stored in 0.8 % saline (0.2 µm syringe filtered) at 4 ^{o}C for further use.

3.5. Optimization of culture condition for biomass production

In biomass production, we have optimized three parameters *i.e*. pH, temperature and agitation to produce high amount of biomass. The pH range selected for the optimization of optimum condition was from 3.5 to 5.5 in the interval of 0.5, the temperature range was from 24 ^{o}C to 32 ^{o}C in the interval of 2 ^{o}C, while the agitation range for shaking condition was from 90 to 210 in the interval of 30 rpm.

Initially, about 1.5 $g.L^{-1}$ of dry weight of mycelium pellet was inoculated into defined growth medium (100 ml) and grown in shaking incubator under selected condition. The chemical compositions of defined growth medium are given in Table 3.4 (Kirk et al., 1978; Wen et al., 2010; Castellet-Rovira et al., 2018). After 3, 6, 9, 12 and 15th day of inoculation, the biomass of each fungus was determined by dry weight method. Here, the biomass from the flask of growing culture was filtered through Whatman filter and kept into glass plate in hot air oven at 60 ºC for overnight (Fig. 3.2). After drying of biomass, weight of the biomass measured on weighing balance to analyse the initial biomass size.

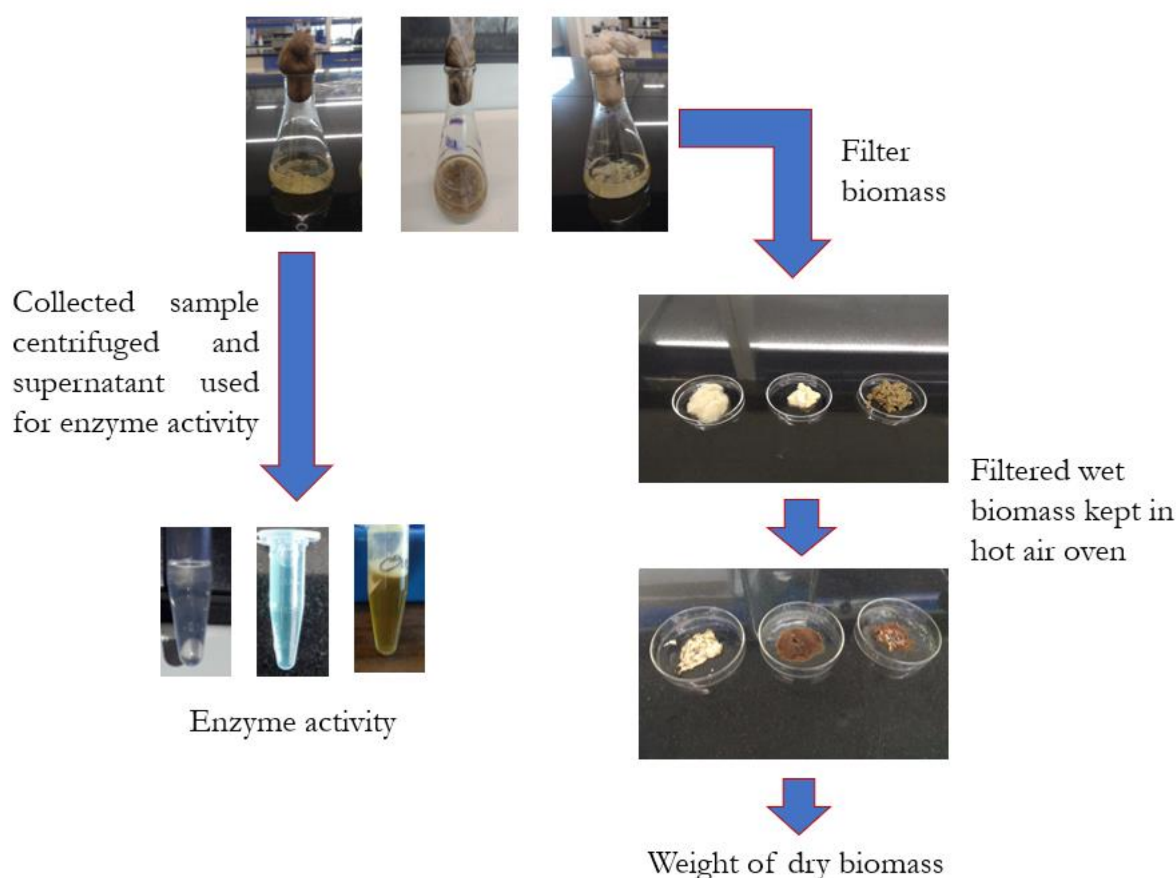

Fig. 3.2. Overview of analysis of dry weight biomass and extracellular enzyme activity estimation of selected fungi

Table 3.4. Chemical composition of growth medium for *G. lucidum, T. versicolor* and *P. chrysosporium*

Sr. no.	Chemical component	Concentration used ($g.L^{-1}$)
1	Glucose	10
2	Ammonium tartrate	2.0
3	Yeast extract	2.0
5	Peptone	2.0
6	KH_2PO_4	2.0
7	$MgSO_4.7H_2O$	0.5
8	$CaCl_2. 2H_2O$	0.1
9	Thiamine-HCl	1 mg
	Trace element	
10	Nitriloacetate	0.3
11	$MnSO_4.2H_2O$	0.05
12	$ZnSO_4.7H_2O$	0.043
13	$CuSO_4.7H_2O$	0.05
14	$FeSO_4.7H_2O$	0.01
15	$Na_2MoO_4.2H_2O$	0.01
16	NaCl	0.1

3.6. Analysis of extracellular ligninolytic enzymes

