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Efficiency Analysis of a Hospital Effluent Treatment Plant In Reducing Genotoxicity and Cytotoxicity of Hospital Wastewaters

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ABSTRACT:

In many developing countries including India, the major part of hospital wastewater is discharged in surface watercourses or in public sewers or percolates into underlying groundwater aquifers with no or only partial treatment. Indeed, some of the substances found in wastewaters are genotoxic and are suspected to be a possible contributor to certain cancers observed in last decades. A properly designed Effluent Treatment Plant (ETP) is, therefore, a feasible solution to avoid hazardous consequences resulting from discharge of untreated hospital wastewaters. The present study was, thus, planned to analyse the efficiency of an ETP installed at an Indian hospital in lowering the mutagenic and cytotoxic potential of hospital effluents as compared to untreated effluents. Short term microbial bioassays; Ames test and *Saccharomyces cerevisiae* respiration inhibition assay were used to evaluate the genotoxicity and cytotoxicity of untreated as well as treated effluents of the hospital. Results indicate that untreated hospital effluents possessed the potential to contaminate the surface and even underground water, thereby making it unfit for irrigation and drinking. In contrast, the treated water samples were found to be slightly genotoxic and mildly cytotoxic, thus inferring that genotoxicity is reduced after the treatment process. This confirms that the ETP of the hospital under study is effective in terms of genotoxicity and cytotoxicity reduction to considerable level.

Keywords: Cytotoxicity, Effluent treatment plant, Genotoxicity, Hospital wastewaters, Short term microbial assays

[I] INTRODUCTION

Every anthropogenic activity generates some waste. Health care services are one among such anthropogenic activities wherein water quality is affected during the course of its use and wastewater is generated. Health-care wastewater is mainly liquid waste, containing some solids produced by humans (staff and patients) or during health-care-related processes, including cooking, cleaning and laundry [26]. The quantity of wastewater produced in health-care facilities such as hospitals is best measured by water consumption. Water consumption by hospitals

depends heavily on factors such as the kind of healthcare services provided, number of beds, accessibility to water, climatic situation, level of care and local water-use practices [27]. Depending upon such factors, the amount of waste water discharged from hospitals may vary from hospital to hospital but it has been estimated at 400 to 120 liters/bed/day [8]. Tsakona et al. [25] reported an estimate on per capita production of waste water in hospital to be 1000 liters/person/day.

The effluent from healthcare facilities contain more drug-resistant pathogens, a greater variety of chemicals, solvents, disinfectants and more hazardous materials like pharmaceuticals and radionuclides than domestic sewage [23]. While in some countries (e.g., Japan, China, Greece, US) [14,16,20] wastewater from big hospitals is pretreated on-site, in many developing countries including India, the major part of hospital wastewater is discharged in public sewers or in surface watercourses or percolates into underlying groundwater aquifers with no or only partial treatment [7]. Many of the chemical compounds present in hospital effluents resist normal wastewater treatment. Residues of pharmaceuticals can be found in all wastewater treatment plant (WWTP) effluents, due to their inefficient removal in the conventional systems [4,13,15,22,24]. They end up in surface waters where they can influence the aquatic ecosystem and may accumulate through the food chain. Concerns regarding the exposure of humans are mostly associated with exposure through drinking water produced from contaminated surface water [20].

Indeed, some of the substances found in wastewaters are genotoxic and are suspected to be a possible contributor to certain cancers observed in last decades [11]. Looking at greater importance of the issue, in recent years few researches have been carried out to evaluate the genotoxicity of hospital effluents [1,6,7,11].The existence of treatment plants in hospitals is rare but commendable and effective according to available literature. Therefore, in the present study an attempt has been made to compare the genotoxicity and cytotoxicity of untreated and final treated effluent discharges of a hospital site located in Jaipur, Rajasthan (India) and thus analysing the efficacy of the effluent treatment plant (ETP) of the said hospital. The bioassays used to investigate genotoxicity of these effluents were *Salmonella* mutagenicity assay /Ames assay (Prokaryotic assay) and *Saccharomyces cerevisiae* respiration inhibition assay (Eukaryotic bioassay).

