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Research Article

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Cytotoxicity Assessment of Nanoparticulate Doxycycline (DH LNP) and Rifampicin against Human Macrophage (U 937) Cell Line

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ABSTRACT

The present study was conducted in order to investigate the cytotoxic effect of Doxycycline (DH), Rifampicin (RIF) and their nanoparticulate formulations (DH LNP and NP RIF) in human U937 macrophages. These drugs are commonly used for the treatment of brucellosis. The DH was found cytotoxic for the cells at 10 µg /ml whereas at concentrations ranging from 0.5 - 5 µg /ml it proved non-toxic. The DH LNP was found to be safe for macrophages between the concentrations of 0.5 - 2 µg/ml however it proved toxic at 5 µg/ml and above. The RIF was non-cytotoxic between the range of 0.5 to 10 µg /mL whereas it was cytotoxic at 20 µg/mL & above. The NP RIF proved safe for macrophages up to a concentration of 5 µg/mL whereas, at 10 µg/mL and above it proved cytotoxic. The comparison of results revealed a greater cytotoxicity of nanoparticulate formulations of drugs than the plain drugs. The combination of DH + RIF was non-cytotoxic between the range of 0.5 to 20 µg /mL, however, it proved cytotoxic at 25 µg/mL & above. Similarly, the combination of nanoparticulate drugs DH LNP + NP RIF was found to be safe for macrophages up to the concentration of 20 µg/ml while it proved cytotoxic at concentration of 25 µg/ml and above. Both the methods employed for assessment of cytotoxicity i.e. trypan blue dye exclusion technique and MTT assay were found equally effective.

Key words: *Cytotoxicity, Doxycycline, Human 937, Nanoparticle, Rifampin,*

INTRODUCTION

Doxycycline is an antibiotic that is used in the treatment of a number of types of infections caused by bacteria and protozoa. It is useful for bacterial pneumonia, acne, chlamydia infections, early Lyme disease, cholera and syphilis. It is also useful for the treatment of malaria when used with quinine and for the prevention of malaria. Doxycycline can be used either by mouth or intravenously[1].

Rifampicin, also known as rifampin, is an antibiotic used to treat several types of bacterial infections, including tuberculosis, *Mycobacterium avium* complex, leprosy, and Legionnaire's disease.

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It is almost always used along with other *anti*biotics, except when given to prevent *Haemophilus influenzae* type b and meningococcal disease in people who have been exposed to those bacteria[2].

Brucellosis is major zoonotic disease and represents a serious health effects in human and animals, worldwide including India. Standard regimens for brucellosis, which include doxycycline plus streptomycin or rifampin, a combination of one of these drugs with ciprofloxacin has also been used in recent years[3,4]. The organisms of the genus Brucella are known to have a high degree of "in vitro" susceptibility to a number of antimicrobial agents[5]. However, their location, which is predominantly intracellular, protects them from the actions of many antibiotics. This fact, together with its good intracellular penetration and ease of administration, made it a drug of great interest for the treatment of brucellosis[6]. However, when it was used in combination with doxycycline there was a clear synergism and a good therapeutic efficacy. In 1986, the Expert Committee on Brucellosis of the Food and Agriculture Organization-World Health Organization recommended the combination of doxycycline-rifampin as the treatment of choice for human brucellosis[7].

There has been a growing interest in the recent years on application of drug delivery systems for targeted and site specific delivery of drugs. The emergence of nanotechnology has further influenced the drug delivery sector, affecting just about every route of administration from oral to injectable. The technology allows site specific delivery, lowers drug toxicity, reduces cost of treatment, improves bioavailability and extends the economic life of proprietary drugs[8]. From the first liposome proposed by Gregoriadis *et al.*[9] till today, there has been an explosion in the number of nanodevices suitable for drug delivery, which are either made up of lipids or composed of polymers (liposmes and microspheres). Application of nanotechnology in designing targeted drug delivery systems for infectious diseases like human immunodeficiency virus (HIV), leishmaniasis, malaria, tuberculosis is therefore being increasingly considered[10]. Hence present study was planned to investigate the cytotoxicity of doxycycline and rifampicin with nanoparticulate drug delivery system on human macrophages.