On the basis of best optimized condition of biomass production, the activities of these enzymes were measured on 3, 6, 9, 12 and 15th days. At zero/initial day, the enzyme activity was not detected. Here, the sample from the test and control flasks were periodically taken out on 3, 6, 9, 12 and 15th days. 2 ml supernatant was taken from each cultured flask aseptically and filtered

through Whatman filter paper and after the filtration, sample was centrifuged at 10000 rpm for 10 minutes. Now, the supernatant obtained after centrifugation was used as enzyme for activity in different selected assay. The reaction mixture of enzyme assay are given in Table 3.5.

Table 3.5. Chemical composition and reaction mixture used for enzyme activity assay

Enzyme activity assay	Reagent	Stock concentration	Volume used in enzyme assay (in ml)
Laccase	ABTS	0.5 mM	0.1
	Sodium acetate buffer	0.1 M	0.3 ml
Manganese peroxidase	Phenol red	0.01 %	0.1
	Sodium lactate	0.25 mM	0.1
	$MnSO_4$	2 mM	0.05
	BSA	0.5 %	0.2
	H_2O_2	20 mM	0.05
	NaOH	2 N	0.04
Lignin peroxidase	Veratryl alcohol	10 mM	0.25
	Sodium tartrate buffer	125 mM	0.5
	H_2O_2	2 mM	0.25

Laccase assay

Add 0.6 ml enzyme extract, 0.1 ml ABTS and 0.3 ml sodium acetate buffer, pH 5.0 in 2 ml centrifuge tube and incubated at 30 ºC. The reaction mixture was incubated according to the adopted method and the absorbance was taken at UV-Vis spectrophotometer at 420 nm. In this assay, ABTS act as substrate for laccase (Papinutti et al., 2003).

Manganese peroxidase assay

Add 0.5 ml enzyme extract, 0.05 ml phenol red, 0.1 ml sodium lactate, 0.05 ml $MnSO_4$, 0.2 ml BSA, 0.05 ml H_2O_2 and 0.04 ml NaOH, 4.5 pH in 2 ml centrifuge tube and incubated at 30 ºC. The reaction mixture was incubated according to the adopted method and the absorbance was taken at UV-Vis spectrophotometer at 610 nm. In this assay, phenol red act as substrate for manganese peroxidase (Kuhawara et al., 1984).