[II] MATERIALS AND METHODS

2.1. Sampling Site

The samples were collected from a hospital site viz. Santokba Durlabhji Memorial Hospital (SDMH) cum Medical Research Institute during January, 2012 and July, 2012 and repeated at two consecutive days in a month. SDMH is the most modern hospital of its kind in the state of Rajasthan. It has a bed capacity of 310 including 31 ICC/CCU beds and 161 beds for special care of neo-natals. This hospital has an ETP encompassing provision for filtration and chlorination of effluent.

Brief plan of ETP at SDMH:

The ETP operating in SDMH is based on activated sludge process. Waste water from whole hospital premises is first collected into a ‘collection tank’ or ‘sewage tank’. The wastewater from collection tank is then propelled to a ‘reactor tank’ by submersible pumps. In reactor tank air is supplied through an air blower in order to provide oxygen for the aerobic microbes. After a specific retention time the sewage is transferred into a ‘settler tank’ where the solids settle down and are separated from wastewater. The process of sludge settlment is enhanced by the addition of ‘Alum’ [hydrated potassium aluminium sulfate {KAl(SO4)2•12H2O}]. It works as a sludge dewatering agent and helps in flocculation of solids. The settled sludge is pumped back into

the reactor tank to maintain specific microbes' density in incoming wastewater and after 2-3 cycles the sludge is removed from the system. The waste sludge is then deposited into soil (landfills) after ozonization.

The clear overflow is then pumped into a 'balance tank' or 'level tank' where chlorination of treated wastewater is done following filtration. Different doses of sodium hypochlorite are provided in balance tank to ensure proper disinfection of treated effluent. Volume of waste water being treated in the ETP of SDMH is about 50,000 litres per day and is used for gardening purposes in hospital itself and rest of the treated wastewater is disposed in municipal sewerage.

2.2. Sample Collection

The samples were collected both before and after treatment. Untreated sample was taken from the sewer of the hospital, where the entire water from the hospital is collected before being treated. Treated wastewater was collected from the outlet from where it comes out of treatment plant. Samples from SDMH hospital were collected during its maximum activity period (usually 8:00 am-6:00pm). Until tested, the samples were stored at 4°C. These samples were then tested for their mutagenic and cytotoxic potential in their crude state without being concentrated.

2.3. Bioassays

Ames Assay (Salmonella/ Microsome Reversion Assay)

The tester strains of Salmonella typhimurium viz. TA98 and TA100 were obtained from Microbial Type Culture Collection & Gene Bank (MTCC), Institute of Microbial Technology (IMTEC), Chandigarh, India. The assay was carried out using the plate incorporation method described by Ames et al. [2] and revised by Maron and Ames [17]. The samples were analysed with and without hepatic S9 fraction. Introduction of mammalian liver enzymes into prokaryotic system incorporates the aspect of mammalian metabolism into the in vitro test. Uninduced swiss albino Mice were used to prepare standard S9 mixture. It was prepared according to the protocol described by Maron and Ames [17]. Sodium azide (5µg/plate) was used as positive control and sterile distilled water was used as negative control. Five concentrations of individual samples (2%, 5%, 10%, 50% and 100% of original samples) were tested and all the plates were run in duplicates. Each set of experiments was repeated twice. Average numbers of spontaneous revertants per plate produced with TA98 and TA100 in the absence of metabolic activation were counted to be 55±8 & 139±3 CFU respectively for January samples and 49±4 & 115±46 respectively for July samples and; in the presence of metabolic activation, spontaneous revertants were 92±6 & 146±6 CFU, respectively for January samples and 90±18 & 142±26 for July samples.

The S. typhimurium TA98 and TA100 strains were grown at 37°C, with shaking, for 10 hours to obtain a final concentration of 10^9 bacterial cells. 0.1 ml of this fresh culture was mixed with 0.2 ml of His/Bio solution, 0.1 ml of sample, 0.5 ml of buffer or 0.5 ml of S9 mix and total volume was made upto 1.0 ml by autoclaved distilled water. This mixture was then shaken and poured on plates containing about 25 ml of minimal glucose agar medium. The test concentrations were selected from a set of standard doses for liquids. The plates were immediately covered with paper to protect photosensitive chemicals present in test compounds. Plates were then inverted and placed in a dark incubator for 48 hrs at 37°C. The revertant colonies were clearly visible with a uniform background lawn of auxotrophic bacteria. After 48 hrs the revertant colonies on the test and control plates were counted. All tester strains were maintained and stored according to standard methods [19]. The strain genotypes (Histidine requirement, *rfa* mutation, *uvr*B and R-factor) were confirmed immediately after receiving the cultures and every time a new set of frozen permanents was prepared and used.

