



Results and Discussion

4.1. Method development and standardization

In the development of HPLC methods for the detection and quantification of cyclophosphamide, etoposide and paclitaxel, we standardized the condition for the better elution of these compounds. Initially, we determined the maximum absorbance (λ max) of the target antineoplastic drug(s) by UV-Vis spectrophotometer. The λ max for cyclophosphamide after the scanning was 199 nm, while 229 nm for both paclitaxel and etoposide as given in Table 4.1. Further, we developed three different HPLC method using C₁₈ column (250 mm×4.6 mm×5 μ m) to detect and quantify the target antineoplastic compounds. The optimized mobile phase were acetonitrile and water (70:30) with 25 °C column temperature and flow rate 1.0 ml.min⁻¹ at wavelength 199 nm for cyclophosphamide. The RT for best elution in case of cyclophosphamide was 03:32.3 $\pm$ 0.41 min. (Table 4.2). While the optimized mobile phase for etoposide and paclitaxel detection were also acetonitrile and water in ratio 80:20 with 25 °C column temperature at 229 nm wavelength and 1.0 ml.min⁻¹ flow rate. The retention time (RT) for best elution in case of etoposide and paclitaxel were 2:40.1 $\pm$ 0.48 min. and 3:49.3 $\pm$ 0.28 min. respectively. The run length for all these methods was 10 min. The optimized conditions and parameters were tabulated and presented in Table 4.2.

4.2. Validation of methods

4.2.1. Linearity, Accuracy and Precision

The linearity of the method is directly proportional to the correlation of the concentration of the drug molecule standard versus the result (concentration) obtained using the method developed for the same compound. The calibration graph shows the linear relationship between different concentration range of cyclophosphamide (10, 50, 100, 250, 500, 750 μ g.ml⁻¹) with correlation coefficient (r^2) value 0.989872 (Fig. 4.1). Similarly, etoposide also shows the linear relationship between different concentration range (10, 50, 100, 250, 500, 750 μ g.ml⁻¹) with r^2

value 0.999914 (Fig. 4.2). Fig. 4.3. shows the linear relationship between different concentration range of paclitaxel (10, 50, 100, 250, 500, 750 $\mu\text{g.ml}^{-1}$) with r^2 value 0.999788.

All the test analytes cyclophosphamide, etoposide and paclitaxel show liner relationship and thus they have agreeable linearity 0.98, 0.99 and 0.99 respectively.

To calculate the accuracy, the percentage of reproducibility at all six concentration levels of cyclophosphamide, etoposide and paclitaxel peak area were calculated depicted in Table 4.3. This indicates the applicability and reliability of the method developed for analysis of these three antineoplastic compounds. The triplicate recovery data of each compound was similar at all the concertation analysed in term of peak area.

The precision of proposed method was measured by the repetition of homogenous sample concentration of paclitaxel, cyclophosphamide and etoposide under proposed condition and the results were tabulated and presented in Table 4.4. In this study, the peak area and retention time were analysed of a single concentration of each compound ten times repeats. The concentration used for precision validation was 500 $\mu\text{g.ml}^{-1}$. Here, among ten cases, as per the optimized RT 3:32 min. for cyclophosphamide observed similar in seven samples and peak area 1936 mAs observed in six sample, it was range from 1934 to 1939 mAs. These values shown not a large difference in peak area and RT. In etoposide, the optimized RT was 2:40 min. observed about similar in six samples and peak area was in range from 19408 to 194011 mAs. It was almost similar in all ten samples, there was no big difference in the peak area and RT of etoposide also. The optimized RT of paclitaxel was 3:49 min. was observed similar in six samples and peak area was observed in range from 18629 to 18630 in all ten samples. There was very close peak area and RT value in all cases.

Table 4.1. λ_{max} of cyclophosphamide, etoposide and paclitaxel absorbance by scanning on UV-VIS spectrophotometer

Sr. no.	Antineoplastic compound	Wavelength (In nm)	Maximum absorbance (OD)
1	Cyclophosphamide	199	2.223 ± 0.008
2	Etoposide	229	3.650 ± 0.035
3	Paclitaxel	229	3.954 ± 0.013

Table 4.2. Optimized HPLC conditions for the detection of cyclophosphamide, etoposide and paclitaxel in water sample

Antineoplastic compound	Run Time (In min.)	Mobile Phase		Column	Column oven Temperat ure ($^{\circ}$ C)	Wavele ngth (In nm)	Retention time (RT) (in min.)	Co- relation coefficient (r^2 value)	Flow rate (ml.min. $^{-1}$)
		Acetonitrile (A)	Water (B)						
Cyclophosphamide	10	70	30	C ₁₈ (250mm $\times$ 4.6mm $\times$ 5 μ m)	25	199	3:32.3	0.989872	1.0
Etoposide	10	80	20	C ₁₈ (250mm $\times$ 4.6mm $\times$ 5 μ m)	25	229	2:40.1	0.999914	1.0
Paclitaxel	10	80	20	C ₁₈ (250mm $\times$ 4.6mm $\times$ 5 μ m)	25	229	3:49.3	0.999788	1.0

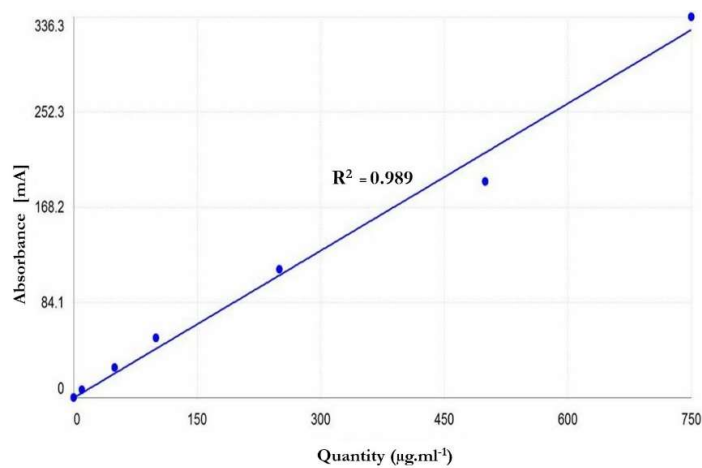


Fig. 4.1. Calibration graph of cyclophosphamide in HPLC at developed conditions

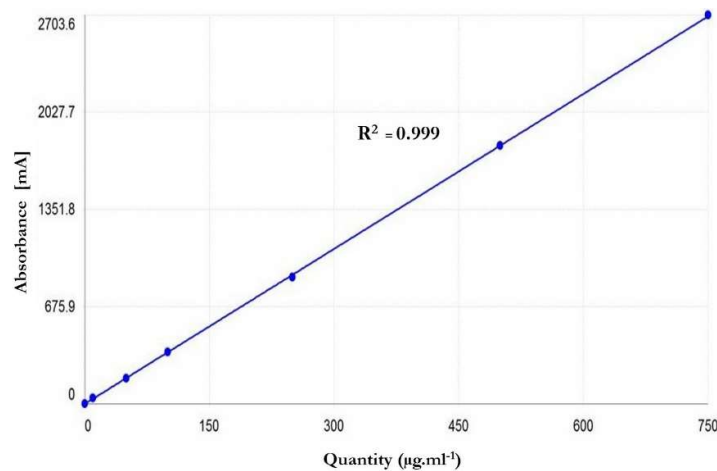


Fig. 4.2. Calibration graph of etoposide in HPLC at developed conditions

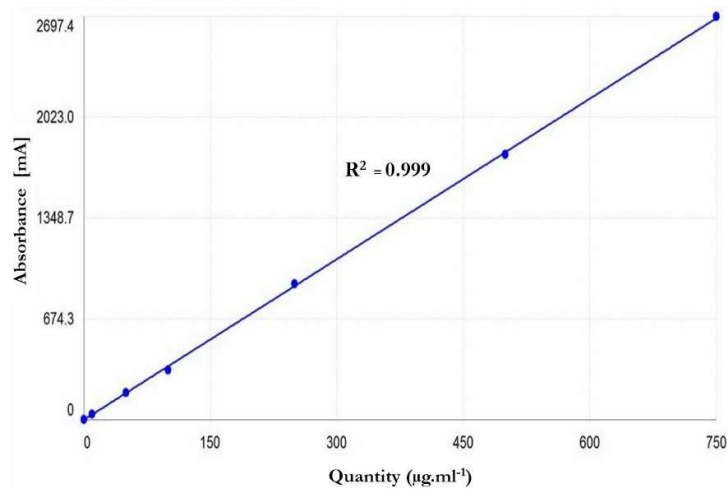


Fig. 4.3. Calibration graph of paclitaxel in HPLC at developed conditions

Table 4.3. Reproducibility data of cyclophosphamide, etoposide and paclitaxel in developed HPLC method

Name of compound	Spiked Level of compound (in $\mu\text{g}.\text{ml}^{-1}$)	Replicate 1 (Peak area in mAs)	Replicate 2 (Peak area mAs)	Replicate 3 (Peak area mAs)
Cyclophosphamide	10	52.3	52.9	52.5
	50	272.9	274.4	273.1
	100	546.5	546.4	546.8
	250	1174	1174.2	1175.8
	500	1936.4	1937.2	1936.6
	750	2981.1	2996.5	2918.2
Etoposide	10	331.1	329.8	330.6
	50	1852.9	1850.7	1856.1
	100	3778.6	3754.5	3779
	250	9067	9072.2	9071.8
	500	18629.2	18632.4	18629.9
	750	26738.3	26738	26738.7
Paclitaxel	10	377.4	371.6	376
	50	1848	1848.6	1814.6
	100	3771.7	3752.1	3768.9
	250	10488.5	10479	10488.3
	500	19409	19409.7	19405.2
	750	31629.3	31632	31629.8

Table 4.4. Precision data of cyclophosphamide, etoposide and paclitaxel on developed HPLC methods

No. of Replicate	Cyclophosphamide (500 µg.ml ⁻¹)		Etoposide (500 µg.ml ⁻¹)		Paclitaxel (500 µg.ml ⁻¹)	
	Peak area	RT	Peak area	RT	Peak area	RT
	(in mAs)	(in min.)	(in mAs)	(in min.)	(in mAs)	(in min.)
1	1936.1	3:32.2	18629.2	3:49.2	19409.2	2:40.5
2	1936.4	3:32	18630.4	3:49.3	19408.8	2:40.1
3	1934.6	3:32.8	18630.2	3:47	19408.9	2:41.8
4	1936.2	3:32.3	18629.3	3:49.6	19410.4	2:40.6
5	1937	3:36.1	18629.5	3:48.9	19407.7	2:40.4
6	1938.4	3:34	18629.6	3:49.2	19408.1	2:47.2
7	1935.4	3:32.4	18630.1	3:50.8	19409.7	2:41.2
8	1935.8	3:32.1	18629.8	3:49.1	19411.2	2:42
9	1934.8	3:33.4	18629.5	3:49.3	19409.6	2:40.5
10	1935.9	3:32.9	18629.9	3:50.6	19409.2	2:40.8

4.2.2. Sensitivity, Specificity and Robustness

The assessment of sensitivity of optimized method for cyclophosphamide, etoposide and paclitaxel was based on limit of detection *i.e.* 10 µg.ml⁻¹ for each. These methods showing the minimum 10 µg.ml⁻¹ of amount or concentration of above three antineoplastic compounds was detected and the limit of quantification was 1 µg.ml⁻¹ or less.

The specificity of all three compounds was determined by comparing the blank and standard sample solutions by injecting 20 µl sample of blank and standard separately into the injecting loop of HPLC system. In Fig. 4.4, first chromatogram representing the blank or control chromatogram, while rest three chromatogram showing peak for cyclophosphamide of

concentration 250, 500 and 750 $\mu\text{g.ml}^{-1}$ at RT 3:32.3 minutes. Similarly in Fig. 4.5, first chromatogram representing the blank or control chromatogram, while rest three chromatogram showing peak for etoposide of concentration 250, 500 and 750 $\mu\text{g.ml}^{-1}$ at RT 2:40.1 minutes. In Fig. 4.6, first chromatogram representing the blank or control chromatogram, while rest three chromatogram showing peak for paclitaxel of concentration 250, 500 and 750 $\mu\text{g.ml}^{-1}$ at RT 3:49.3 minutes. All these chromatograms indicated the purity of analytes peak and confirms the specificity of methods.

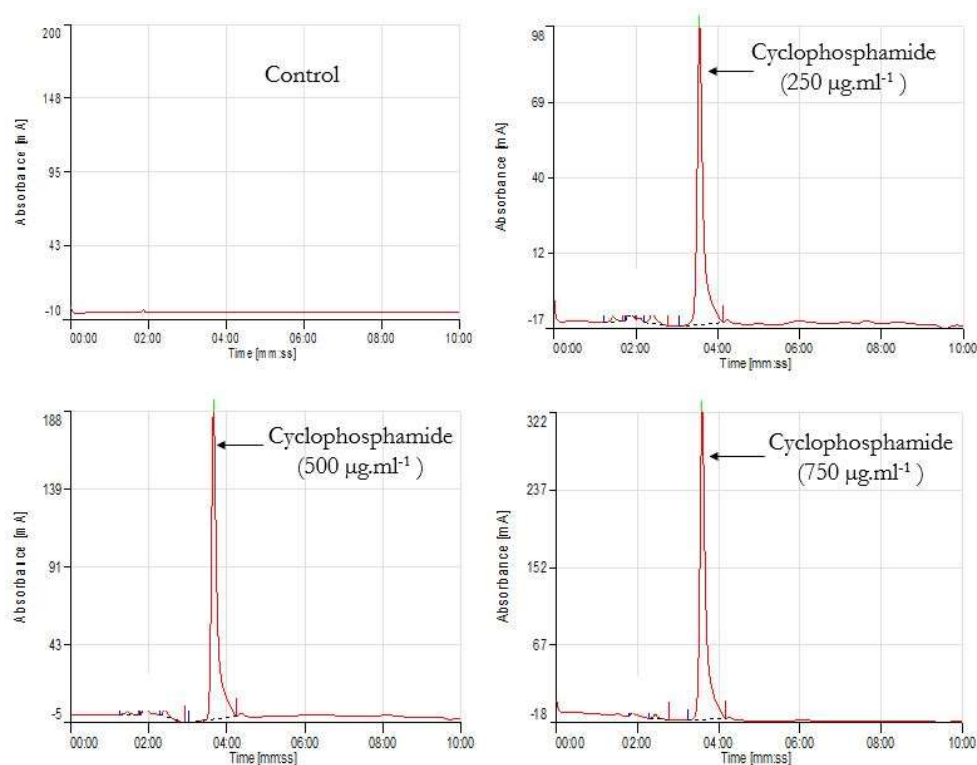


Fig. 4.4. HPLC chromatogram peak of cyclophosphamide for control and different concentration

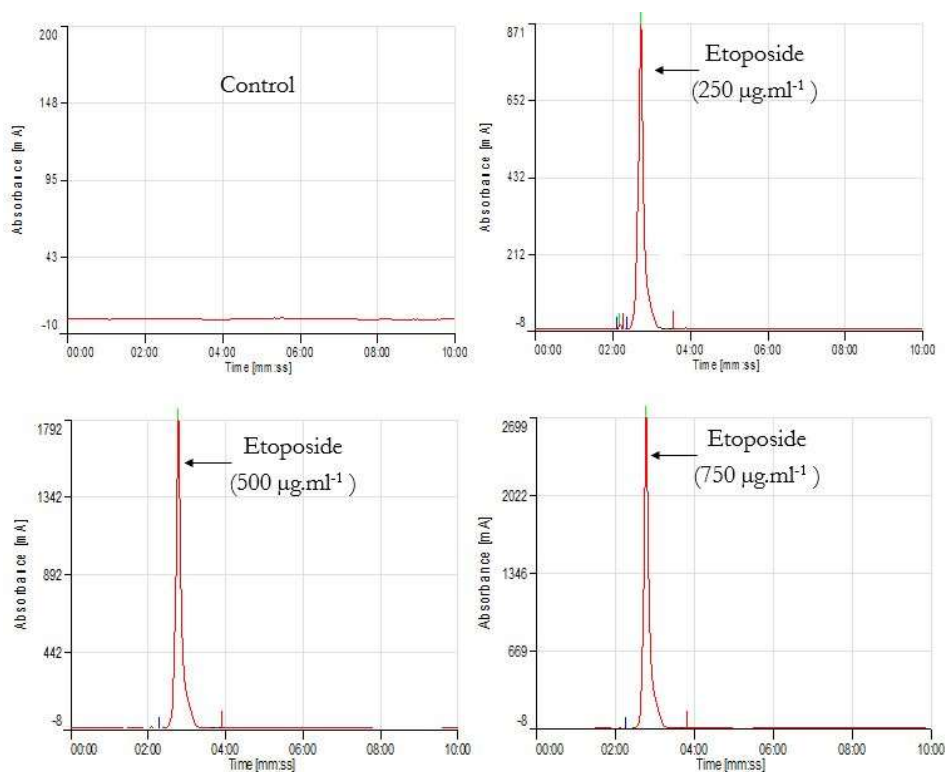


Fig. 4.5. HPLC chromatogram peak of etoposide for control and different concentration