Lignin peroxidase assay

0.25 ml enzyme extract, 0.25 ml veratryl alcohol, 0.5 ml sodium tartrate buffer and 0.25 ml H_2O_2 in 2 ml centrifuge tube and incubated at 30 ºC. The reaction mixture was incubated according to the adopted method and the absorbance was taken at UV-Vis spectrophotometer at 310 nm. In this assay, veratryl alcohol act as substrate for lignin peroxidase (Arora and Gill, 2001).

The enzyme activity for these enzymes was expressed as $IU.L^{-1}$. One unit is defined as 1 µmol substrate oxidized per min. by the enzyme (Wen et al., 2010).

3.7. Biodegradation of selected antineoplastic compounds by white rot fungi

Initially, an individual stock solution of three antineoplastic compounds (cyclophosphamide, etoposide and paclitaxel) were prepared of 300 $mg.L^{-1}$ concentration separately and filtered through 0.22 µm syringe filter. Then, each drug from stock solution was spiked with the defined medium under aseptic condition. The final concentration of drugs in each treatment and control flask was 300 $\mu g.L^{-1}$. In last, 1.5 $g.L^{-1}$ (dry weight) of fungal mycelium was inoculated in the experimental flasks.

The degradation experiment flasks were divided into 3 control *i.e.* one for blank or negative control (medium + antineoplastic compound spiked) to asses physiochemical degradation of

spiked compound, second for heat killed or abiotic control (medium + heat killed fungus + antineoplastic compound spiked) to asses the biosorption process rate of fungi for antineoplastic compound and third for biotic or experimental control (medium + antineoplastic compound spiked + live WRF inoculated) to asses degradation of drugs by fungi (Castellet-Rovira et al., 2018). In heat killed control fungi were inoculated before the autoclave of medium.

The experiment was run for continuously 15 days for each fungal culture. All flasks (experiment and control) were run in triplicate. The experiment flasks were run on different culture condition for each fungus according to their best optimized culture condition for production of biomass end extracellular enzyme. Here, *T. versicolor* treatment flasks were run at 25 ºC, 4.5 pH and 150 rpm, *G. lucidum* treatment flasks were run at 30 ºC, 5.0 pH and 150 rpm and *P. chrysosporium* treatment flask were run at 30 ºC, 4.5 pH and 180 rpm for 15 days in shaking incubator under dark to prevent photodegradation. However, on 0, 3, 6, 9, 12 and 15 days of inoculation, 1 ml sample from each experiment and control flasks were taken out to analyse the degradation rate of antineoplastic compounds by fungi on LC-MS.

Simultaneously, with biodegradation experiment the effect of cyclophosphamide, etoposide and paclitaxel on the growth of fungi were also analysed. Here, another control was used *i.e.* biomass control (defined medium + fungi), which incubated in the same conditions as mentioned above for degradation of antineoplastic compound. In the last day of experiment, biomass of control and experimental flask were filtered and dried at 60 ^{o}C temperature in hot air oven. After this, dry weight of biomass was calculated by the given formula to analyse the negative effect of antineoplastic drugs on fungal growth (Eq^{n}. 1).

$$Effect\ of\ antineoplastic\ compound\ on\ growth\ of\ fungi: Total\ dry\ weight\ biomass\ of\ control - Total\ dry\ weight\ biomass\ of\ experimental\ flask \qquad (1)$$