Saccharomyces cerevisiae Respiration Inhibition Assay

Previous studies have shown that bioassays utilizing *S. cerevisiae* represent a useful tool to detect the toxic effects of hospital wastewaters on eukaryotic cells [21]. A commercial brand of dried baker's yeast (*S. cerevisiae*) was used for this assay. The Baker's yeast assay [3] was carried out by preparing a 1% (v/v) suspension of yeast in sterilized saline solution (0.85% NaC1) as the suspending fluid. The yeast suspension was stirred for 15 min to break up yeast floc. Test samples (0.2 mL) were added to 0.8 mL of yeast suspension and incubated for 30 min at 30°C with shaking. Different concentrations of samples (1%, 2.5%, 5%, 25%, 50% and 100%) were analysed; 0.001% & 0.1% concentrations were also tested when there was more than 50% reduction in cell respiration at the 1% concentration. To this, 0.1 mL of INT (2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride) solution (0.2%) and 0.1 mL of 10% solution of yeast extract were added to each sample and the mixture incubated in the dark at 30°C for 1 hr with shaking. The reaction was stopped with 0.1 mL of 37% formaldehyde. Tetrazolium salts act as artificial electron acceptors along electron transport system (ETS) during respiration of microbial cells, becoming reduced to form insoluble formazans; therefore, microbial respiration can be assessed using INT reduction to the microscopically spotted end product, INT-formazan. The proportion of respiring cells was determined as follows: one or two loopfuls of the yeast suspension were spread on a glass slide, air dried, counterstained with 0.025% malachite green and blotted after 1 min; 500 cells were examined with bright field microscopy (100x) and the number of respiring cells (green cells with red INT-formazan crystals) and non respiring cells (green cells) were determined.

All reagents used in both assays were of analytical grade, supplied by Himedia Laboratories Limited and Sigma-Aldrich (Mumbai, India).

2.4. Calculations

Data Analysis for Ames Test

The most common method of evaluation of data from the mutagenicity assay is the ‘‘two fold rule’’ according to which doubling of spontaneous reversion rate at one or two test chemical concentrations constitutes a positive response [19]. This rule specifies that if a test compound doubles or more than doubles mean spontaneous mutation frequency obtained on the day of testing, then the compound is considered significantly mutagenic. Using this procedure the following criteria were used to interpret results:

- Positive—A sample was considered mutagenic if it produced a reproducible, dose-related increase in the number of revertant colonies in one or more strains of S. typhimurium. A sample was considered weak mutagenic if it produced a reproducible dose-related increase in the number of revertant colonies in one or more strains but the number of revertants was not double the background number of colonies.
- Negative— A sample was considered non-mutagenic if no dose-related increase in the number of revertant colonies was observed in at least two independent experiments.
- Inconclusive—If a sample could not be identified clearly as mutagenic or non-mutagenic, the results were classified as inconclusive (e.g. if there was only one elevated count).

For all samples that showed dose dependent increase in the number of revertant colonies, mutagenicity ratios were calculated. Mutagenicity ratio is the ratio of average induced revertants on test plates (spontaneous revertants plus induced revertants) to average spontaneous revertants on negative control plates (spontaneous revertants) (Mathur et al. 2005).

For statistical analysis, independent two tailed t-test was performed. Revertants were taken as the dependent variable and dose as the independent

variable; keeping time (January and July, 2012) and strains (TA 98 and TA 100) fixed. A comparison-wise P values of <0.05 were considered to be statistically significant. The Statistical Package for Social Sciences (SPSS), release 18.0 was used for statistical analysis and MS-Excel 2007 was used for graphical representations (concentration-response curves).