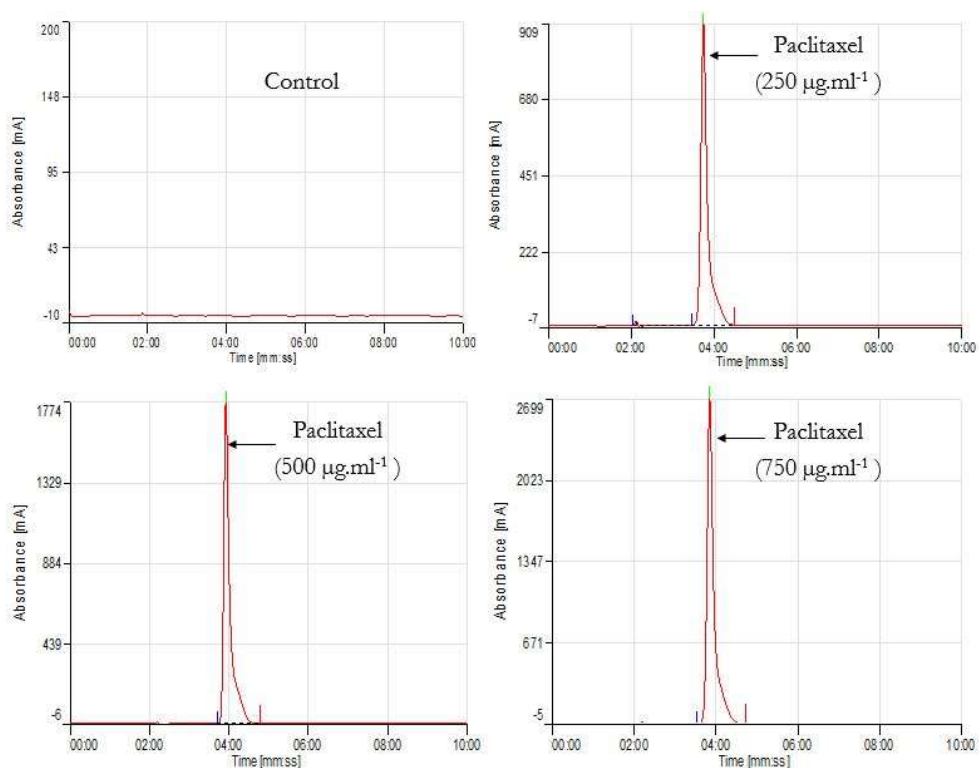


Fig. 4.6. HPLC chromatogram peak of paclitaxel for control and different concentration

The robustness of developed HPLC method was evaluated by changing the parameters and conditions of proposed method such as mobile phase, temperature, and flow rate and are summarized in Table 4.5. Here, the selected range of temperature was from 20 to 30 °C, flow rate was 0.8 to 1.2 ml.min⁻¹ and mobile phase ratio of acetonitrile and water was from 50:50 to 90:10. On these the parameters the peak area, peak height and RT of antineoplastic compound was optimized for each compound. The highest elution of cyclophosphamide was observed at 25 °C temperature, 1.0 ml flow rate and 70:30 mobile phase, while in case of etoposide and paclitaxel was observed at 25 °C temperature, 1.0 ml flow rate and 80:20 mobile phase. In other conditions the results were obtained for these antineoplastic compounds but their recovery or elution rate was very less as compared to the optimized conditions.

Table 4.5. Robustness of developed HPLC method for cyclophosphamide, etoposide and paclitaxel

Antineoplastic compound	Parameter optimized		Peak area (in mAs)	RT (in min.)
Cyclophosphamide	Temperature (°C)	25	3431.1 ±0.26	3:32.3 ±0.41
	Flow rate (ml.min. ⁻¹)	1.0		
	Mobile phase (Acetonitrile:Water)	70:30		
Etoposide	Temperature (°C)	25	26738.3 ±0.33	2:40.1 ±0.48
	Flow rate (ml.min. ⁻¹)	1.0		
	Mobile phase (Acetonitrile:Water)	80:20		
Paclitaxel	Temperature (°C)	25	31629.9 ±0.36	3:49.3 ±0.28
	Flow rate (ml.min. ⁻¹)	1.0		
	Mobile phase (Acetonitrile:Water)	80:20		

In 2003, Larson and his group developed HPLC method for the simultaneous analysis of cyclophosphamide and paclitaxel along with three other antineoplastic compounds (IF, 5-FU and doxorubicin) with the use of C₁₈ column. The mobile phase used for this analysis were acetonitrile and water in combination with buffer in ratio of 22.75:77.25 at wavelength 195 nm and 1.2 ml.min⁻¹ flow rate in isocratic mode. The minimum detection limit of cyclophosphamide and paclitaxel were 0.5 µg.ml⁻¹ and 2.0 µg.ml⁻¹ at retention time of 10.52 min. and 38.59 min. respectively (Larson et al., 2003). The method developed in present study are more reliable to the Larson methods in term of mobile phase use, require less retention time for elution and high sensitivity etc. Sottani et al. (2005) developed HPLC-MS method for the detection for cyclophosphamide and IF from the urine sample health worker. The LOQ of cyclophosphamide and IF in urine was 0.02 and 0.4 µg.L⁻¹ respectively. In 2008, Sottani et al. developed another RP-HPLC method using ESI-MS for the simultaneous detection of cyclophosphamide, IF, epirubicin, doxorubicin and daunorubicin from human urine sample. The LOQ for cyclophosphamide, IF and epirubicin was 0.2 µg.L⁻¹, while for daunorubicin and doxorubicin was 150 ng.L⁻¹ and 300 ng.L⁻¹ respectively (Sottani et al., 2008). Another liquid chromatography (LC-ESI-MS/MS) was developed and validated by Nussbaumer et al. (2010) for the detection of 10 antineoplastic compound (cyclophosphamide, etoposide, IF, epirubicin, doxorubicin, gemcitabine, cytarabine, irinotecan and vincristine) from the hospital waste effluent. The analysis was carried out with acetonitrile, water and formic acid as mobile phase at 0.2 ml.min⁻¹ flow rate in isocratic mode. The limit of quantification for these drugs on developed method was in range of concentration from 0.25 to 2 µg.L⁻¹. Ferrando-Climent et al. (2013) developed UPLC-MS/MS method using SPE for the detection of cyclophosphamide, etoposide and paclitaxel along with seven other antineoplastic compounds from hospital and municipal wastewater samples and validated the same. The sensitivity or LOD of developed method for these compounds was from 0.8 to 24 ng.L⁻¹. Here, the mobile phase use for elution of these 10 antineoplastic compounds were 0.1 % formic acid with acetonitrile at 0.4 ml.min⁻¹

in gradient mode. This method is highly reliable, sensitive and stable as compared to presently developed methods.

4.3. Morphology and growth of fungi

White rot fungi are basidiomycetes fungi and provide a whitish morphological appearance on their growth. Initially, the collected WRF strains were grown on petri plates for their subculturing. After five to six day of subculture process, the optimum growth was achieved, and the mycelium colonies of fungi were visible on agar petri plates. Among these three fungi, every fungus has their specific morphological appearance as indicated in Fig. 4.7. Furthermore, the growing fungi from petri plates were transferred into selected broth medium for mycelium pellet formation and biodegradation experiment. In broth medium with shaking condition, the morphology of each fungus was different to the static condition. In shaking condition, several spherical beads of mycelium were produced in medium are shown in Fig. 4.8, each fungus has different and specific spherical structure of mycelium pellet in compared to other two fungi. *G. lucidum* grown as spherical bead having hairy structure on their surface, *T. versicolor* grown as spherical beads without any structure on their surface, while *P. chrysosporium* grown as round ball like without any structure on their surface.



Fig. 4.7. Growth of selected WRFs on petri plates (a) *G. lucidum*, (b) *T. versicolor* and (c) *P. chrysosporium*



Fig. 4.8. Growth of selected WRFs under shaking conditions (a) *G. lucidum*, (b) *T. versicolor* and (c) *P. chrysosporium*

4.4. Optimized culture conditions for biomass and enzyme production

The production of biomass and maximum extracellular enzyme activity of *G. lucidum*, *T. versicolor* and *P. chrysosporium* were estimated under different culture conditions. When the fungi utilized the medium for their growth, consequently the amount of biomass in experimental flasks was also increased in all fungi. This results in increase in the number of cells and their enzyme production. Although, WRFs are highly prominent source to secrete extracellular enzyme, which are highly responsible for the mineralisation of recalcitrant compounds by their oxidation-reduction mechanism. As we found from prior arts, *T. versicolor*, *G. lucidum* and *P. chrysosporium* are also able to produce these enzymes. In present study, we have determined the extracellular enzyme *i.e.* laccase, manganese peroxidase and lignin peroxidase enzyme activity from these three growing fungi culture. The activity of these enzymes was assessed by three different methods or assay that we have discussed in methodology section.

4.4.1. Optimized conditions for biomass and enzyme production by *G. lucidum*

In case of *G. lucidum*, the dry weight of biomass of *G. lucidum* was calculated on 0, 3, 6, 9, 12 and 15 days of experiment. The highest amount of biomass and enzyme activity was estimated at 30 °C temperature, 5.0 pH and 150 rpm. Here, the initial mycelium biomass size used was 1.505 g.L⁻¹ and it was increased to 5.599 ±0.14 g.L⁻¹, 8.958 ±1.02 g.L⁻¹, 3.871 ±0.042 g.L⁻¹, 3.229 ±0.17 g.L⁻¹ and 3.084 ±0.011 g.L⁻¹ on 3, 6, 9, 12 and 15 days respectively (Fig. 4.9). However, at zero/initial day of experiment, no enzyme activity was observed. The laccase activity of *G. lucidum* was 94.3 ±0.47 IU.L⁻¹ on 3rd day, 214.4 ±0.8 IU.L⁻¹ on 6th day, 137.5 ±0.61 IU.L⁻¹, on 9th day, 76.4 ±0.34 IU.L⁻¹ on 12th day and 38.2 ±0.46 IU.L⁻¹ observed on 15th day. Manganese peroxidase activity of *G. lucidum* was 31.6 ±1.8 IU.L⁻¹ on 3rd day, 64.22 ±0.74 IU.L⁻¹ on 6th day, 42.6 ±3.72 IU.L⁻¹ on 9th day, 31.96 ±2.22 IU.L⁻¹ on 12th day and 18.44 ±0.66 IU.L⁻¹ observed on 15th day. Lignin peroxidase activity of *G. lucidum* was 7.78 ±0.68 IU.L⁻¹ on 3rd day, 16.3 ±0.47 IU.L⁻¹ on 6th day, 6.7 ±0.11 IU.L⁻¹ on 9th day, 3.52 ±1.04 IU.L⁻¹ on 12th day and 2.36 ±0.24 IU.L⁻¹ observed on 15th day. These results indicate, the highest biomass of *G. lucidum* was shown as 8.958 ±1.02 g.L⁻¹ on 6th day. Whereas, the maximum laccase, manganese peroxidase and lignin peroxidase activity were shown as 214.4 ±0.8 IU.L⁻¹, 64.22 ±0.74 IU.L⁻¹ and 16.3 ±0.4 IU.L⁻¹ at 6th day respectively (Fig. 4.10).

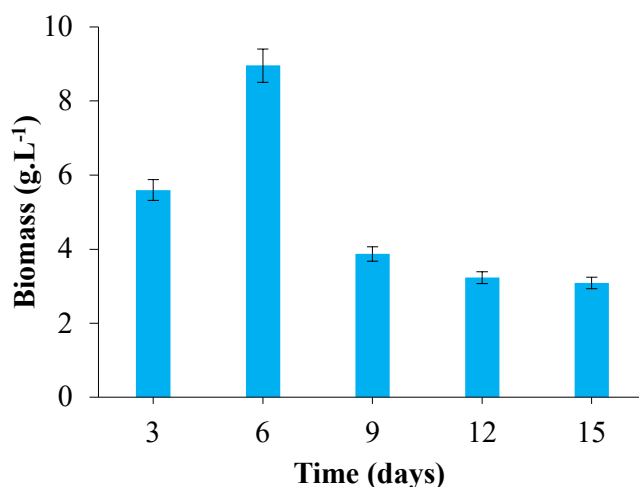


Fig. 4.9. Dry weight biomass analysis under optimized conditions using *G. lucidum*

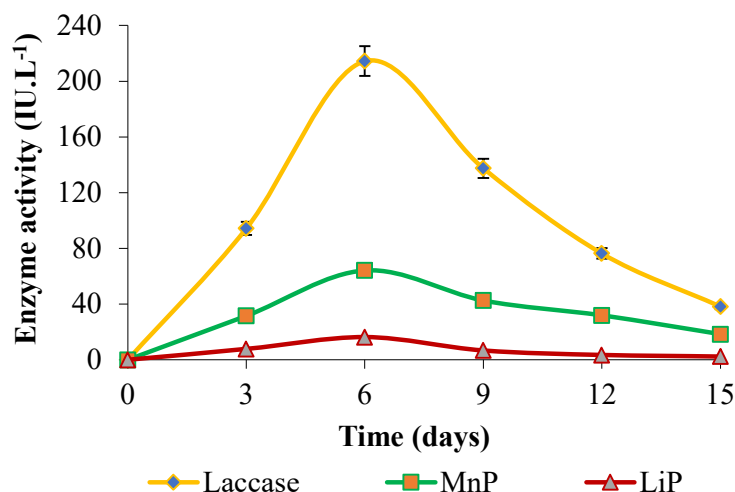


Fig. 4.10. Extracellular enzyme activity analysis under optimized condition using *G. lucidum*

4.4.2. Optimized condition of *T. versicolor* for biomass and enzyme activity

In case of *T. versicolor*, the highest amount of biomass and enzyme activity was estimated at 26 °C temperature, 4.5 pH and 150 rpm. The initial mycelium biomass size of *T. versicolor* used was 1.552 g.L⁻¹ and it was increased to 2.359 ±0.032 g.L⁻¹, 4.212 ±0.018 g.L⁻¹, 10.84 ±0.812 g.L⁻¹, 7.062 ±0.26 g.L⁻¹ and 4.032 ±0.025 g.L⁻¹ on 3, 6, 9, 12 and 15 days respectively (Fig. 4.11). However, laccase enzyme activity of *T. versicolor* was 70.4 ±5.12 IU.L⁻¹ on 3rd day, 148.1 ±5.37 IU.L⁻¹ on 6th day, 265.4 ±12.32 IU.L⁻¹ on 9th day, 104.8 ±1.44 IU.L⁻¹ on 12th day and 56.3 ±4.16 IU.L⁻¹ observed on 15th day. The manganese peroxidase activity was estimated as 15.42 ±0.4 IU.L⁻¹ on 3rd day, 18.89 ±0.073 IU.L⁻¹ on 6th day, 30.32 ±0.96 IU.L⁻¹ on 9th day, 14.28 ±0.2 IU.L⁻¹ on 12th day and 8.46 ±1.04 IU.L⁻¹ observed on 15th day, while lignin peroxidase activity was 12.5 ±1.07 IU.L⁻¹ on 3rd day, 22.14 ±1.04 IU.L⁻¹ on 6th day, 36.72 ±1.64 IU.L⁻¹ on 9th day, 7.5 ±1.35 IU.L⁻¹ on 12th day and 2.98 ±0.42 IU.L⁻¹ was observed on 15th day. These results indicate, the highest biomass of *T. versicolor* was shown as 10.84 ±0.812 g.L⁻¹ on 9th day. Whereas, the maximum laccase, manganese peroxidase and lignin

peroxidase activity were shown as $265.4 \pm 12.32 \text{ IU.L}^{-1}$, $30.32 \pm 0.96 \text{ IU.L}^{-1}$ and $36.72 \pm 1.64 \text{ IU.L}^{-1}$ at 9th day respectively (Fig. 4.12).