3.8. LC-MS analysis

For the degradation analysis, at 0, 3, 6, 9, 12 and 15 days of incubation 1 ml sample were taken from each experiment and control flask. Each sample was filtered through 0.22 µm syringe filter before injecting into LC-MS for further analysis. After the filtration of sample, the quantification of cyclophosphamide and etoposide were carried out in LC-MS instrument at Advanced Instrument Research Facility at Jawaharlal Nehru University New Delhi, India. During the detection of antineoplastic compounds concentration in LC-MS, three data set were assessed for the analysis of removal during treatment process which are given below in the following equation 2, 3 and 4:

$$Total\ removal\ rate\ (\%): \frac{Concentration\ of\ drugs\ at\ 0\ days - Concentration\ of\ drugs\ at\ 15th\ day\ in\ biotic\ control}{Concentration\ of\ drugs\ at\ 0\ day} \times 100 \quad (2)$$

$$Total\ sorption\ rate\ (\%): \frac{Concentration\ of\ drugs\ at\ 0\ days - Concentration\ of\ drugs\ at\ 15th\ day\ in\ heat\ killed\ control}{Concentration\ of\ drugs\ at\ 0\ day} \times 100 \quad (3)$$

$$Total\ biodegradation\ rate\ (\%): Total\ removal\ rate\ (\%) - Total\ sorption\ rate\ (\%) \quad (4)$$

3.9. Degradation kinetics study

The kinetics of degradation of cyclophosphamide, etoposide and paclitaxel by *G. lucidum*, *T. versicolor* and *P. chrysosporium* was investigated to analyse the half-time and removal rate. The degradation of these compounds followed the pseudo-first-order kinetics model to evaluate '$t_{1/2}$' and degradation rate. The following equation (5) was applied for this study (Bankole et al., 2020):

$$\ln\left(\frac{S0}{S}\right) = kt \quad (5)$$

where,

S_0 = substrate (antineoplastic compound) initial concentration

S = substrate concentration at time 't'

k = substrate degradation constant

3.9.1. Estimation of reducing sugar and pH

The glucose concentration and pH of the experimental flask was measured to predict the effect of these parameter on rate of degradation after variation in their set values. *G. lucidum, T. versicolor* and *P. chrysosporium* were grown in defined growth medium (Table 3.4), where the initial concentration of glucose in each flask was 10 $g.L^{-1}$. The consumption of glucose was analysed by the analysis of reducing sugar from the sample and it was estimated by DNS method (Fig. 3.3). DNS method involved DNSA (30 g of sodium potassium tartrate in 50 ml double distilled water, then 20 ml of 2 M NaOH added to it and mix gently. After this, 1 g of DNS added and final volume 100 ml was adjusted). During glucose estimation initially, sample from growing fungal culture was filtered through muslin cloth contained jar. Then, 2 ml of filtered sample taken in a test tube and 2 ml DNS reagent added to it. After this, test tube was kept in water bath at 100 ^{o}C temperature for 10 min. (Fig. 3.3). In last sample was cool down at room temperature and the absorbance was taken at 540 nm (Garriga et al., 2017). The estimation of glucose was performed on 3, 6, 9, 12 and 15 days of experiment for each culture.

The pH value during biodegradation of antineoplastic compounds was measured at 0, 3, 6, 9, 12 and 15 days from the incubation start and compared the effect of pH variation in rate of degradation to their initial set value (Fig. 3.3).

The time-course-degradation profile of cyclophosphamide, etoposide and paclitaxel with each fungal culture were analysed on the basis of degradation rate with particular fungus and their pH and glucose levels presence in the experiment and control samples.