Interpretation of Test Results for S. cerevisiae Respiration Inhibition Assay

Results of Baker's yeast assay were expressed in terms of percent inhibition of respiring cells compared with negative controls. Effective Concentrations resulting 20% and 50% inhibition (EC_{20} and EC_{50}) in the percentage of respiring cells, respectively, were calculated using logistic regression. Statistical analysis of concentration – response curves to calculate EC_{20} and EC_{50} values for Baker's yeast assay were performed by probit method of logistic regression using a MS excel software XLSTAT 2012; Addinosoft.

[III] RESULTS AND DISCUSSION

As genotoxic pharmaceutical compounds, including cytostatic agents, are discharged in wastewaters, the mutagenic potential of wastewaters from hospitals is a domain of interest. The hospital wastewater is a complex matrix of hazardous compounds which warrants treatment before discharge into the environment. Therefore, in present study genotoxic and cytotoxic potential of untreated and treated hospital effluents were measured and compared for effectiveness of treatment.

3.1. Ames assay

The results of the Salmonella mutagenicity assay with untreated and treated wastewaters from SDMH are summarized in Table 1 as the mutagenicity ratio of average induced reversions to spontaneous reversions. The untreated samples showed concentration-dependent significant mutagenicities with mutagenicity ratios greater than 2.0 (Table 1; Fig. 1 & 2) during both sampling periods. The specific mutagenic activity of these samples as measured with strain TA98 (1884±7 and 2460±88 induced revertants respectively in January and July at 100% concentration of sample, in the absence of S9 hepatic fraction) and with strain TA100 (2338±6 and 2405±12 induced revertants respectively in January and July at 100% concentration of sample, in the absence of S9 hepatic fraction) indicated that the untreated wastewater samples from SDMH were strongly genotoxic (Fig. 1 & 2). The addition of S9 mix further increased the number of revertant colonies with strain TA98 (2363±45 and 3137±24 induced revertants respectively in January and July at 100% concentration of sample) and TA100 (2355±12 and 2754±15 induced revertants respectively in January and July at 100% concentration of sample).

The treated sample from SDMH effluent treatment plant was found to be slightly genotoxic with mutagenicity ratio, less than 2.0 with TA100 (at 2% concentration both in absence and presence of metabolic activation and at 5% concentration of July samples without metabolic activation) and a little higher than 2.0 at successive concentrations with both the strains during both sampling seasons (Table 1). Concentration- response curves calculated for increasing exposures to treated effluents revealed 479±2 and 474±16 induced revertants at 100% concentrations with strains TA98 and TA100, respectively, for January samples and; 428±22 and 373±19 induced revertants at 100% concentrations with strains TA98 and TA100, respectively, for July samples in the absence of S9 fraction (Fig. 1 & 2). In the presence of hepatic S9 fraction there were 595±20 and 571±17 revertant colonies at 100% effluent concentrations occurred with strains TA98 and TA100, respectively for January samples and; 499±47 and 471±4 revertant colonies produced with strains TA98 and TA100, respectively for July samples.

Thus, it could be observed that during both sampling periods, wastewater samples (both

untreated and treated) had similar significant mutagenic activities with comparable concentration response profiles, suggesting no seasonal influence on the genotoxicity of samples from hospitals. Overall, the results obtained with the Ames assay indicated that hospital wastewaters could have strong and significant mutagenic potentials before preliminary treatment. The findings of this study are comparable to earlier studies [7,9,11], which proved that hospital waste waters possessed considerable mutagenic activity. 55% and 68% of tested hospital wastewater samples were found mutagenic by Jolibois et al. [9] and Jolibois and Guerbet [11] respectively in Salmonella fluctuation assay whereas Gupta et al. [7] found all untreated wastewater samples significantly mutagenic at 100µl dose in standard plate incorporation method of Ames assay. Gupta et al. [7] reported dose-dependent mutagenicity similar to the present study. They also observed improved mutagenicity level after the addition of S9 mix.