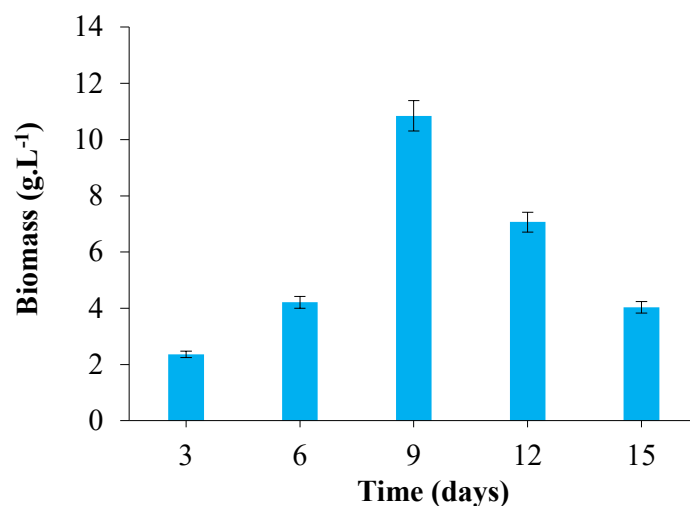


Fig. 4.11. Dry weight biomass analysis under optimized condition using *T. versicolor*

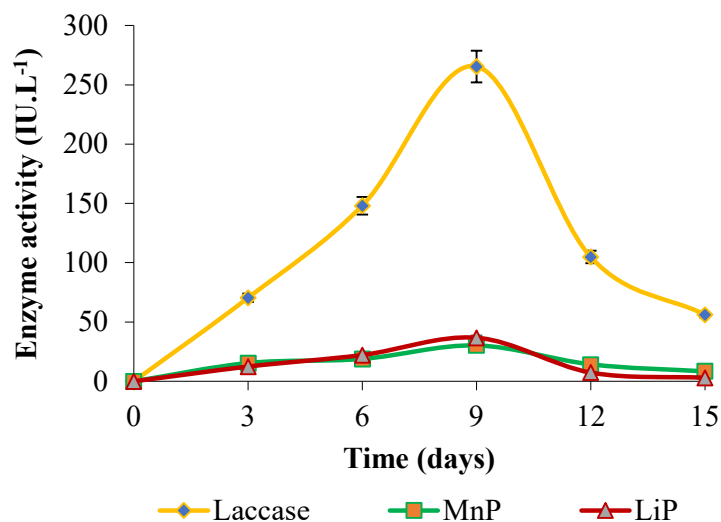


Fig. 4.12. Extracellular enzyme activity analysis under optimized condition using *T. versicolor*

4.4.3. Optimized condition of *P. chrysosporium* for biomass and enzyme activity

In case of *P. chrysosporium*, the highest amount of biomass and enzyme activity were estimated at 30 °C temperature, 4.5 pH and 180 rpm. The initial mycelium biomass size of

P. chrysosporium used for experiment was 1.496 g.L^{-1} and it was increased to $4.442 \pm 0.28 \text{ g.L}^{-1}$, $9.232 \pm 0.015 \text{ g.L}^{-1}$, $15.148 \pm 0.423 \text{ g.L}^{-1}$, $8.079 \pm 0.09 \text{ g.L}^{-1}$ and $4.728 \pm 0.024 \text{ g.L}^{-1}$ on 3, 6, 9, 12 and 15 days respectively (Fig. 4.13). However, the enzyme activity of *P. chrysosporium*, for laccase was $22.7 \pm 0.71 \text{ IU.L}^{-1}$ on 3rd day, $39.5 \pm 0.25 \text{ IU.L}^{-1}$ on 6th day, $72.1 \pm 1.97 \text{ IU.L}^{-1}$ on 9th day, $18.8 \pm 1.34 \text{ IU.L}^{-1}$ on 12th day and $4.13 \pm 0.58 \text{ IU.L}^{-1}$ observed on 15th day. The manganese peroxidase activity was $72 \pm 4.15 \text{ IU.L}^{-1}$ on 3rd day, $112.8 \pm 2.24 \text{ IU.L}^{-1}$ on 6th day, $186.2 \pm 5.44 \text{ IU.L}^{-1}$ on 9th day, $89.44 \pm 2.56 \text{ IU.L}^{-1}$ on 12th day and $21.6 \pm 1.02 \text{ IU.L}^{-1}$ observed on 15th day, while lignin peroxidase activity was $54.8 \pm 2.92 \text{ IU.L}^{-1}$ on 3rd day, $88.14 \pm 2.54 \text{ IU.L}^{-1}$ on 6th day, $146.3 \pm 4.81 \text{ IU.L}^{-1}$ on 9th day, $61.2 \pm 3.14 \text{ IU.L}^{-1}$ on 12th day and $27.14 \pm 0.6 \text{ IU.L}^{-1}$ was on 15th day. These results indicate, the highest biomass of *P. chrysosporium* was shown as $15.148 \pm 0.423 \text{ g.L}^{-1}$ on 9th day. Whereas, the maximum laccase, manganese peroxidase and lignin peroxidase activity were shown as $72.1 \pm 1.97 \text{ IU.L}^{-1}$, $186.2 \pm 5.44 \text{ IU.L}^{-1}$ and $146.3 \pm 4.81 \text{ IU.L}^{-1}$ at 9th day respectively. In this fungus, the maximum activity was shown for enzyme manganese peroxidase as well for lignin peroxidase as compared to *G. lucidum* and *T. versicolor*, where laccase activity was shown as maximum (Fig. 4.14).

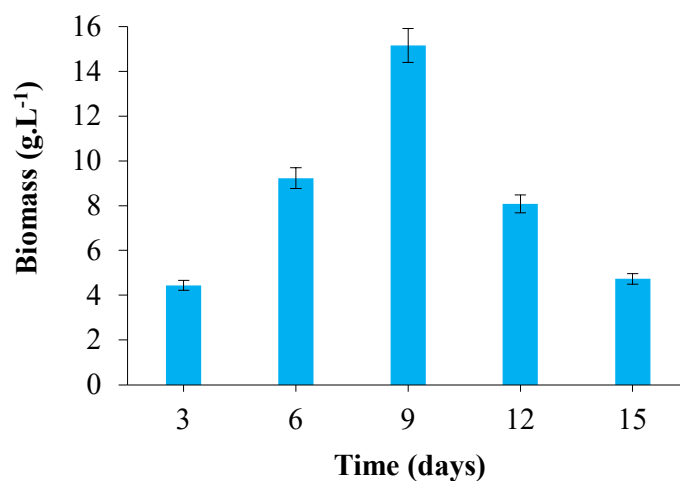


Fig. 4.13. Dry weight biomass analysis under optimized condition using *P. chrysosporium*

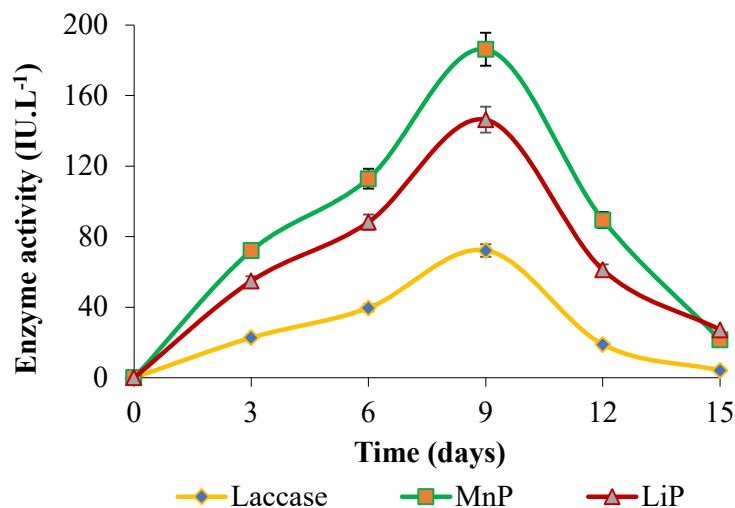


Fig. 4.14. Extracellular enzyme activity analysis under optimized condition using *P. chrysosporium*

In 2008, Wang and their group optimized the culture conditions for maximum extracellular enzyme activity of *P. chrysosporium*. They analysed the activity of two enzyme manganese peroxidase and lignin peroxidase. The fungal culture shown maximum enzyme activity at 30 °C and 4.5 pH. The maximum activity for enzyme lignin peroxidase and manganese peroxidase was analysed as 541 and 88.5 IU.L⁻¹ respectively at 8th day (Wang et al., 2008). Junnarkar et al. (2016), studied the production of lignin peroxidase from *P. chrysosporium*. The maximum enzyme activity was calculated as 33.3 U.mg⁻¹ on 6th day at the optimum condition (30 °C and pH 3.5). Another study performed for the optimization of culture conditions for the production of lignin peroxidase and manganese peroxidase enzymes from *P. chrysosporium* by Couto et al. (2006). According to their analysis, the optimum condition for the maximum production of lignin peroxidase was 34 °C temperature and 4.2 pH, while for manganese peroxidase was 32 °C temperature and 4.5 pH. Jayasinghe et al. (2008), studied the optimization of growth condition for the biomass production of *G. lucidum*. According to their analysis, *G. lucidum* was obtained their maximum biomass at 30 °C and pH 5.0. Another study was performed by Nasreen et al. (2005), for the analysis of biomass production of *G. lucidum*

on different culture conditions. They analysed, the highest biomass production at 25 °C and pH 5.0 in PDB growth medium as compared to kirk medium and kirk modified medium. Hariharan and Nambisan (2013), optimized the growth condition for the production of extracellular enzyme laccase, lignin peroxidase and manganese peroxidase activity from fungus *G. lucidum*. In their study, they observed maximum enzyme activity at 8th day of inoculation and the enzyme activity for laccase, lignin peroxidase and manganese peroxidase was calculated as 498, 895, 2998 IU.mL⁻¹ respectively at 27 °C and pH 5.0.

Another study performed by Agrawal et al. (2017), for the production and investigation of extracellular enzyme activity from WRFs *G. lucidum*. In this strain, the enzyme activity for laccase, lignin peroxidase and manganese peroxidase was calculated as 1154, 1175 and 15035 IU.L⁻¹ respectively. Snajdr and Baldrian (2007), determined the conditions for the production of extracellular enzyme activity of *T. versicolor* fungus. The maximum activity of enzyme laccase and manganese peroxidase was calculated as 370 and 8.7 IU.L⁻¹ at 35 °C. Another study was performed by Qin et al. (2017), for the estimation of laccase activity from *T. versicolor* growing culture. They grow fungus culture at 28 °C, 6.0 pH and the maximum laccase activity was observed as 1071 IU.L⁻¹ on 9th day. These reports indicates that, the enzyme activity shown by the previous studies is higher as compared to the present study, but the culture conditions were different in each finding. The enzyme activity and biomass estimation of *G. lucidum*, *T. versicolor* and *P. chrysosporium* was also estimated by the several researchers during the degradation of different pharmaceutical compounds.

A study investigated the laccase enzyme activity of *T. versicolor* during treatment of two antineoplastic compounds bleomycin and vincristine. The laccase activity of normal fungal culture without antineoplastic compounds was observed as 25 IU.L⁻¹, whereas the activity in case of bleomycin and vincristine treatment culture was reduced to 14 and 20 IU.L⁻¹ respectively. In their study, they observed the laccase activity of *T. versicolor* was influenced

by bleomycin and vincristine (Jureczko et al., 2021). In another study, Ferrando-Climent et al. (2015), grown *T. versicolor* at 25 °C and 4.5 pH for the treatment of antineoplastic compounds azathioprine, cyclophosphamide, etoposide, tamoxifen and paclitaxel. In their study, they estimated the laccase enzyme activity of *T. versicolor* and the maximum laccase activity was calculated as 350 U.L⁻¹ on 9th day. Vasiliadou et al. (2016), performed a study for the removal of pharmaceutical compounds using *T. versicolor* and *G. lucidum*. In their study, they monitored the laccase, manganese peroxidase and lignin peroxidase enzyme activity of both fungal cultures. The selected WRFs were grown at 25 °C, 4.5 pH and 150 rpm. The reported enzyme activity of laccase, manganese peroxidase and lignin peroxidase in *T. versicolor* was 250, 20 and 50 IU.L⁻¹ respectively, while in *G. lucidum* it was 20, 40 and 10 IU.L⁻¹ respectively.

4.5. Biodegradation of cyclophosphamide, etoposide and paclitaxel

In biodegradation study, the removal efficiency of *P. chrysosporium*, *T. versicolor* and *G. lucidum* fungi for the degradation of cyclophosphamide, etoposide and paclitaxel were estimated. The treatment process involved both degradation and biosorption for the removal of these antineoplastic compounds. The removal efficiency of etoposide was higher as compared to cyclophosphamide and paclitaxel in each case of fungi. Degradation analysis was performed on 3, 6, 9, 12 and 15 days of fungi inoculation (Table 4.6).

In case of *G. lucidum*, the degradation rate of cyclophosphamide was 24.72 % on 3rd day and it was increased to 71.50 % on 6th day. After 6th day, the degradation of cyclophosphamide was very less *i.e.* 0.07 % and total degradation achieved was 71.57 % after 15 days of experiment (Fig. 4.15). The biosorption removal percentage of cyclophosphamide with *G. lucidum* in heat killed control was about 4 %, so the total removal percentage achieved for cyclophosphamide was 75.56 % (Table 4.6). This rate of degradation was significantly different to a study performed by Castellet-Rovira et al. (2018), where *G. lucidum* shows the degradation of

cyclophosphamide less than 40 %, although laccase activity was also present in sample *i.e.* 100 U.L⁻¹. The total removal percentage of etoposide by *G. lucidum* was >99 %. After 3 days, the degradation of etoposide was 58.27 % and it was increased to 98.49 % at 6th day of treatment process (Fig. 4.15). Further, it was slightly increased to 98.94 % at 9th day. The biosorption rate of etoposide with *G. lucidum* was 0.75 %, that was quite low as compared to cyclophosphamide (Table 4.6). The total removal rate of paclitaxel by *G. lucidum* was about 21 %. Here, the biodegradation percentage for paclitaxel was achieved 8.23 % on 3rd day of immobilization and it was increased to 19.54 % on 6th day. After 6th day the degradation rate was slightly increased *i.e.* 0.63 % and further no more degradation was achieved for paclitaxel (Fig. 4.15). The sorption removal percentage of paclitaxel with *G. lucidum* was about 1.67 %, while maximum degradation percentage was 20.17 % (Table 4.6). Here, these results shows that, the *G. lucidum* achieved their maximum degradation for cyclophosphamide, etoposide and paclitaxel at 6th day but in etoposide it was quite high as compared to cyclophosphamide and paclitaxel.