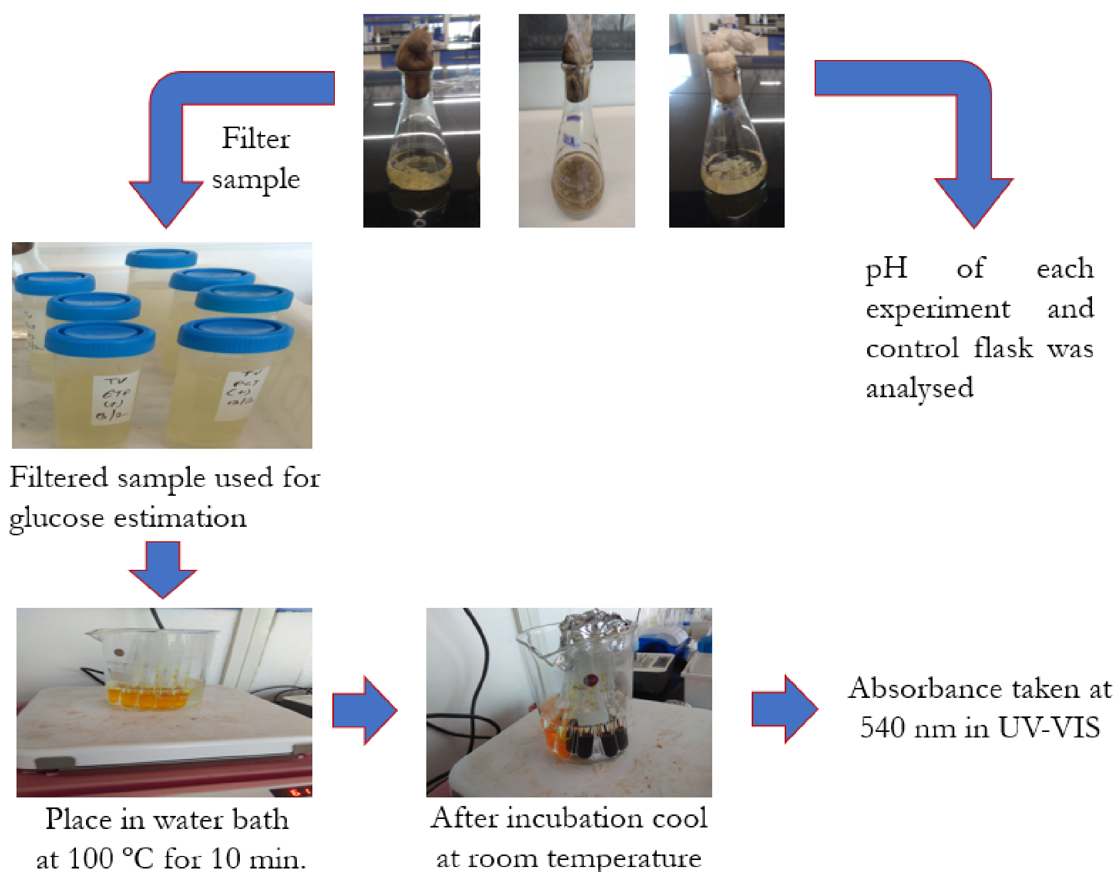

Fig. 3.3. Estimation of reducing sugar and pH of the *G. lucidum*, *T. versicolor* and *P. chrysosporium* during biodegradation experiment

3.10. Toxicity analysis

3.10.1. Cytotoxicity of native compounds

The cytotoxicity of selected antineoplastic was carried out on Raw 264.7 cell line. MTT assay was performed to analyse the cytotoxic effect of native forms of antineoplastic compounds. The 100 ml medium was prepared using the composition DMEM (10 g.L^{-1}), sodium bicarbonate (3.7 g.L^{-1}) and phenol red (15 mg.L^{-1}) dissolve into double distilled water. After the mixing of medium components, pH was adjusted to 7.2 to 7.4. Finally add, 11 ml FBS to make 10 % FBS containing medium and filtered the medium through filter assembly in sterile environment or biosafety cabinet-2 using vacuum pump. After the filtration, 1 ml from 100x stock penicillin-streptomycin solution into prepared medium was added, after this 1 ml medium

from freshly prepared medium flask was taken out and added into a 5 ml LB (Luria broth) tube. The medium was kept at 4 °C and LB tube at 37 °C temperature with 150 rpm in the incubator for overnight to contamination check. Beside the medium preparation, three different stock solution of cyclophosphamide, etoposide and paclitaxel were prepared of 1000 $\mu g.ml^{-1}$ in absolute DMSO and syringe filtered with 0.2 µm filter. After the preparation of stock of compounds, the dilution of stock was made into 250, 200, 150, 100, 50, 50, 25, and 10 $\mu g.ml^{-1}$ for each antineoplastic compound.