There were also observations which indicated that Effluent Treatment Plant in SDMH was successful in significantly reducing the number of revertant colonies. Moreover, it is evident by t-test analysis in conjunction with concentration response curves and mutagenicity ratios declination that there was a significant difference between samples from SDMH before and after treatment in terms of number of revertant colonies produced indicating considerable reduction in genotoxicity levels of samples after treatment in ETP (Table 2). The studies conducted by Jolbois and Guerbet [10] and Gupta et al. [7] were again in accordance, which reported that the wastewater treatment plants were able to reduce the genotoxicity of effluents in comparison to influents or untreated wastewaters. Further, the addition of hepatic fraction increased the number of revertants in all cases, indicating that mammalian enzymes can convert some of the pro-mutagenic compounds into active mutagenic metabolites. However, it is difficult to compare these results with other studies, because many parameters can influence the genotoxicity test response (composition of the sample, hospitals size and their degree of activity, nature of the medicines used in treatments, nature of tests being performed, nature of the genotoxicity tests, etc.) and there are only few hospital waste water studies available in literature.

3.2. Baker's yeast respiration inhibition assay

The results obtained in *Saccharomyces cerevisiae* Respiration Inhibition Assay with SDMH wastewater samples are illustrated in Table 3. For this assay also, there was not much difference observed in data obtained during the months of January and July. The untreated SDMH samples were reported to have EC_{20} and EC_{50} values 0.36% (0.72 µL) and 13.34% (26.67 µL) respectively, for January samples and 0.15% (0.30 µL) and 4.4% (8.79 µL) respectively, for July samples. After treatment, the samples revealed higher EC_{20} [2.36% (4.71 µl) and 1.12% (2.23 µl), respectively for January and July samples] and EC_{50} values [33.95% (67.90 µl) and 23.84% (47.68 µl), respectively for January and July samples]. Further, concentration response curves (Fig. 3) clearly show lesser percentage of respiration inhibition in yeast cells when exposed to treated samples in comparison to untreated samples.

These observations again prove that treatment plant installed at SDMH is effective in removing toxicants from hospital waste water resulting in lower cytotoxicity after treatment. This infers that treated waste waters can be regarded safe for their disposal in municipal sewers which can further be treated in municipal wastewater treatment plant before being discharged into environmental water bodies. The results obtained with the eukaryotic Baker's yeast assay in this study were generally in agreement with the prokaryotic Ames assay. Hence, this assay can be used in conjunction with Ames Salmonella assay for screening waste water

discharges for their probable mutagenicity and cytotoxicity routinely. No study had yet been performed to test cytotoxicity of hospital waste waters using this respiration inhibition assay. However, detection of cytotoxicity caused due to presence of heavy metals in environmental waste waters has successfully been done by Codina et al. [5]. Results of their study showed that this assay was potentially useful in detection of chemical toxicity of metals in waste waters.

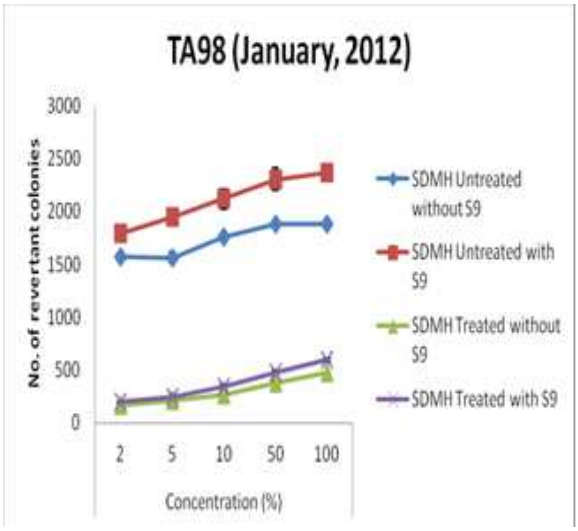
Table: 1. Mutagenicity Ratios (MR)[1] of *Salmonella* TA 98 and TA 100 in Ames test on hospitals wastewater samples

Sampling site	Concentration (%)	Mutagenicity Ratio TA98				Mutagenicity Ratio TA100			
		January, 2012		July, 2012		January, 2012		July, 2012	
		-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
SDMH (U)	2	+	+	+	+	+	+	+	+
	5	+	+	+	+	+	+	+	+
	10	+	+	+	+	+	+	+	+
	50	+	+	+	+	+	+	+	+
	100	+	+	+	+	+	+	+	+
SDMH (T)	2	+	+	+	+	-	-	-	-
	5	+	+	+	+	+	+	-	+
	10	+	+	+	+	+	+	+	+
	50	+	+	+	+	+	+	+	+
	100	+	+	+	+	+	+	+	+