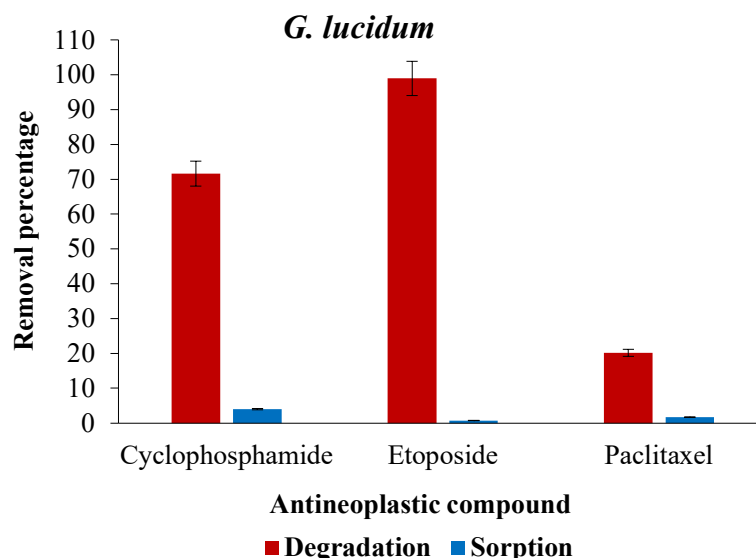


Fig 4.15. Total removal efficiency of *G. lucidum* for cyclophosphamide, etoposide and paclitaxel

The removal of cyclophosphamide, etoposide and paclitaxel by *T. versicolor* was highly different in term of degradation and biosorption percentage. The total removal rate of cyclophosphamide was negligible *i.e.* 1.47 % along with biosorption rate. After 15 days of experiment, the degradation rate was 1.04 % and biosorption rate was 0.43 %. The degradation rate of etoposide was quite high as compared to cyclophosphamide. The total removal rate of etoposide was about 97 %, while maximum biodegradation rate achieved was 79.93 % (Fig. 4.16). After 3 days, the degradation of etoposide with *T. versicolor* was observed and it was about 31.47 %, further the rate was increased up to 79.82 % on 6th day. After 6 days very less concentration of etoposide was reduced *i.e.* 0.11 % and it was almost negligible. Further, no more removal was observed in the collected sample were analysed on LC-MS. These results indicates that, the maximum degradation for etoposide was achieved by *T. versicolor* on 6th day. The biosorption rate of etoposide with *T. versicolor* was also very high. It was about 18.02 % indicated in the LC-MS data of collected samples from biosorption control flask (Table 4.6). The paclitaxel degradation was start after 3 days and at 6th day the degradation rate of paclitaxel with *T. versicolor* was about 3.12 % and it was increased to 5.91 % and 6.07 % on 9th and 15th day respectively (Fig. 4.16). After 9 days, very minor concentration of paclitaxel was degraded. The biosorption rate of paclitaxel with *T. versicolor* was 2.36 %. So, the total removal rate was about 8.43 % after 15 days of treatment process (Fig. 4.16). The biodegradation of cyclophosphamide and etoposide was studied by Ferrando-Climent et al. (2015). According to their study, the removal of cyclophosphamide was about 1 % and they reported no removal of cyclophosphamide by *T. versicolor*, even after laccase activity was showing in sample, while the etoposide was totally removed from the sample. Another study was also conducted for the removal of cyclophosphamide by Castellet-Rovira et al. (2018) and they investigate that the cyclophosphamide was removing but the removal rate was below 40 % of their initial concentration. Ferrando-Climent et al. (2015) also performed a study for removal of paclitaxel

from hospital wastewater by the help of *T. versicolor* but they were not founded paclitaxel presence in the sample and the degradation study of paclitaxel was not confirmed.

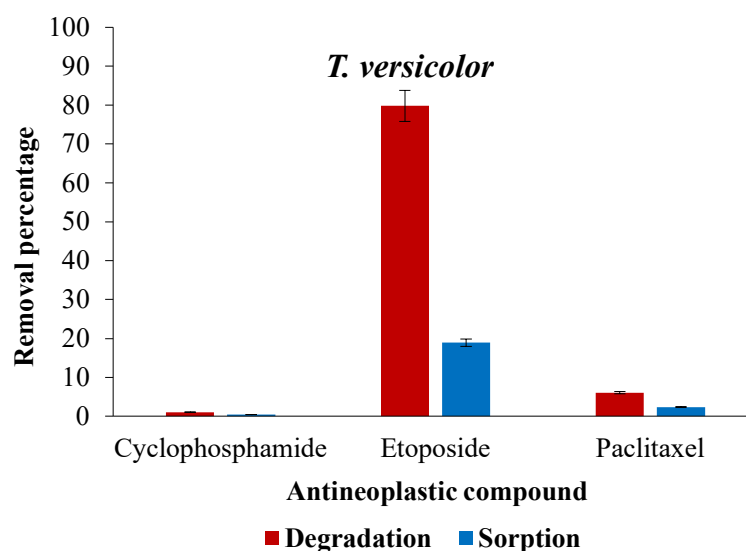


Fig. 4.16. Total removal efficiency of *T. versicolor* for cyclophosphamide, etoposide and paclitaxel

In case of *P. chrysosporium*, the degradation of cyclophosphamide was also negligible. No biodegradation of cyclophosphamide was achieved during biodegradation with *P. chrysosporium* after the 15 days of experiment. However, some concentration of cyclophosphamide from the samples was reduced by the biosorption process. The biosorption rate of cyclophosphamide by *P. chrysosporium* was about 23.77 % (Fig. 4.17). The degradation of etoposide was quite different, it was analysed about 32.64 % on 6th day and it was increased to 44.71 %, 69.57% and 76.85 % on 9, 12 and 15 days respectively. The maximum degradation rate was 76.85 % achieved after 15 days of degradation experiment, while total removal percentage was about 98 % (Table 4.6). The biosorption rate of etoposide in *P. chrysosporium* was about 21.25 %, it was high as like cyclophosphamide biosorption rate (Fig. 4.17). This fungus also shown degradation for paclitaxel, but it was very low. The degradation of paclitaxel with *P. chrysosporium* was quite different in comparison to other two compounds. Here, no

degradation was analysed on 3rd day, while after 3rd day the degradation was start and it was increased to 2.63 % on 6th day, 4.37 % on 9th day and 4.91 % on 15th day respectively. The total biodegradation percentage of paclitaxel with this fungus was 4.91 % and the biosorption percentage was 1.42 %, so the total removal percentage of paclitaxel was 6.33 % (Fig. 4.17). These results indicates that, *P. chrysosporium* have ability only for the removal of etoposide, while degradation of some percentage of paclitaxel was also achieved but their rate was very low. In cyclophosphamide, the removal was only shown through biosorption process, there was no biodegradation, but it shows the highest biosorption removal efficiency among all the selected fungi.

Table 4.6. Biodegradation of selected antineoplastic compounds with *G. lucidum*, *T. versicolor* and *P. chrysosporium*

Antineoplastic compounds		Total degradation rate (%)				
(Initial concentration 300 µg.L ⁻¹)		(Average of triplicate)				
		3 days	6 days	9 days	12 days	15 days
<i>Ganoderma lucidum</i>	Cyclophosphamide	24.72	71.50	71.57	71.57	71.57
	Etoposide	58.27	98.49	98.94	98.94	98.94
	Paclitaxel	8.23	19.54	20.17	20.17	20.17
<i>Trametes versicolor</i>	Cyclophosphamide	0	0	1.04	1.04	1.04
	Etoposide	31.47	79.82	79.93	79.93	79.93
	Paclitaxel	0	3.12	5.91	6.07	6.07
<i>Phanerochaete</i>	Cyclophosphamide	0	0	0	0	0
<i>Chrysosporium</i>	Etoposide	18.79	32.64	44.71	69	76.85
	Paclitaxel	0	2.63	4.37	4.91	4.91

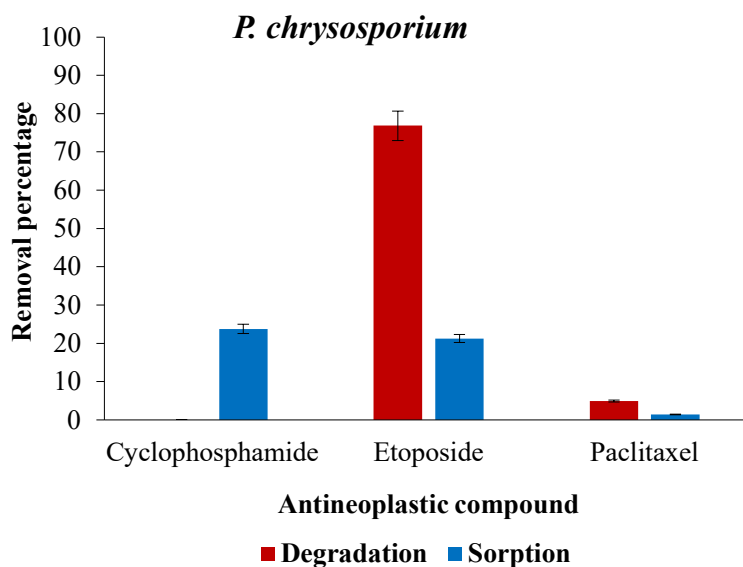


Fig. 4.17. Total removal efficiency of *P. chrysosporium* for cyclophosphamide, etoposide and paclitaxel

Table 4.7. Total efficiency of *G. lucidum*, *T. versicolor* and *P. chrysosporium* for the removal of cyclophosphamide and etoposide

WRFs	Antineoplastic compound	Total removal rate (In percentage)	Biosorption rate (In percentage)	Biodegradation rate (In percentage)
<i>G. lucidum</i>	Cyclophosphamide	75.56 %	3.99 %	71.57 %
	Etoposide	99.69 %	0.75 %	98.94 %
	Paclitaxel	21.84 %	1.67 %	20.17 %
<i>T. versicolor</i>	Cyclophosphamide	1.47 %	0.43 %	1.04 %
	Etoposide	98.75 %	18.93 %	79.82 %
	Paclitaxel	8.43 %	2.36 %	6.07 %
<i>P. chrysosporium</i>	Cyclophosphamide	23.77 %	23.77 %	No degradation
	Etoposide	98.1 %	21.25	76.85 %
	Paclitaxel	6.33 %	1.42 %	4.91 %

The present result indicates that, among these three WRFs, *G. lucidum* have highest potential for the removal of cyclophosphamide, etoposide and paclitaxel, while *T. versicolor* and *P. chrysosporium* shows the removal efficiency only for etoposide and very less for paclitaxel but not for cyclophosphamide (Table 4.6). The removal of paclitaxel was shown by all the fungi but the removal rate was very low and in case of *T. versicolor* and *P. chrysosporium*, their removal was less than 10 %, it was quite insignificant removal.

Apart from cyclophosphamide, etoposide and paclitaxel, several other antineoplastic compounds also have been removed from these WRFs by the various researchers (Table 2.5). *G. lucidum* and *T. versicolor* were used for the elimination of IF and iopromide also. The *T. versicolor* removed iopromide about 47 % of their initial concentration ($174.4 \mu\text{g.L}^{-1}$) and out of which about 85 % removal was observed through degradation and 15 % was through biosorption. The removal of IF was only about 25 % from their initial concentration ($39.1 \mu\text{g.L}^{-1}$) and from which only less than 10 % was removed through degradation. *G. lucidum* removed IF and iopromide less than 40 % of their initial concentration and in both cases, the biodegradation was less than 60 % of their total removal (Castellet-Rovira et al., 2018). Another study performed by Ferrando-Climent et al. (2015) by the use of *T. versicolor* for removal of tamoxifen, azathioprine and IF. In their study, the removal of tamoxifen and IF was performed in both hospital wastewater and synthetic sample, where removal of azathioprine was only performed in hospital wastewater sample. The initial concentration in hospital wastewater sample of tamoxifen, IF and azathioprine were $0.0445 \mu\text{g.L}^{-1}$, $0.077 \mu\text{g.L}^{-1}$ and $0.055 \mu\text{g.L}^{-1}$ respectively, while in synthetic sample initial concentration of tamoxifen and IF were $0.0003 \text{ mg.ml}^{-1}$ and 0.01 mg.ml^{-1} respectively. The tamoxifen was removed about 48 % in hospital wastewater sample, while in synthetic sample it was removed about 92 % after 1 hour and 99 % after 9 days of experiment, in which about 82 % after 24 hours and 94 % after 9 day was removed through biosorption process. The removal of IF was

only achieved in real hospital wastewater sample and removal rate was 61 %, whereas no removal was observed for IF in synthetic sample. The azathioprine was totally removed (100 %) from real wastewater sample.

Jureczko et al. (2021) studied biodegradation of vincristine and bleomycin by *T. versicolor*, *F. fomentarius*, *H. fasciculare*, *P. nidulans* and *P. ostreatus*. They used 10 mg.L⁻¹ as initial concentration of vincristine and bleomycin. The total removal efficiency of *T. versicolor*, *F. fomenatrius* and *H. fasciculare* for vincristine was achieved 94 % after 4 days and 97 % after 14 days, while in *P. oestrus* the efficiency was 52.5 %. However, no removal of vincristine was observed in *P. niulans* mediated treatment. The biosorption rate of vincristine with *T. versicolor*, *F. fomentarius* and *H. fasciculare* was analysed as 14 % to 29 % of total removal (97 %) of their concentration. The removal of bleomycin was observed only with *T. versicolor* and *H. fasciculare*. The removal rate of bleomycin analysed with *T. versicolor* was 36 % after 9 days and 64 % after 14 days, while in *H. fasciculare* was 58.5 % observed after 14 days of experiment. However, no removal of bleomycin was observed with *F. fomenatrius*, *P. oestrus* and *P. niulans*. The biosorption rate of bleomycin with *T. versicolor* was about negligible, while in *H. fasciculare* it was about 29 % observed. *P. chrysosporium* was used to remove diclofenac (Zhang and Geißen, 2010), tetracycline and oxytetracycline (Wen et al., 2010). Lucas et al. (2018) performed a study on elimination of four antibiotics through biosorption process of three different WRFs. The WRFs strain used for removal of diclofenac, carbamazepine, venlafaxine and iopromide were *T. versicolor*, *G. lucidum* and *I. lacteus*. They found highest biosorption rate of diclofenac in case of all fungi i.e. 42 %, 34 % and 26 % observed in case of *T. versicolor*, *G. lucidum* and *I. lacteus* respectively, while total biodegradation was 54 %, 64 % and 71 % observed in case of *T. versicolor*, *G. lucidum* and *I. lacteus* respectively. The biosorption rate was also shown for rest three antibiotics, but it was very less as compared to diclofenac. For carbamazepine, the biosorption was 8 %, 13 % and

5 %, while biodegradation was 50 %, 23 % and 57 % observed in case of *T. versicolor*, *G. lucidum* and *I. lacteus* respectively. In iopromide, the biosorption rate was 8 %, 18 % and 5 %, while biodegradation was 39 %, 13 % and 22 % observed in case of *T. versicolor*, *G. lucidum* and *I. lacteus* respectively. The biosorption rate of venlafaxine was 6 %, 11 % and 6 %, and biodegradation was 49 %, 19 % and 9 % observed in case of *T. versicolor*, *G. lucidum* and *I. lacteus* respectively. Another study was also investigated by Jureczko et al. (2021) for analysis of biosorption efficiency of two antineoplastic compounds vincristine and bleomycin on five WRFs strains. The WRF strain used for this investigation were *F. fomentarius*, *H. fasciculare*, *P. nidulans*, *P. ostreatus*, and *T. versicolor*. They used alive or dead biomass for the analysis of biosorption efficiency and the found variation in efficiency for both dead and alive biomass in case of each fungus. In case of alive biomass, the biosorption efficiency of bleomycin was 23 %, 15 %, 11 %, 6 % and 0 % with *T. versicolor*, *P. ostreatus*, *P. nidulans*, *H. fasciculare* and *F. fomentarius* respectively, while vincristine shown 14 %, 16 %, 20 %, 7 % and 7 % efficiency with *T. versicolor*, *P. ostreatus*, *P. nidulans*, *H. fasciculare* and *F. fomentarius* respectively. In case of dead biomass, the biosorption efficiency of bleomycin was 35 %, 23 %, 0 %, 29 % and 38 % with *T. versicolor*, *P. ostreatus*, *P. nidulans*, *H. fasciculare* and *F. fomentarius* respectively, while vincristine shown 17 %, 14 %, 8.6 %, 16 % and 13 % efficiency with *T. versicolor*, *P. ostreatus*, *P. nidulans*, *H. fasciculare* and *F. fomentarius* respectively. This study reports the highest biosorption efficiency of bleomycin in alive biomass with *T. versicolor*, while in dead biomass was shown with *F. fomentarius*. The highest biosorption efficiency of vincristine was shown with *P. nidulans*, while in dead biomass was shown with *T. versicolor*.