3.10.2. Cell seeding

After the sterility check of prepared medium, Raw 264.7 cells were grown in the prepared medium using a T-75 flask at 37 °C and 5 % CO_2 in an incubator (Das et al., 2012). When the sufficient growth of adherent cells achieved, the cells from the growing flask were washed out with the help of autoclaved 1x PBS in a sterile centrifuge tube and centrifuged at 2500 rpm for 5 min. for pellet collection. Then, remove the supernatant and mix the pellet gently by adding 5 ml of fresh media into tube. After the mixing of cells, take out 100 µl cell suspension and 400 µl of 0.4 % trypan blue. The cell suspension with trypan blue was used for cell count in the haemocytometer. The concentration or number of cells was counted by using diluent factor.

3.10.3. *In-vitro* cytotoxicity assay for native antineoplastic compounds

Primarily, 200 µl (1×10^4) of Raw 264.7 cells were seeded into each well of 96 well plate (flat bottom). The plate was kept in incubator at 37 °C with 5 % CO_2 for 24 hours. After 24 hours, plate was taken out from the incubator and 50 µl of drugs or compound sample (final concentration into well were 250, 200, 150, 100, 50, 25, and 10 $\mu g.ml^{-1}$) added into wells of the plate. DMSO was used as solvent control, the higher concentration (5000 $\mu g.ml^{-1}$) of antineoplastic compound was used as positive control and fresh medium was used as a negative control. Each well in the plate was containing the final volume 250 µl. All the controls and test

samples were added in triplicate into wells. After adding the sample, the plate was kept at 37 °C with 5 % CO_2 for 48 hours drugs treatment. After the 48 hours, medium from each well was removed and 200 µl fresh medium without FBS and 25 µl MTT (5 $mg.ml^{-1}$) were added. Then, the plate kept at 37 °C with 5 % CO_2 for 4 hours. After the 4 hours of incubation, MTT containing media from each well was removed and 200 µl DMSO into each well added as indicating in Fig 3.4. In last, the plate was covered with foil to avoid direct effects of light and shake in shaker for 15 minutes at 37 °C temperature. Finally, absorbance of the plate was measured at 590 nm in the microtitre plate reader.

The following equation was used to determine cytotoxicity of antineoplastic compounds:

$$\text{\% of Cytotoxicity} = 100 - \left[\frac{\text{Absorbance (Sample)}}{\text{Absorbance (Control)}}\right] \times 100$$

The IC_{50} (Inhibitory concentration) of drugs were calculated by the equation $y = mx+c$ using MS-Excel software.

3.11. Toxicity of transformed products

Toxicity of intermediates or transformed products of cyclophosphamide, etoposide and paclitaxel formed during biodegradation were analysed by MTT assay same as used for cytotoxicity study of native form of these compounds with some modification. The toxicity was estimated to visualize the cytotoxic effect of transformed products on cell viability. During the biodegradation analysis experiment, simultaneously the samples for toxicity analysis of transformed products were also taken from the control and experiment flasks. 1 ml sample from each flask was taken and filtered through 0.22 µm syringe filter in biosafety cabinet under aseptic conditions. The filtered sample was used to determine the cytotoxicity of transformed products on cell viability of Raw 264.7 cells. The sample collected from normal growing fungus flask was used as negative control to assess the cytotoxic effect of metabolites produced by fungus during their life cycle and effect of medium composition presented in the flask.