Table: 2. *P* – values[2] (two-tailed) obtained in t- test analysis of Ames test data indicating significant difference between samples collected before and after treatment

Concentration (%)	TA98				TA100			
	Jan		Jul		Jan		Jul	
	without S9	with S9	without S9	with S9	without S9	with S9	without S9	with S9
2	0.000	0.001	0.000	0.001	0.000	0.000	0.001	0.002
5	0.001	0.001	0.000	0.001	0.000	0.001	0.001	0.001
10	0.000	0.002	0.000	0.001	0.000	0.001	0.000	0.001
50	0.000	0.002	0.001	0.000	0.000	0.000	0.000	0.000
100	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000

1 MR; +, Ratio greater than 2.0 indicating possible mutagenicity; -, Ratio less than 2.0 indicating non mutagenicity.
2 All values are significant; $P<0.05$



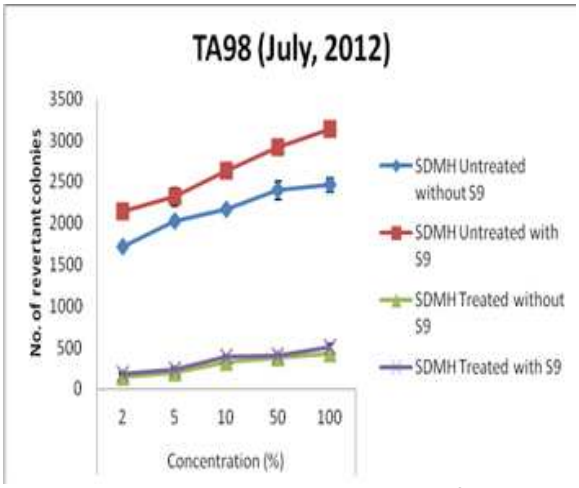
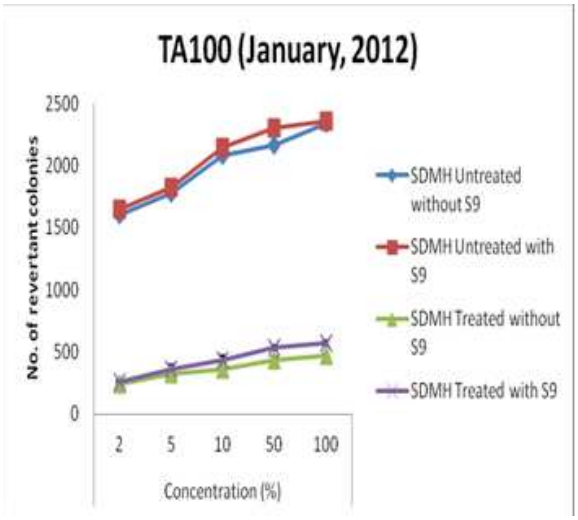


Fig: 1. Concentration-response curves for SDMH wastewaters with strain TA98 in absence and presence of metabolic activation



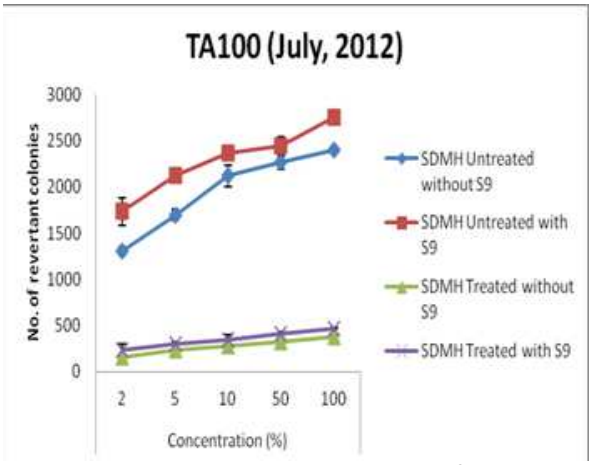


Fig: 2. Concentration-response curves for SDMH wastewaters with strain TA 100 in absence and presence of metabolic activation