4.6. Negative effect of antineoplastic compounds on fungi

The dry weight biomass of control and experimental flask was calculated to analyse the negative effect of cyclophosphamide, etoposide and paclitaxel on growth of fungi. There was

no effect of these compounds observed on all three fungi. They were produced the same amount of biomass size with each antineoplastic compounds that was produced during the optimization of biomass production before the treatment process as shown in Fig. 4.9, 4.11 and 4.13. A study performed by Jureczko et al. (2021), for the biomass estimation of five WRFs (*F. fomentarius*, *H. fasciculare*, *P. nidulans*, *P. ostreatus* and *T. versicolor*) during treatment of antineoplastic compounds bleomycin and vincristine. In their study they investigated that, the effect of bleomycin and vincristine was different in each case of fungal culture. In some fungi, the biomass was increased as compared to the control biomass growth, while in some case the biomass or growth was inhibited as compared to the control biomass growth. The WRFs *P. nidulans* was not able to remove bleomycin and vincristine but their biomass was increased to the control. However, vincristine was not exhibiting negative effect on the growth of all fungi. So, they concluded that, each pharmaceutical compound has a particular influence on the growth of each fungal growth and their degradation ability does not depend on the biomass inhibition.

4.7. Estimation of pH and reducing sugar

During the biodegradation study, simultaneously the pH value and glucose concentration were estimated. Here, we have analysed the pH value and estimated the glucose concentration of experimental flask on every day of sampling. On the basis of the variation in these parameters, we provide a prediction towards relation of pH and glucose concentration on rate of degradation.

4.7.1. pH

Initially, the pH of growth medium was adjusted according to the best growing condition of fungi for the degradation of pharmaceutical. The pH of *G. lucidum* was adjusted 5.0, while for *T. versicolor* and *P. chrysosporium* it was 4.5 adjusted at initial day of experiment. The pH of

all the experimental and control flask were checked or analysed at every movement of the sampling *i.e.* 0, 3, 6, 9, 12 and 15 days. The pH value of *G. lucidum* was observed 5.0, 3.72 ± 0.08 , 4.39 ± 0.09 , 6.74 ± 0.1 , 6.92 ± 0.06 and 6.30 ± 0.13 at 0, 3, 6, 9, 12 and 15 days respectively (Fig. 4.18 (a)). The pH value of *T. versicolor* was observed 4.5, 4.46 ± 0.07 , 5.02 ± 0.1 , 4.52 ± 0.14 , 5.87 ± 0.08 and 6.5 ± 0.1 at 0, 3, 6, 9, 12 and 15 days respectively (Fig. 4.18 (b)), whereas the pH value *P. chrysosporium* was observed 4.5, 3.76 ± 0.8 , 3.87 ± 0.12 , 6.04 ± 0.1 , 4.98 ± 0.12 and 5.86 ± 0.14 on 0, 3, 6, 9, 12 and 15 days respectively during the degradation experiment (Fig. 4.18 (c)).

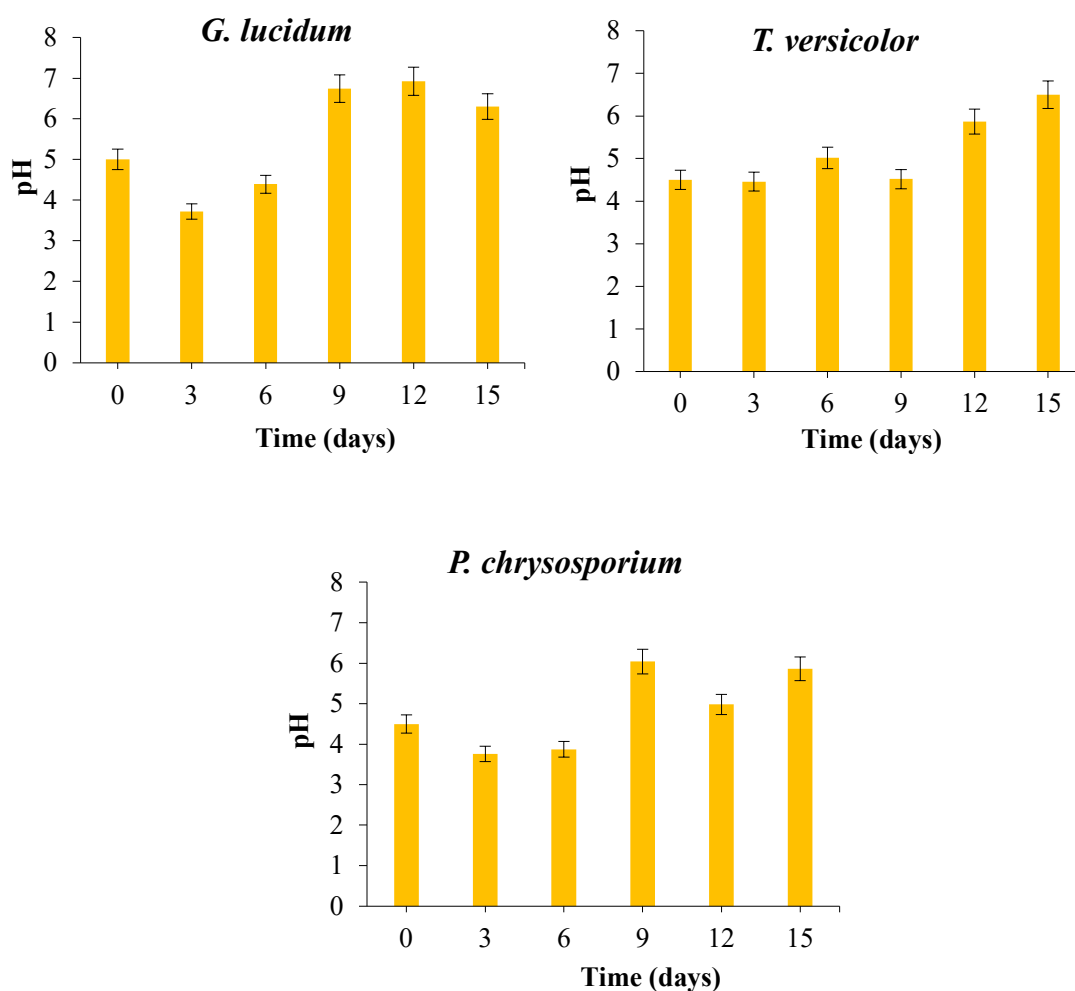
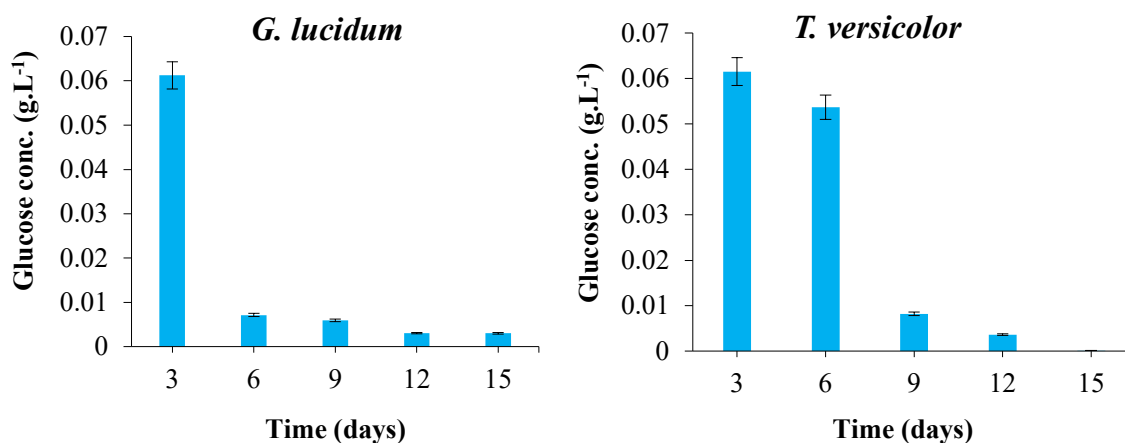


Fig. 4.18. pH analysis during degradation of antineoplastic compounds using (a) *G. lucidum* (b) *T. versicolor* (c) *P. chrysosporium*

4.7.2. Glucose estimation

During degradation, in growth medium the glucose was used as carbon source for fungi. The initial glucose concentration used at zero day was 10 g.L⁻¹ in each flask. On every day of sampling along with other analysis, glucose concentration was also estimated in term of reducing sugar. In case of *G. lucidum*, it was calculated as 0.06121 ±0.0084 g.L⁻¹, 0.00714 ±0.0002 g.L⁻¹, 0.005962 ±0.00028 g.L⁻¹, 0.00306 ±0.00003 g.L⁻¹ and 0.00302 ±0.000022 g.L⁻¹ on 3, 6, 9, 12 and 15 days respectively (Fig. 4.19 (a)). In *T. versicolor*, the glucose concentration was calculated as 0.0615 ±0.00274 g.L⁻¹, 0.5367 ±0.00098 g.L⁻¹, 0.0822 ±0.00038 g.L⁻¹, 0.00362 ±0.00052 g.L⁻¹ and 0.0012 ±0.00007 g.L⁻¹ on 3, 6, 9, 12 and 15 days respectively (Fig. 4.19 (b)). The glucose concentration in *P. chrysosporium* was calculated as 0.00551 ±0.0004 g.L⁻¹, 0.00393 ±0.00036 g.L⁻¹, 0.0022 ±0.00019 g.L⁻¹, 0.00016 ±0.00002 g.L⁻¹ and 0.00011 ±0.00001 g.L⁻¹ on 3, 6, 9, 12 and 15 days respectively (Fig. 4.19 (c)). These results indicate, the maximum concentration of glucose was utilized by the fungi at their initial stage of degradation. Among these three fungi, the highest glucose concentration was utilized by *P. chrysosporium* from start to end of the experiment.



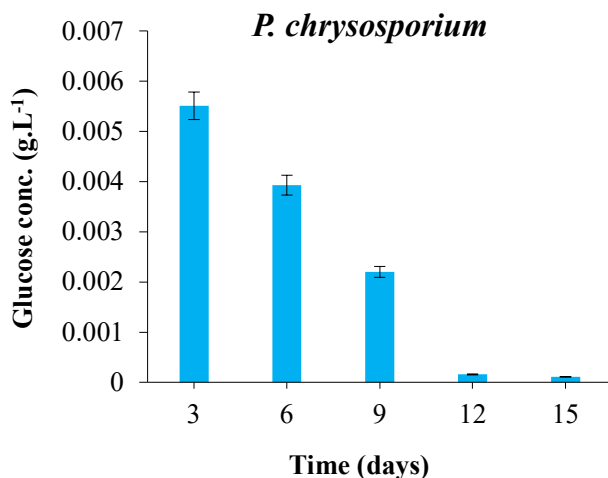


Fig. 4.19. Pattern of glucose utilization by (a) *G. lucidum* (b) *T. versicolor* and (c) *P. chrysosporium* during degradation of antineoplastic compounds

4.7.3. Time-course degradation relationship with pH and glucose

In *G. lucidum* treatment flasks, the rate of degradation was achieved for all three compounds but the percentage was different. The highest degradation of etoposide, cyclophosphamide and paclitaxel achieved was 98 %, 71 % and 20 % respectively. Although, the variation in pH value of *G. lucidum* in each antineoplastic case was similar. Initially, the pH value was decreased from 5.0 to 3.72 ± 0.08 and further, it was continuously increased till 15 days. The *G. lucidum* achieve their maximum efficiency for the degradation of cyclophosphamide, etoposide and paclitaxel at pH 4.39 ± 0.09 . However, the maximum utilization of glucose concentration was achieved only at the end of 3rd day and after that only very less amount was present (Fig. 4.20). So, the effect of glucose concentration on degradation rate is not confirm during this treatment process.

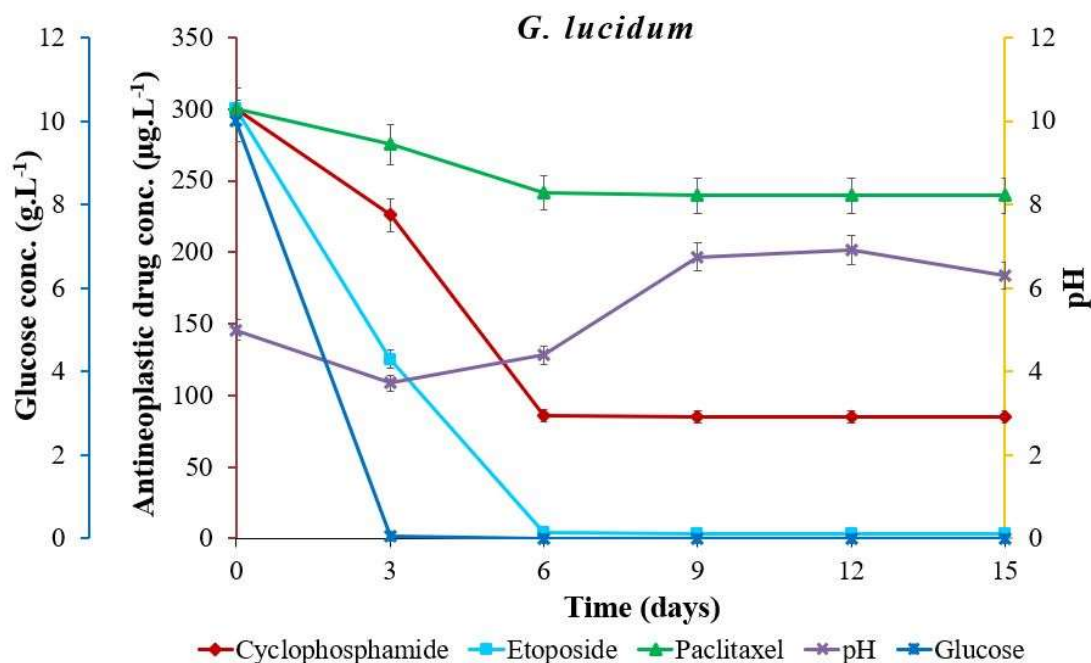


Fig. 4.20. Time course of cyclophosphamide, etoposide, paclitaxel degradation along with pH and glucose by *G. lucidum*

In *T. versicolor* the highest activity for the degradation of cyclophosphamide, etoposide and paclitaxel was highly different to *G. lucidum*. In this fungus, the significant degradation was achieved only for etoposide. However, very minute degradation of paclitaxel was also achieved but no biodegradation was existing for cyclophosphamide in this study. The initial pH of *T. versicolor* mediated treatment flask was 4.5 and it was increased to 5.02 ± 0.1 on 6th day. The maximum degradation rate of etoposide was also observed at 6th day. These results indicate, the maximum activity of *T. versicolor* was shown at pH 5.02 ± 0.1 for degradation of etoposide. The removal of paclitaxel was shown after 15 days *i.e.* 6.07 % at pH 5.87 ± 0.08 but it was not much significant. The initial glucose this treatment was also high. Here, the maximum amount of glucose was utilized at end of 3 days, but after 3 days the glucose utilization reduced and become very less (Fig. 4.21). In this treatment also, the effect of glucose concentration on rate of degradation is not confirmed.