MATERIAL AND METHODS

Antimicrobial compounds tested: Total seven antimicrobial agents formed from the plain and nanoparticulate Doxycycline and Rifampicin were obtained from Institute of Chemical Technology, Matunga, Mumbai, were as follows, Plain Doxycycline hydrochloride (DH), Doxycycline Hydrochloride Lipomer (DH LNP), Plain rifampicin (RIF), Nanoparticulate Rifampicin (NP RIF) , Combination Doxycyline + Rifampicin (DH + RIF), Combination Doxycyline Lipomer + Nanoparticulate Rifampicin (DH LNP + NP RIF), Plain Rifampicin + Nanoparticulate (RIF + NP RIF)

Human macrophage cell line (U-937): Human macrophage cell line of histiocytic lymphoma origin U-937 was procured from National Centre for Cell Science (NCCS), University of Pune campus, Pune and maintained in the Department of Microbiology, Bombay Veterinary College as per standard protocol provided by NCCS.

Maintenance and propagation of U937 Human Macrophage cell line : Propagation and maintenance of U-937 human macrophage cell line was carried out as per the protocol provided by NCCS. Briefly, the cells were centrifuged at 1000 rpm for 5 min and supernatant was discarded. The pellet was resuspended in RPMI 1640 growth medium supplemented with 10 per cent fetal calf serum (FCS) and 2 mM L-glutamine. The density of the cells was adjusted to 1 x 10^5 viable cells /ml and cells were distributed in tissue culture flasks, followed by incubation at 37^{o}C in 5 per cent carbon-dioxide atmosphere in the CO_2 incubator. The growth medium was changed after every 2 to 3 days.

Assessment of cell viability : Viability of cells was assessed by trypan blue dye

exclusion technique. A 100 µl cell suspension was mixed with equal volume of working solution of trypan blue dye and after incubation at room temperature for 3 min., viable cells (unstained) were counted using Neubauer's chamber. The number of viable cells was calculated by following formula.

No. of viable cells = N X 10^4 X d Where N = Average number of viable cells counted in four WBC chamber of hemocytometer and d = dilution factor.

The viability of U937 human macrophages was assessed each time when the cells were sub cultured by trypan blue dye exclusion technique. Dead cells appeared blue with disrupted borders while living cells remained unstained. In general the viability of cells was found to be in the range of 90-95 per cent at each sub culturing (Plate 1).

Cytotoxicity assay : All the drug formulations included in the efficacy studies were subjected to cytotoxicity tests in order to find out non-toxic dose of the formulation. The cytotoxicity of different concentrations of DH, DH LNP, RIF, NP RIF and their combinations for U937 human macrophages was assessed by Trypan blue dye exclusion technique (Plate 2. A & B) and MTT assay as described below. The cytotoxicity was judged on the basis of percent viability of cells and drug concentrations where percent cell viability was 80 per cent and above after contact time of 24 hr were considered non-cytotoxic.

Assessment of cytotoxicity of drugs : Cytotoxicity of different concentrations of antimicrobial formulations for macrophges was assessed by trypan blue dye exclusion technique and MTT tetrazolium salt assay. The antimicrobial formulations were diluted in RPMI 1640 medium to obtain concentrations corresponding to 0.5, 1, 2, 5, 10, 20, 50 and 100 µg/ ml. A 100 µl of each of the diluted antimicrobial preparation was mixed with equal volume of cell suspension (2 x 10^6 cells/ml) in 96 well flat bottom tissue culture plate, mixed thoroughly and incubated at 37^{o}C in incubator. An untreated cell control consisting 100 µl RPMI 1640 medium containing 2 x 10^6 cells/ml without any antimicrobial compound was included in the test. Three replications of each concentration were included in the assay and the cytotoxicity was assessed at 24 hr of incubation.