Table: 3. EC_{20} and EC_{50}Values[3] for hospitals waste waters in Baker's Yeast bioassay

S. No.	Sample site		EC_{20}		EC_{50}	
			(µL*)	(%)	(µL*)	(%)
1	SDMH Untreated	Jan	0.72	0.36	26.67	13.34
		Jul	0.30	0.15	8.79	4.4
2	SDMH treated	Jan	4.71	2.36	67.90	33.95
		Jul	2.23	1.12	47.68	23.84

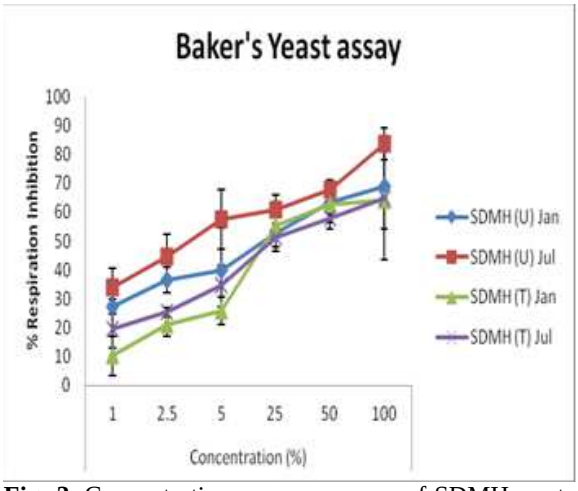


Fig: 3. Concentration-response curve of SDMH waste water samples in Baker's Yeast respiration inhibition assay

3 EC_{20} and EC_{50}: values showing 20% and 50% reduction of respiring yeast cells.

*Volumes of samples added to distilled water to make total volume upto 200µL used for the assay.

[IV] CONCLUSION

Concluded from the above results it can be interpreted that untreated hospital effluents possessed the potential to contaminate the surface and even underground water, thereby making it unfit for irrigation and drinking. In contrast, the treated water samples were found to be slightly genotoxic and mildly cytotoxic, thus inferring that genotoxicity is reduced after the treatment process. This confirms that the ETP at SDMH is efficient enough in terms of genotoxicity and cytotoxicity reduction.

The pollution loads from the hospitals are increasing and therefore cannot be treated together with the municipal effluents. Hence, there is a need to rethink the disposal of hospital waste water into municipal treatment plants. The results of this study affirm that a properly designed Effluent Treatment Plant is a feasible solution to avoid hazardous consequences resulting from discharge of untreated hospital wastewaters. An advanced and efficient on-site treatment of hospital wastewater is, therefore, strongly recommended instead of dumping the wastewater directly into municipal sewers. This study also builds up a basic groundwork to acquire more information about the prevalence and levels of mutagenic and cytotoxic agents in hospital wastewaters. Extensive monitoring programs should further be performed in order to discover possible sources of genotoxic and mutagenic substances. More work need to be done in the analytical field to identify and quantify the main compounds responsible for the genotoxicity.

This study has also demonstrated that it is possible to carry out the ecotoxicological risk assessment of hospital effluents both before and after treatment by the use of standardized bioassays. Being simple, quick, sensitive, cost effective and relatively easy to perform, short term bioassays can assess harmfulness of effluents conveniently. The growing interest in these tests is due to the fact that despite the

existence of different mechanisms of toxicity and sensitivities of different organisms, a substance that is toxic in one organism often can be used as an indicator of toxicity to other organisms [12]. The bioassays employed in present study can be used as monitoring tools to pre-screen waste waters for the presence of chemicals with genotoxic and cytotoxic potential. Thus, this study allows a quantitative risk characterization of hospital wastewaters and; any water utility equipped to perform bacteriological examination of water, can utilize this procedure as a part of standard tests for quality examination of wastewaters. However, while short-term microbial assays have the potential to be extrapolated to higher organisms in vivo animal studies are still required to validate their use in support of the assessment of health care wastewaters toxicity, before approving them for routine examination.

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