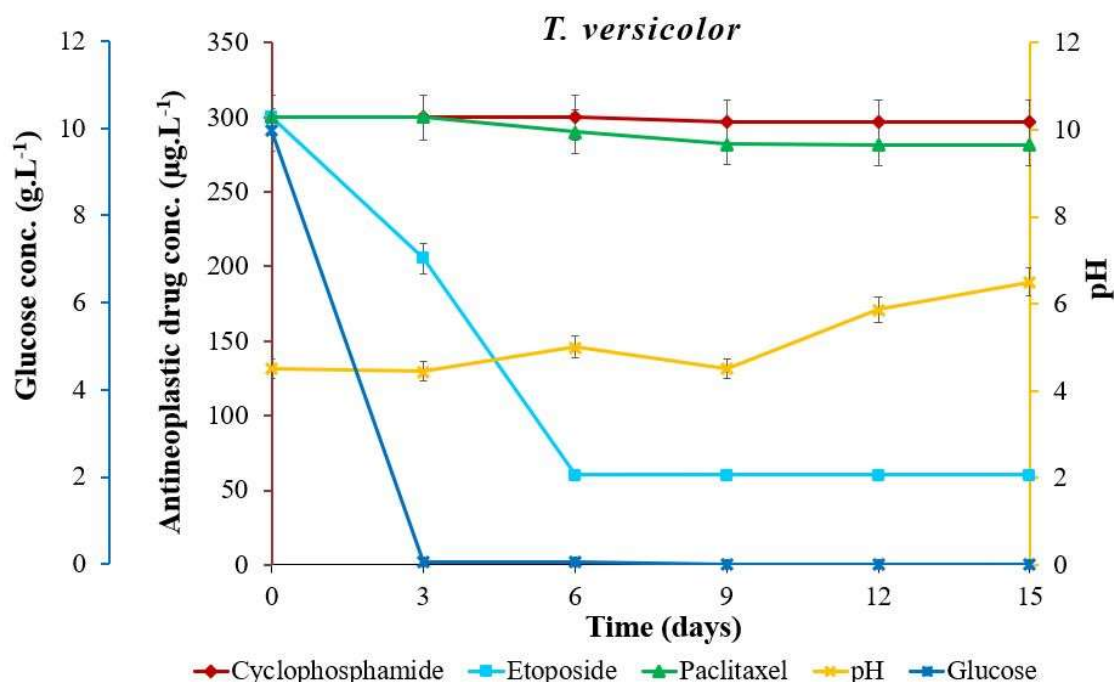


Fig. 4.21. Time course of cyclophosphamide, etoposide, paclitaxel degradation along with pH and glucose by *T. versicolor*

In *P. chrysosporium* the degradation was only shown for etoposide. Some activity was also shown for paclitaxel *i.e.* 4.91 % after 15 days but it not significant. Here, the initial value of growing treatment flask was 4.5 and it was reduced to 3.76 ± 0.8 and 3.87 ± 0.12 on 3rd and 6th day but after this the pH value was increased. The highest degradation activity of etoposide was shown at 15th day and the pH of experimental flask at 15th day was 5.86 ± 0.14 . These results indicates that the maximum degradation activity of *P. chrysosporium* was achieved at pH 5.86. Whereas the glucose utilization in *P. chrysosporium* was maximum at 3rd day and after this very minute concentration of glucose was present in the culture (Fig. 4.22). So, same as like other two fungi, the relation of glucose on degradation activity is not confirmed during this treatment process. Among all three WRFs, the maximum and fast glucose was utilized by the *P. chrysosporium*.

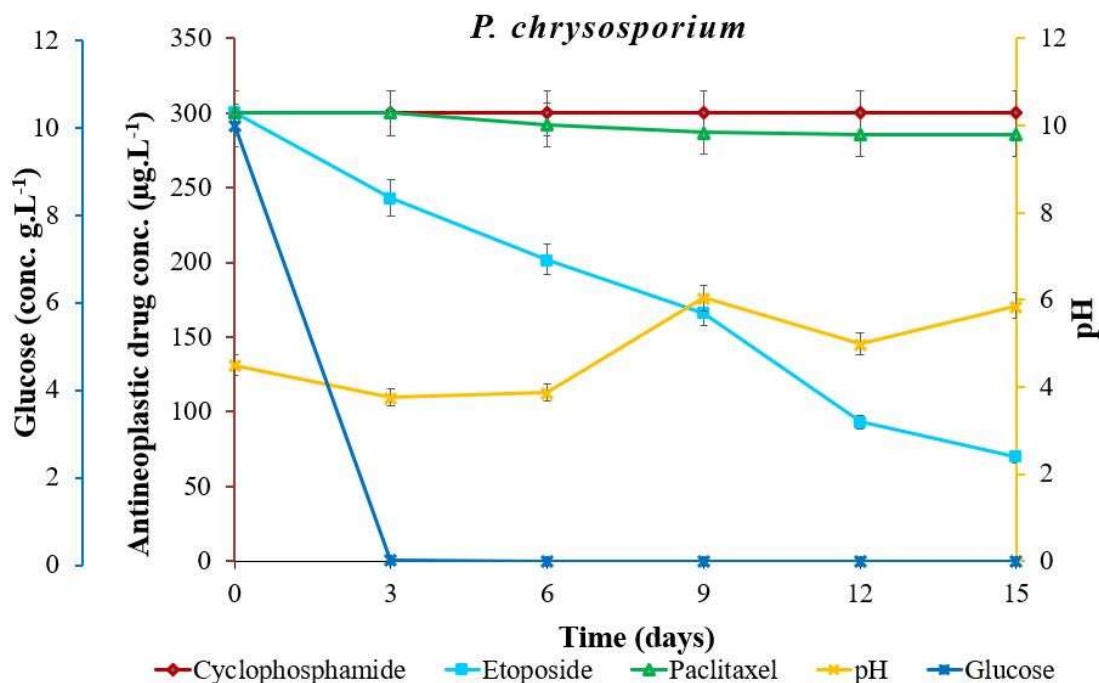


Fig. 4.22. Time course of cyclophosphamide, etoposide, paclitaxel degradation along with pH and glucose by *P. chrysosporium*

According to these findings, the maximum removal efficiency of *G. lucidum* for cyclophosphamide, etoposide and paclitaxel achieved at pH 4.39 ± 0.09 . In *T. versicolor* it was demonstrated at pH 5.02 ± 0.1 for etoposide. The initial pH of the *P. chrysosporium* treatment flask was 4.5, which was reduced to 3.76 ± 0.8 and 3.87 ± 0.12 on the third and sixth days, respectively. The highest etoposide removal efficiency was observed on the 15th day, and the pH of the experimental flask on the 15th day was 5.86 ± 0.14 . So, the maximum removal efficiency of *P. chrysosporium* was achieved at a pH of 5.86. Only at the end of the third day the maximum utilization of glucose was observed, and after that, only a very small amount was present in the sample. However, because the degradation rate was highest in *G. lucidum*, *T. versicolor* on sixth day and in *P. chrysosporium* continuous degradation till 15th day, the current study cannot co-relate the degradation efficiency with the glucose concentration utilization. Ferrando-Climent et al. (2015), also performed investigation on time-course

biodegradation of antineoplastic compounds by *T. versicolor* with simultaneous glucose consumption analysis. Similar to the present study, glucose consumption was utilized in the initial days or second day of the degradation experiment. Another study conducted by Castellet-Rovira et al. (2018), they analysed the glucose concentration during the treatment of antineoplastic compounds with six WRFs (*T. versicolor*, *I. lacteus*, *S. rugosoannulata*, *G. lucidum*, *A. erebia*, *G. luteofolius*). In their study, they measured glucose concentration only at the start and end day of experiment. In the end of experiment, they reported no glucose concentration was present in the sample of each fungal culture and the pH remained stable *i.e.* 4.5.

4.8. Degradation kinetics and kinetics model study

In present work during the degradation, the biodegradation kinetics of antineoplastic compounds by these three WRFs was also evaluated. There is degradation rate depends on the concentration of antineoplastic compounds, so herein only pseudo-first-order reaction was applied for kinetics study. Zero-order and Second-order reaction were not eligible for this degradation study because in these reactions the rate of reaction is not depends or directly proportional to the substrate or antineoplastic compound concentration.

In *G. Lucidum* treatment, all three antineoplastic compounds had followed the pseudo-first-order kinetics with r^2 values 0.8774, 0.902 and 0.8774 cyclophosphamide, etoposide and paclitaxel respectively. Among these three compounds, the maximum degradation was achieved for etoposide on 3rd day and the degradation rate constant ' k ' value was 0.5453 day⁻¹, whereas the rate constant of cyclophosphamide and paclitaxel was 0.1564 day⁻¹ and 0.0285 day⁻¹ respectively. The theoretical half-time ($t_{1/2}$) of drug degradation value calculated for etoposide was 1.27113 day, while for cyclophosphamide it was 4.43189 days. The half-time of paclitaxel was highly different to etoposide and paclitaxel. Here, the value of paclitaxel was

24.31 days as it was degraded only 20.17 % of the initial concentration ($300 \mu\text{g.L}^{-1}$) till 15 days (Fig. 4.23).

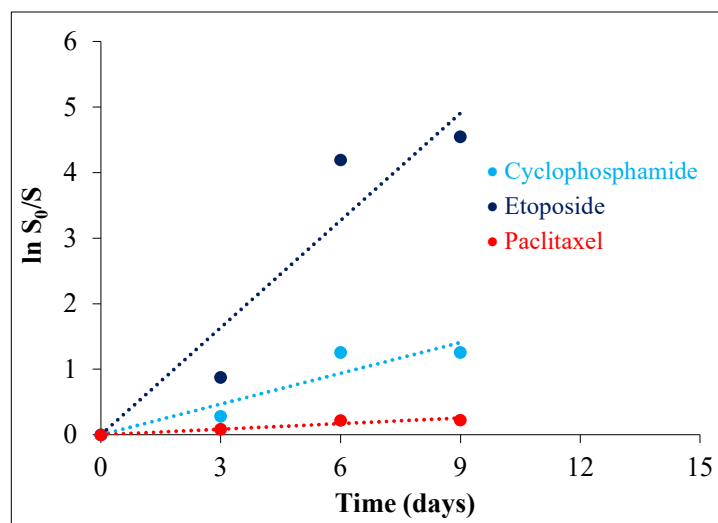


Fig. 4.23. Pseudo-first-order kinetics model for degradation of cyclophosphamide, etoposide and paclitaxel with *G. lucidum*

In *T. versicolor* treatment experiment, the degradation of etoposide was quite comparable with *G. lucidum*, while degradation of cyclophosphamide and paclitaxel was highly different. In this treatment, the etoposide was degraded up to 79.82 % at 6th day but for cyclophosphamide and paclitaxel the rate was 1.04 %, 6.07 % till 15 days. Etoposide and paclitaxel followed pseudo-first-order degradation path with r^2 values 0.8981 and 0.8911 respectively, but cyclophosphamide degradation path showed r^2 value only 0.7455 for the same kinetics. The degradation rate constant was maximum for etoposide *i.e.* 0.2386 day^{-1} , while for cyclophosphamide and paclitaxel the ' k ' value was 0.0008 day^{-1} and 0.0055 day^{-1} . These low kinetics constant of cyclophosphamide and paclitaxel was achieved because both the compounds were nearly unaffected by this WRF. The calculated half-time value of the initial concentration ($300 \mu\text{g.L}^{-1}$) for cyclophosphamide was 866.434 days (more than two years) and

lowest for etoposide *i.e.* 2.90506 days with this fungus. Half-time calculated for paclitaxel was 126.027 days (Fig. 4.24).

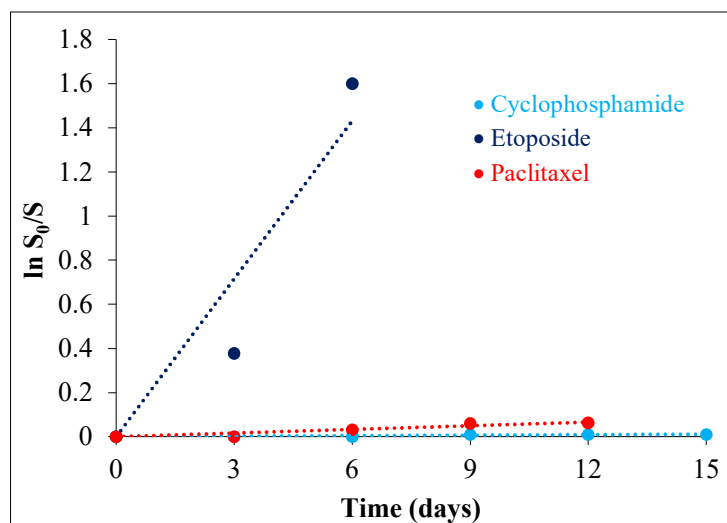


Fig. 4.24. Pseudo-first-order kinetics model for degradation of cyclophosphamide, etoposide and paclitaxel with *T. versicolor*

In *P. chrysosporium* degradation, the cyclophosphamide was not degraded till 15 days of experiment, but the degradation kinetics of etoposide investigated successfully with pseudo-first-order degradation kinetics. Here, 76.85 % degradation of etoposide was achieved with *P. chrysosporium* within 15 days of experiment. The r^2 value of etoposide was calculated as 0.9425 and degradation rate constant ' k ' achieved was 0.0896 day^{-1} with half time of degradation 7.73602 days, when initial drug concentration was $300 \mu\text{g.L}^{-1}$. Paclitaxel was degraded only 4.91 % after 15 days and the kinetics investigated suggested pseudo-first-order degradation path for this removal with r^2 value 0.9104. The degradation rate constant and half-time value for paclitaxel in this experiment were 0.0043 day^{-1} and 161.197 days respectively (Fig. 4.25).

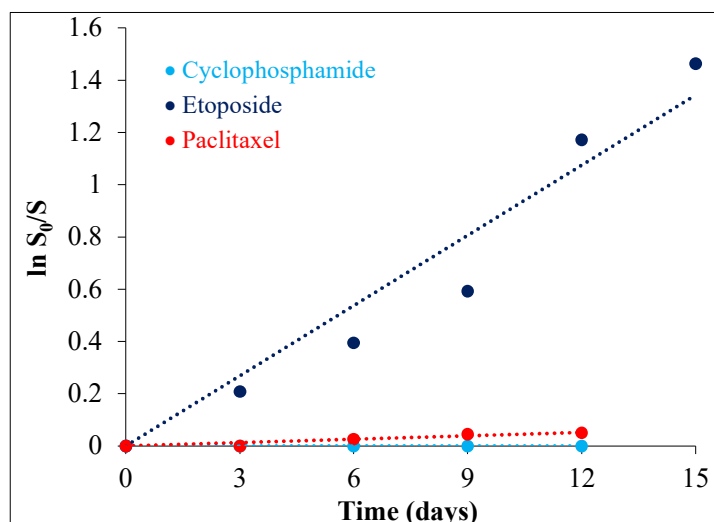


Fig. 4.25. Pseudo-first-order kinetics model for degradation of cyclophosphamide, etoposide and paclitaxel with *P. chrysosporium*

Among these three WRFs, the maximum degradation rate constant was calculated in treatment of etoposide with *G. lucidum* i.e. 0.5453 day^{-1} whereas, the lowest degradation rate constant was shown by *T. versicolor* for treatment of cyclophosphamide i.e. 0.0008 day^{-1} . The degradation kinetics of allethrin by fungus *Fusarium proliferatum* was studied by Bhatt et al. (2020). They investigated that the fungal strain was completely removed allethrin from the sample in 5 days. The fungal culture followed the pseudo-first-order degradation kinetics. The $t_{1/2}$ was calculated as 26.05 h with rate constant (k) value 0.0193 h^{-1} . Another study was performed by Bankole et al. (2020), on the investigation of biodegradation kinetics of three pharmaceutical compounds i.e. diclofenac, celecoxib and ibuprofen by using WRFs *G. lucidum* and *Laetiporus sulphureus*. They followed both pseudo-first-order kinetics model and pseudo-second-order kinetics model for the degradation kinetics analysis. In pseudo-first-order kinetics, the $t_{1/2}$ of diclofenac, celecoxib and ibuprofen were calculated as 0.99, 0.91 and 0.98 days respectively with ' k ' constant value 0.29, 6.72 and 4.2 day^{-1} respectively. A study investigated by Zhang et al. (2019), for the degradation of sulfadiazine degradation with *P. chrysosporium*. The degradation study followed the pseudo-order-reaction kinetics for the

analysis. They performed degradation at different pH range *i.e.* 3.7, 4.7, 5.7 and 6.7. The degradation '*k*' constant values on above pH for sulfadiazine were calculated as 0.04 d⁻¹, 0.43 d⁻¹, 0.46 d⁻¹ and 0.33 d⁻¹ respectively. These studies indicates that the degradation rate constant of a pharmaceutical compound different or vary in treatment with each fungus and only pseudo-first-order kinetics model is applicable for degradation kinetics analysis of pharmaceutical compounds with WRF.