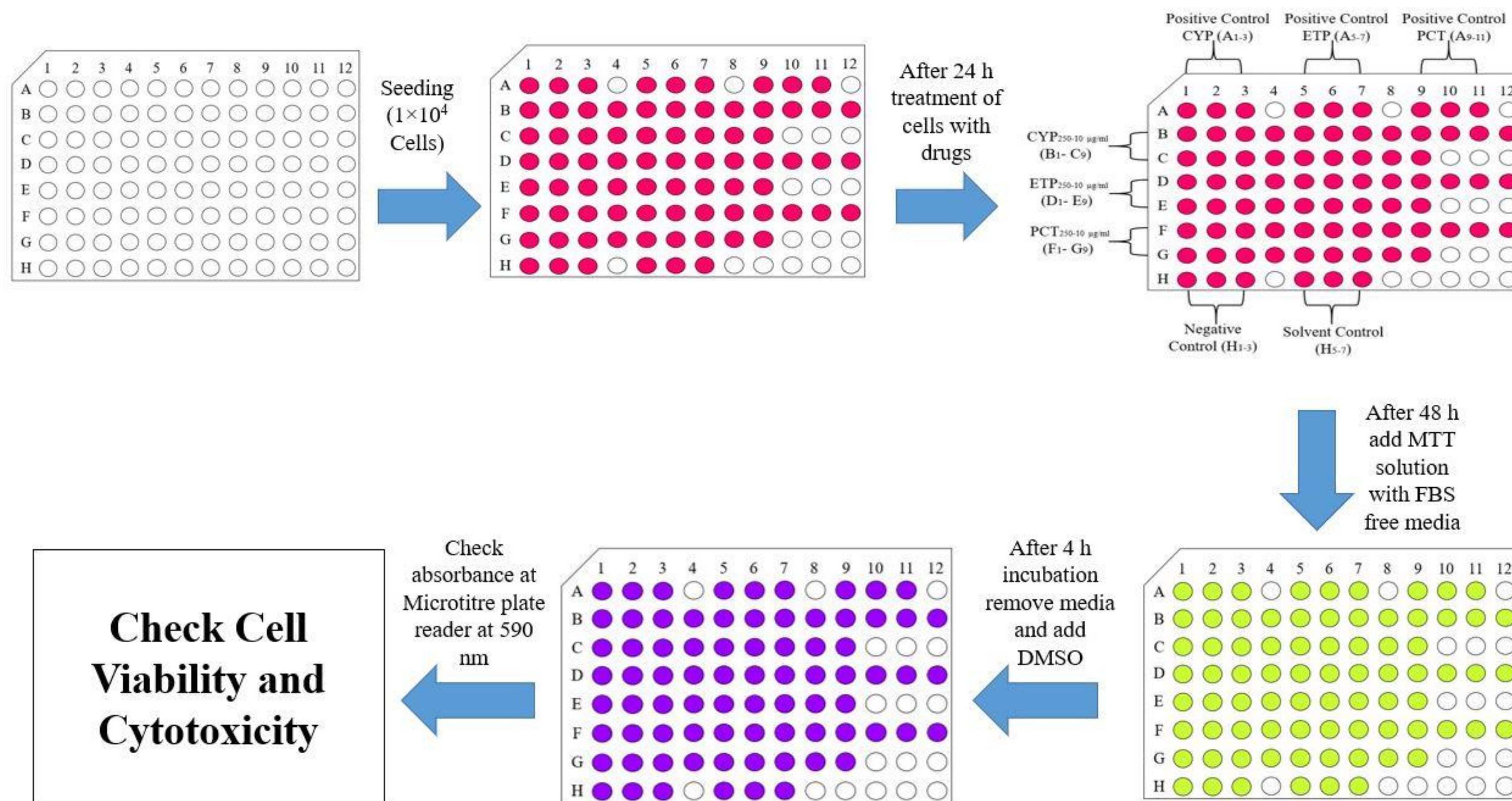

Fig 3.4. Schematic overview of *In-vitro* cytotoxicity analysis of native form of cyclophosphamide, etoposide and paclitaxel on Raw 264.7 cell line