4.9. Cyto-toxicity analysis of native and degraded products of antineoplastic compounds

4.9.1. Cyto-toxicity of native antineoplastic compounds

In this study, the MTT assay was performed by using Raw 264.7 cell line to evaluate the cytotoxicity of cyclophosphamide, etoposide and paclitaxel which are widely used for cancer treatment and showing presence in water bodies. The MTT assay was applied to detect (Lojk et al., 2020) cell viability and the percentage of cytotoxicity on Raw 264.7 cells with different doses of cyclophosphamide, etoposide and paclitaxel *i.e.* 10, 25, 50, 100, 150, 200 and 250 µg.ml⁻¹. After 48 hours of antineoplastic compound treatment, the viability of cells was determined and the results of cytotoxicity for above mentioned antineoplastic compounds described in Table 4.8. The cytotoxicity percentage of cyclophosphamide on Raw 264.7 cell at concentration 10, 25, 50, 100, 150, 200 and 250 µg.ml⁻¹ were 17.48 %, 26.17 %, 34.22 %, 41.96 %, 50.07 %, 61.69 % and 69.58 % respectively. In case of etoposide the percentage of cytotoxicity on Raw 264.7 cell at an exposure concentration 10, 25, 50, 100, 150, 200 and 250 µg.ml⁻¹ were 42.99 %, 52.22 %, 63.35 %, 74.86 %, 80.23 %, 87.29 % and 92.01 % respectively. While the cytotoxicity of paclitaxel on Raw 264.7 cell at concentration 10, 25, 50, 100, 150, 200 and 250 µg.ml⁻¹ were 31.07 %, 36.49 %, 44.08 %, 59.57 %, 75.25 %, 84.9 % and 88.85 % respectively. At 48 hours of antineoplastic compounds treatment indicated the consistent decrease in cell viability of Raw 264.7 cells with an increase in the concentration of

cyclophosphamide, etoposide and paclitaxel. These three antineoplastic compounds were significantly inhibiting proliferation in Raw 264.7 cell line by exhibiting cytotoxic effects. The IC_{50} value for cyclophosphamide was $145.44 \pm 2.67 \mu\text{g.ml}^{-1}$, etoposide was $15.40 \pm 5.92 \mu\text{g.ml}^{-1}$ and paclitaxel was $69.76 \pm 4.23 \mu\text{g.ml}^{-1}$ on 10, 25, 50, 100, 150, 200 and 250 $\mu\text{g.ml}^{-1}$ concentrations (Table 4.8). IC_{50} of etoposide was the lowest as compared to cyclophosphamide and paclitaxel as indicated in Fig. 4.26 and Table 4.7. So, among these three antineoplastic compounds, etoposide indicating the highest cytotoxicity on Raw 264.7 cells because if the IC_{50} value of the compound is lower, the compounds will be more cytotoxic and it was determined by designing a dose-response curve. The cytotoxic response of different concentrations of these three antineoplastic compounds on Raw 264.7 cell line is shown in Fig. 4.26 (a-c). The inhibitory concentration value showing half-maximal effective concentration obtained from the relative potency of selected antineoplastic compounds and the dose-response curve.

In prior studies, AT and CT of cyclophosphamide, etoposide and paclitaxel were analysed on *V. fischeri*, *B. calyciflorus*, *C. dubia*, *D. rerio*, *L. minor*, *D. magna* and *T. platyurus* (Table 4.9). The toxicity was determined in the form of EC_{50}/LC_{50} . The values of EC_{50}/LC_{50} for cyclophosphamide on *V. fischeri* were $>100 \mu\text{g.ml}^{-1}$ (acute) and $1396 \mu\text{g.ml}^{-1}$ (chronic), *B. calyciflorus* were $1924 \mu\text{g.ml}^{-1}$ (acute) and $89.84 \mu\text{g.ml}^{-1}$ (chronic), *C. dubia* were $986.6 \mu\text{g.ml}^{-1}$ (acute) and $58.03 \mu\text{g.ml}^{-1}$ (chronic), *T. platyurus* was $1396 \mu\text{g.ml}^{-1}$ (acute) and *L. minor* was $>100 \mu\text{g.ml}^{-1}$ (chronic) (Lutterbeck et al., 2016; Białk-Bielińska et al., 2017; Russo et al., 2018). In etoposide, value of toxicity on *B. calyciflorus* were $>120 \mu\text{g.ml}^{-1}$ (acute) and $3.7 \mu\text{g.ml}^{-1}$ (chronic), *C. dubia* was $0.204 \mu\text{g.ml}^{-1}$ (chronic), *D. rerio* was $>100 \mu\text{g.ml}^{-1}$ (acute), *T. platyurus* was $74.85 \mu\text{g.ml}^{-1}$ (acute) and *D. magna* was $0.239 \mu\text{g.ml}^{-1}$ (chronic) (Kovács et al., 2016; Parrella et al., 2014a). In paclitaxel, toxicity was determined on *D. magna* was $>0.074 \mu\text{g.ml}^{-1}$ (acute) (CDER, 1996).

Table 4.8. Determination of cytotoxicity and inhibitory concentration of cyclophosphamide, etoposide and paclitaxel

Antineoplastic compound	Concentration dose ($\mu\text{g.ml}^{-1}$)	Cell cytotoxicity percentage	IC ₅₀ ($\mu\text{g.ml}^{-1}$)
Cyclophosphamide	10	17.48	145.44 $\pm$ 2.67
	25	26.17	
	50	34.22	
	100	41.96	
	150	50.07	
	200	62.69	
	250	69.58	
Paclitaxel	10	31.07	69.76 $\pm$ 4.23
	25	36.49	
	50	44.08	
	100	59.57	
	150	75.25	
	200	84.9	
	250	88.85	
Etoposide	10	42.99	15.40 $\pm$ 5.92
	25	52.22	
	50	63.35	
	100	74.86	
	150	80.23	
	200	87.29	
	250	92.01	

It shows that, the cytotoxic effect of cyclophosphamide and etoposide is lower as well as higher on Raw 264.7 cells as compared to previous studies but in the case of paclitaxel toxicity is lower as compared to prior studies. Researchers determined the AT and CT of these antineoplastic compounds on different animal model and microorganism such as bacteria, algae etc. by estimating different parameters such as luminescence, growth inhibition mortality, immobilisation and reproduction inhibition is given in Table 4.9 but the cytotoxic effect of cyclophosphamide, etoposide and paclitaxel on Raw 264.7 cell is not yet analysed.

In view of the above result, for cyclophosphamide the percentage of cell cytotoxicity was 17.48 % minimum at an exposure concentration $10 \mu\text{g.ml}^{-1}$ and 69.58 % maximum at $250 \mu\text{g.ml}^{-1}$, while the 50 % of cell death occurred at $145.44 \pm 1.67 \mu\text{g.ml}^{-1}$. The linear regression value for cyclophosphamide was 0.9519 (Fig. 4.26 (a)). The etoposide was showing higher cell cytotoxicity that was 42.99 % minimum at concentration $10 \mu\text{g.ml}^{-1}$ and 92.01% maximum at concentration $250 \mu\text{g.ml}^{-1}$, while the 50 percent cell death occurred at $5.40 \pm 0.94 \mu\text{g.ml}^{-1}$. The linear regression value for etoposide was 0.9185 (Fig. 4.26 (b)). In present study, the cytotoxicity percentage of paclitaxel on Raw 264.7 cells was 31.07 % minimum at concentration $10 \mu\text{g.ml}^{-1}$ and 88.85 % maximum at concentration $250 \mu\text{g.ml}^{-1}$, while 50 % of cell death after the treatment of paclitaxel was at concentration $69.76 \pm 4.23 \mu\text{g.ml}^{-1}$. The linear regression value for paclitaxel was 0.9717 (Fig. 4.26 (c)). It reveals that etoposide is more toxic for Raw 264.7 cells as compared to cyclophosphamide and paclitaxel.

This is the first study performed, in which MTT assay was used to detect cytotoxic effect of above antineoplastic compounds on the immune cell line. The test was based on the standard colorimetric assay by the estimation of cell proliferation or cell growth. The present study showed cyclophosphamide, etoposide and paclitaxel are cytotoxic for Raw 264.7 cells while their cytotoxicity is varying accordingly.

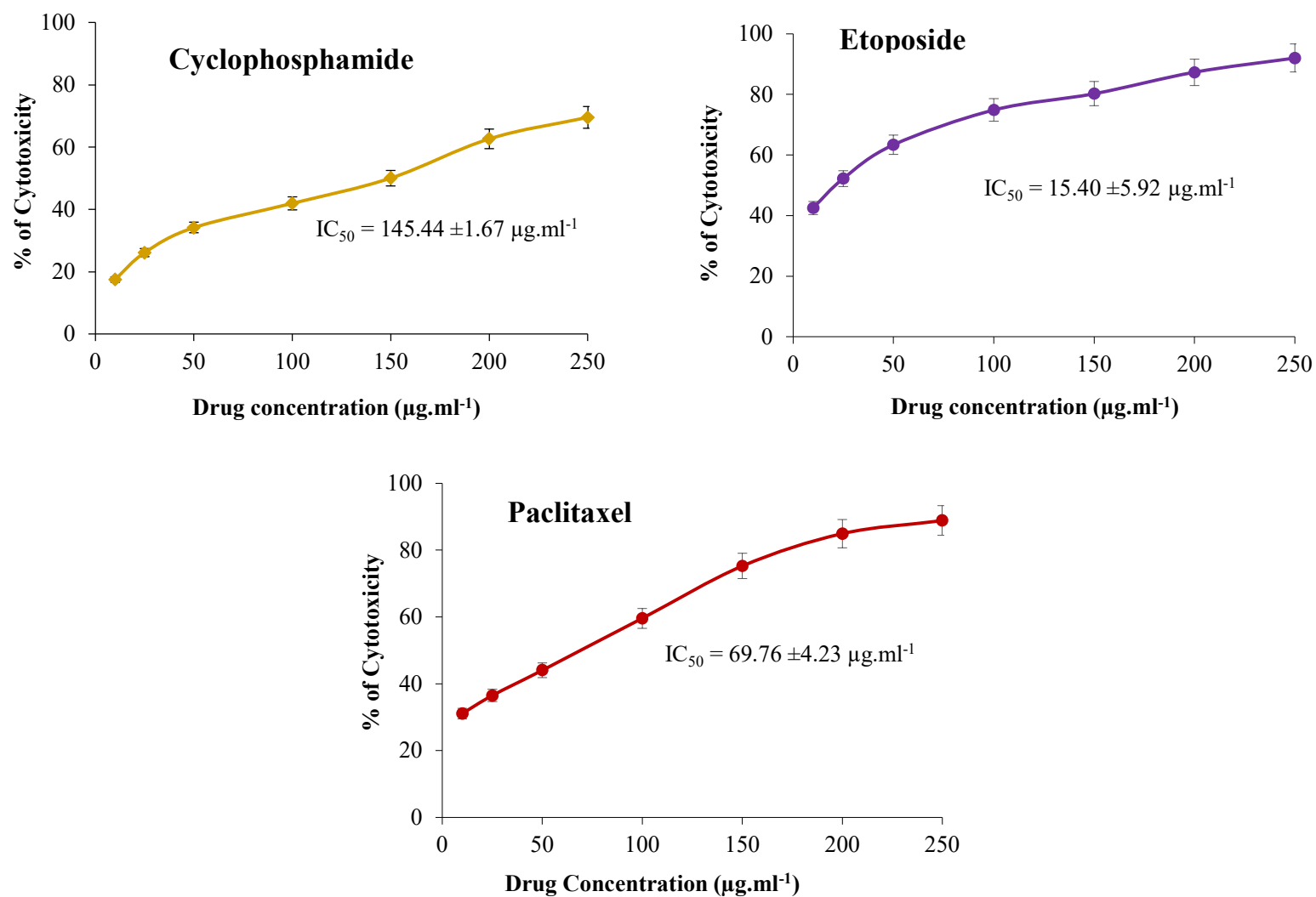


Fig 4.26. Dose-response curve of a) cyclophosphamide b) etoposide and c) paclitaxel

Table 4.9. Toxicity data of cyclophosphamide, paclitaxel and etoposide on different organism (Heath et al., 2020; Russo et al., 2020)

Antineoplastic compounds	Toxicity type	Organism	Toxicity (EC ₅₀ /LC ₅₀) mg.ml ⁻¹	References
Cyclophosphamide	Acute	<i>Vibrio fischeri</i>	>0.1	(Białk-Bielińska et al., 2017)
		<i>Brachionus calyciflorus</i>	1.924 (1.210–3.036)	(Russo et al., 2018)
		<i>Ceriodaphnia dubia</i>	0.986 (0.765–1.272)	(Russo et al., 2018)
		<i>Daphnia magna</i>	>1.0	(Zounková et al., 2007)
			>0.1	(Białk-Bielińska et al., 2017)
		<i>Thamnocephalus platyurus</i>	1.396 (1.304–1.494)	(Russo et al., 2018)
	Chronic	<i>Vibrio fischeri</i>	1.396 (1.304–1.494)	(Lutterbeck et al., 2016)
		<i>Synechococcus leopoliensis</i>	>0.120	(Česen et al., 2016a)
			>0.320	(Česen et al., 2016a)
			>0.1	(Białk-Bielińska et al., 2017)

Results and Discussion

			>0.2	(Russo et al., 2018)
		<i>Brachionus calyciflorus</i>	0.089 (0.067–0.119)	(Russo et al., 2018)
		<i>Ceriodaphnia dubia</i>	0.058 (0.037–0.089)	(Russo et al., 2018)
		<i>Daphnia magna</i>	>0.1	(Grung et al., 2008)
		<i>Lamna minor</i>	>0.1	(Białk-Bielińska et al., 2017)
Paclitaxel	Acute	<i>Daphnia magna</i>	>.00074	(CDER, 1996)
Etoposide	Acute	<i>Brachionus calyciflorus</i>	>0.12	(Parrella et al., 2014b)
		<i>Ceriodaphnia dubia</i>	16 % at 0.120	(Parrella et al., 2014b)
		<i>Daphnia magna</i>	25 % at 0.120	(Parrella et al., 2014b)
			0.030 (0.016–0.040)	(Zounková et al., 2007)
		<i>Thamnocephalus platyurus</i>	0.074 (0.056–0.099)	(Parrella et al., 2014b)
		<i>Danio rerio</i>	>0.1	(Kovács et al., 2016)
	Chronic	<i>Pseudokirchneriella subcapitata</i>	0.250 (0.120–0.460)	(Zounková et al., 2007)

<i>Brachionus</i>	0.0037	(Parrella et al.,
<i>calyciflorus</i>	(0.0027–	2014b)
	0.0053)	
<i>Ceriodaphnia</i>	0.002	(Parrella et al.,
<i>dubia</i>	(0.0015–	2014b)
	0.0025)	
<i>Daphnia magna</i>	0.0023	(Parrella et al.,
	(0.0018–	2014b)
	0.0029)	

The cytotoxic effect shown by antineoplastic compounds in MTT assay cause cell growth inhibition in Raw 264.7 cells which may be occur due to DNA strand breakage or topoisomerase activity. So, the above finding indicates that these antineoplastic compounds considered as potential cytotoxic agent and cause damage of the immune system or body of an organism when come in contact with these compounds.

4.9.2. Cytotoxicity of transformed/degraded products

The cytotoxicity of by-products generated during biodegradation of cyclophosphamide, etoposide and paclitaxel by *G. lucidum*, *T. versicolor* and *P. chrysosporium* was determined by cell viability assay. In this study, MTT assay was performed to investigate the cell viability of Raw 264.7 cells when treated with TPs of cyclophosphamide etoposide and paclitaxel. Toxicity of TPs was analysed of sample collected on 3, 6, 9, 12 and 15 day of degradation experiment. As we discussed in section 4.4, the cyclophosphamide was removed only by *G. lucidum*, so except from this fungus, in rest two fungi mediated treatment flask, the cyclophosphamide was remained same or present in their native form from the beginning to end of the degradation experiment. The toxicity level of cyclophosphamide drug control was 7.68 % and medium

control was 0.44 % for Raw 264.7 cells was observed. In *G. lucidum* mediated treatment, the cell cytotoxicity percentage of cyclophosphamide was 1.42 %, 2.38 %, 2.01 %, 2.41 % and 2.31 % measured on 3, 6, 9, 12 and 15 days respectively. In *T. versicolor* it was 6.72 %, 6.65 %, 6.87 %, 7.06 % and 7.03 measured on 3, 6, 9, 12 and 15 days respectively, while in *P. chrysosporium* it was 7.31 %, 6.87 %, 6.22 %, 6.39 % and 6.17 % analysed on 3, 6, 9, 12 and 15 days respectively. These results of cyclophosphamide indicates that, the cyclophosphamide was remained same in case of *T. versicolor* and *P. chrysosporium* and their toxicity level Raw 264.7 cells was almost similar in both fungi treated samples from beginning to end. There was not much difference reported in the toxicity and this may be due to non-degradation of cyclophosphamide by these fungi. While the toxicity of *G. lucidum* treated sample indicates difference in cell cytotoxicity level in compared to these fungi. Here, the toxicity level of TPs on Raw 264.7 cells was decreased as compared to their native compound toxicity level. At initial degradation, the toxicity level of TPs was less *i.e.* 1.42 % and it was increased to 2.38 % on 6th day. Further, no more variation in toxicity was observed or it was observed almost equal on all day of sampling (Fig. 4.27).

These results analysed that the toxicity level of TPs of cyclophosphamide was reduced after the degradation with this fungus. When the cytotoxicity of native form of cyclophosphamide on Raw 264.7 cell line was analysed, we determined the cell cytotoxicity in the form of IC₅₀. The IC₅₀ value of cyclophosphamide was determined between 10 to 250 µg.ml⁻¹ and the 50 % of cell were affected on concentration of 145.44 ± 2.67 µg.ml⁻¹ (Table 4.8). The current study of toxicity of cyclophosphamide defined by the cell cytotoxicity percentage of treated sample. Here, the concentration of antineoplastic compound in sample was less as compared to previous study and cell cytotoxicity percentage also varying but this may be due to the exposure of concentration level of cyclophosphamide on Raw 264.7 cells. (Ferrando-Climent et al., 2015) performed an investigation on toxicity evaluation of *T. versicolor* mediated degraded sample

of cyclophosphamide on *V. fischeri* but due to non-biodegradation of cyclophosphamide by *T. versicolor*, the toxicity of treated sample was same from beginning to end of experiment.

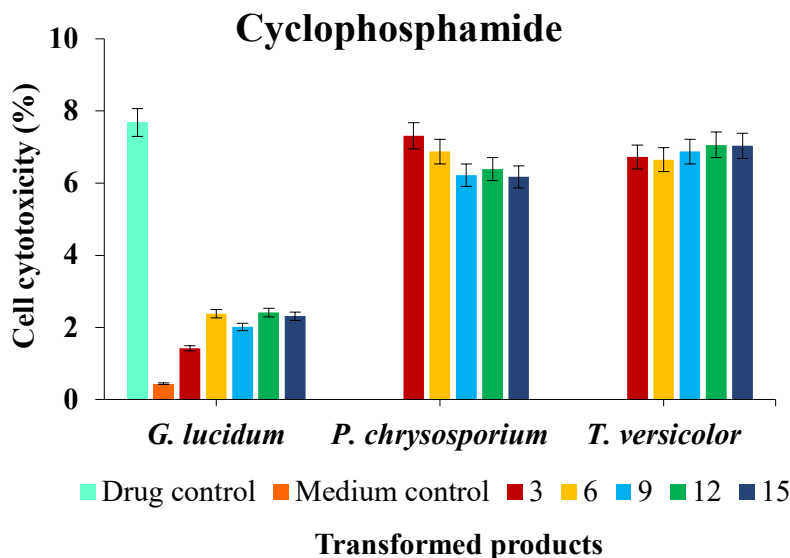


Fig. 4.27. In-vitro cytotoxicity of TPs of cyclophosphamide on Raw 264.7 cells during biodegradation with *G. lucidum*, *P. chrysosporium* and *T. versicolor*

In case of etoposide, each TPs were able to generate toxicity on Raw 264.7 cells but their cell cytotoxicity percentage was varying in each fungi treatment sample according to TPs generated. The toxicity level of etoposide drug control was 18.22 % and medium control was 0.45 % for Raw 264.7 cells was observed. The cytotoxicity percentage of *G. lucidum* mediated TPs of etoposide was observed 0.83 % on 3rd day and it was increased to 1.46 %, 1.43 %, 1.57 % and 1.48 % on 6, 9, 12 and 15 days respectively. Here, the toxicity of etoposide TPs increased after 3 days and further it was analysed almost similar on 6, 9, 12 and 15 days. This may be due to the maximum degradation was achieved on 6 day and after that no new TPs were form and the toxicity was remained same. The cell cytotoxicity percentage of by *P. chrysosporium* mediated TPs of etoposide was observed 4.11 % on 3rd day and it was continuously increased to 4.95 % on 6th day, 7.95 % on 9th day, 8.03 % on 12th day respectively. After this, the toxicity

level of TPs was reduced to 7.02 on 15th day. These results of toxicity provide the highest cell cytotoxicity of TPs of etoposide on Raw 264.7 cells during this treatment process. Here, the toxicity of TPs was already higher at the initial degradation, but it was continuously increased after 3 days. Moreover, the toxicity level was reduced at the end of experiment but still it was higher to their initial rate. The toxicity percentage of *T. versicolor* mediated TPs was analysed 2.19 on 3rd day. After 3 days, the cytotoxicity level was increased from 2.19 % to 2.46 %, further it was reduced to 2.06 % and again it was slightly increased 2.74 %, 2.68 % on 12 and 15 days respectively (Fig. 4.28). These results indicating that, the initial formed TPs were less toxic for Raw 264.7 cells but during biodegradation some more toxic TPs were generated as compared to initial TPs of etoposide produced during this process.

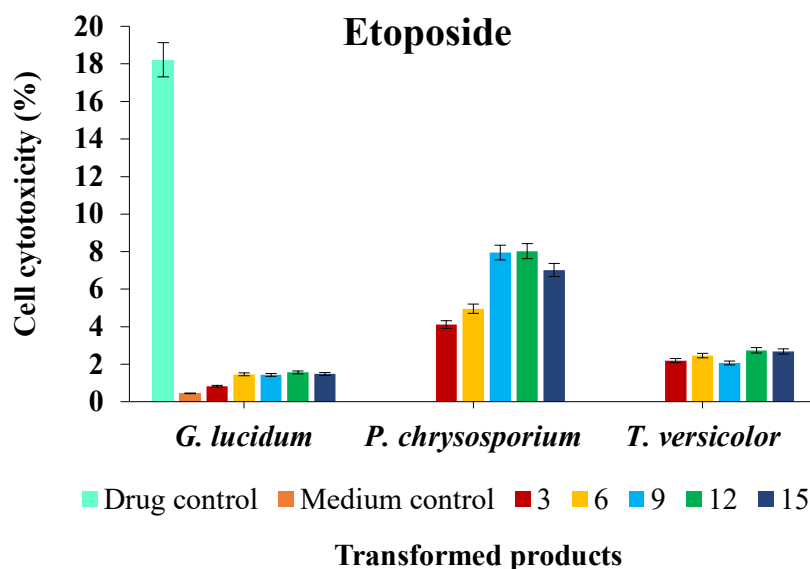


Fig. 4.28. In-vitro cytotoxicity of TPs of etoposide and on Raw 264.7 cells during biodegradation with *G. lucidum*, *P. chrysosporium* and *T. versicolor*

In case of *P. chrysosporium*, the cell cytotoxicity of etoposide was higher *i.e.* 8.03 % analyse from TPs produced during treatment with other two fungi. This indicate that, the *P. chrysosporium* can remove etoposide from the aquatic sample but after degradation it can

generate more toxic by-products of etoposide as compared to *G. lucidum* and *T. versicolor* mediated TPs. The cell cytotoxicity of *G. lucidum* mediated TPs of etoposide exhibited less toxicity in comparison to other two fungi mediated TPs. During the parent compounds toxicity analysis, we determined that the etoposide was highly cytotoxic for Raw 264.7 cells and their toxicity estimated in the form of IC₅₀. The value of etoposide for Raw cells was 15.40 ± 5.92 µg.ml⁻¹ when cells were treated between a range of 10 to 250 µg.ml⁻¹ (Table 4.8).

This indicated that, the native form of etoposide was quite higher toxic for these cells. However, after the degradation with fungi, their TPs were also able to generate cell cytotoxicity and toxicity of level of each TPs was different. Several studies for the investigation of toxicity of cyclophosphamide and etoposide were also performed by the researchers but they carried out their studies as AT and CT on microorganism such as *D. magna*, *V. fischeri*, *B. caliciflorus*, *C. dubia*, *L. minor* etc. in the form EC₅₀/LC₅₀ (Table 4.9). Their toxicity range varied and it was depending on the drug concentration taken for the exposure of microorganism (Česen et al., 2016b; Lutterbeck et al., 2016; Białk-Bielińska et al., 2017 Russo et al., 2018).

In case of paclitaxel, the degradation rate was extremely low. Here, only in *G. lucidum* some concentration of paclitaxel was reduced, while in case of other fungi, the degradation of paclitaxel was less than 7 %. Therefore, the cell cytotoxicity of paclitaxel and TPs formed during treatment with fungi was analysed on Raw 264.7 cells. The toxicity level of paclitaxel drug control was 11.33 % and medium control was 0.45 % for Raw 264.7 cells observed. In *G. lucidum*, it was estimated 10.31 % on 3rd day. At this movement of sampling, the degradation rate was very low, so mainly the toxicity of native form was present but after this when the degradation rate was increased, toxicity level was slightly decreased continuously up to end of experiment. The cell cytotoxicity percentage on 6, 9, 12 and 15 days was estimated 9.42 %, 8.42 %, 8.38 % and 8.03 % respectively. These result shows that the toxicity of paclitaxel was

reduced, when the degradation rate was increased but due to low degradation it was not much reduced to their initial level. In *T. versicolor*, the cell cytotoxicity percentage was 11.39 % on 3rd day and after that the toxicity level reduced. The cytotoxicity of 6, 9, 12 and 15 days was analysed 10.14 %, 10.07 %, 10.02 % and 10.04 % respectively. The toxicity percentage on 3rd day was high because up to this time, no degradation of paclitaxel was start and only native form of paclitaxel was present. Here, after 3 days, the toxicity level was reduced but it was high to *G. lucidum* mediated samples and this may be due to low degradation rate of paclitaxel with *T. versicolor*. The cell cytotoxicity percentage of *P. chrysosporium* mediated sample of paclitaxel was observed 11.27 % on 3rd day, while after 3 days the value of toxicity was reduced. It was analysed 10.41 %, 10.29 %, 10.16 % and 10.11 % on 6, 9, 12 and 15 days respectively (Fig. 4.29).

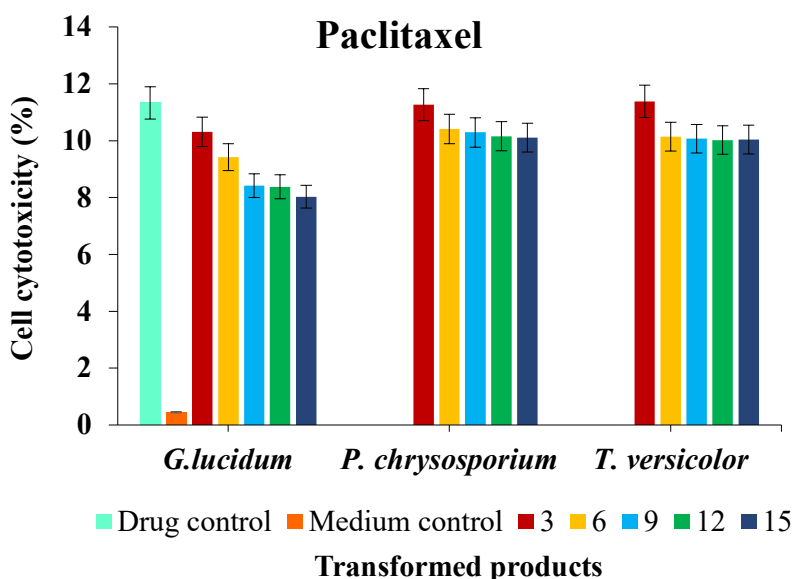


Fig. 4.29. *In-vitro* cytotoxicity of TP's of paclitaxel on Raw 264.7 cells during biodegradation with *G. lucidum*, *P. chrysosporium* and *T. versicolor*

Here, the toxicity percentage was also high on 3rd day, and it may be due to non-degradation of paclitaxel up to 3rd day. After 3 days, the toxicity level was reduced continuously at the end

of treatment experiment. When the cell cytotoxicity of native paclitaxel form was estimated, the IC_{50} value was $69.76 \pm 4.23 \mu\text{g.ml}^{-1}$ between the concentration range from $10 \mu\text{g.ml}^{-1}$ to $250 \mu\text{g.ml}^{-1}$. If we compare to treated sample with native form, the cytotoxicity was varied but it can indicate paclitaxel is cytotoxic for Raw 264.7 cells even if present in very low concentration.

Steinbrecht et al. (2020) analysed toxicity of cyclophosphamide metabolites (4-hydroxy-cyclophosphamide) on two human cancer cell line (MCF-7 and HepG2). It was able to generate cytotoxicity on these two cell lines, but the toxicity response was more observed in MCF-7. The EC_{50} value of MCF-7 was $28 \mu\text{M}$ and HepG2 was $111 \mu\text{M}$ observed. Another study carried out by Fernandes et al. (2020) for toxicity evaluation of cyclophosphamide on *Mytilus galloprovincialis* at concentration of 1000 ng.L^{-1} . Cyclophosphamide was able to generate oxidative stress on murine mussels and cause DNA damage. The chronic toxicity of cyclophosphamide was evaluated on *V. fischeri* (Lutterbeck et al., 2016) as EC_{50} $1396 \mu\text{g.ml}^{-1}$ in conc. range between 1304 to $1494 \mu\text{g.ml}^{-1}$, *Brachionus calyciflorus* as EC_{50} $89.8 \mu\text{g.ml}^{-1}$ in conc. range between 67.2 to $119.4 \mu\text{g.ml}^{-1}$ and *Ceriodaphnia dubia* as EC_{50} $58 \mu\text{g.ml}^{-1}$ in conc. range between 37.4 to $89.9 \mu\text{g.ml}^{-1}$ (Russo et al., 2018). In 2014, Parrella et al. (2014) investigated the chronic toxicity of etoposide on *B. calyciflorus*, *C. dubia* and *Daphnia magna*. The chronic toxicity of etoposide on *B. calyciflorus* measured as EC_{50} value as $3.7 \mu\text{g.ml}^{-1}$ in conc. range between 2.7 to $5.3 \mu\text{g.ml}^{-1}$, *C. dubia* as EC_{50} $0.204 \mu\text{g.ml}^{-1}$ in conc. range between 0.152 to $0.256 \mu\text{g.ml}^{-1}$ and *D. magna* as EC_{50} value $0.239 \mu\text{g.ml}^{-1}$ in conc. range between 0.181 to $0.299 \mu\text{g.ml}^{-1}$ observed.

These results indicate, cyclophosphamide, etoposide and paclitaxel are highly toxic for cell viability even when present in their low concentration. But after the degradation with these

fungi, the toxicity level of these compounds was reduced and their toxicity level in each fungus was varying according to their TPs generated during treatment process.