Trypan blue dye exclusion assay : The cytotoxicity of the antimicrobial compounds for human macrophages was judged based on the per cent viability of cells exposed to different concentrations of drugs. The cell suspension treated with different concentrations of antimicrobial compounds was processed for assessment of cell viability by trypan blue dye exclusion assay as described earlier

MTT Assay : MTT tetrazolium salt (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was performed as per the method of Mosmann[16]. with some modifications. The assay was based on the principle that the viable cells reduced MTT tetrazolium salt forming a coloured product formazan that could be quantified spectrophometrically thereby serving as an indirect measurement of cell viability. The cells treated with drugs and untreated cells were transferred to micro centrifuge tubes and were centrifuged at 1000 rpm for 5 mins. Supernatant was discarded and cells were resuspended in 80 µl of working MTT. A 20 µl of this suspension was transferred to the wells of 96 well flat bottom tissue culture plate. The plate was wrapped with silver foil and incubated at 37^{0}C in CO_2 incubator. After 4 hr of incubation, 150 µl of dimethyl sulphoxide (DMSO) was added to each well (Plate 2 C). The absorbance of each well was measured in an ELISA plate reader at a wavelength of 550 nm. Percent viability was calculated as per following formula.

$$\text{Per cent viability} = \frac{\text{Mean O.D of each drug dilution}}{\text{Mean O.D of control wells}} \times 100$$

Where, O.D. – Optical density

U937 Human macrophage cell line : Human macrophage cell line U937 employed for efficacy studies during present investigation was successfully maintained in the laboratory using RPMI 1640 medium supplemented with 50 µg / ml of gentamicin, 2mM- glutamine and 10 % FCS. Morphologically the cells were rounded, mononuclear with crenated borders and having a rim of cytoplasm. The observations of cell morphology and staining characters in the present study are concordant with those of Kothawale [11].

Cytotoxicity : The drug carriers employed for targeted delivery of drugs are often likely to be toxic for the cells or animals used in experimentation. Further, it is also likely at times that the drug in itself may be toxic for cells. The success of the efficacy studies thus depends on selecting a non - toxic and safe dose such that the cells used in the experiment survive, phagocytose the bacteria as well as internalize the drug loaded carrier molecules. Several researchers have reported toxicity of compounds used as drug carriers *viz.* liposomes (dipalmitoyl phosphatidyl choline (DPPC) and pH-sensitive dioleoyl phosphatidyl ethanolamine (DOPE)), microspheres (poly (lactide-coglycolide) 50:50 (PLGA 50:50 and PLGA 50:50H) [12, 13, 14]. Moreover, the ultimate objective of the efficacy studies was to search an effective drug formulation that is safe and effective for clinical use. The cytotoxicity assays thus, form an integrant component of efficacy studies. We employed two methods for assessment of cytotoxicity of drug formulations; trypan blue dye exclusion technique [15] and MTT assay[16] and found both of them to be equally effective.

Cytotoxicity of DH and DH LNP : The results of cytotoxicity of various concentrations of DH and DH LNP for macrophages are presented in Table 1 & Fig. A. It is evident from the results that the DH was cytotoxic for the cells at 10 µg /ml when exposed for 24 hrs. The other concentrations of DH evaluated between the range of 0.5 - 5 µg /ml were found to be non-toxic for macrophages. The DH LNP between the concentrations ranging from 0.5 - 2 µg/ml was found to be safe and no cytotoxicity was noticed. However, at concentration of 5 µg/ml and above the DH LNP proved toxic for macrophages. It is obvious from the comparison of results of cytotoxicity tests of DH and DH LNP that the DH LNP showed greater cytotoxicity than DH.

Table -1: Assessment of cytotoxicity of DH and DH LNP for U937 human macrophage cell line

Drug Concentration (µg/ml)	Per Cent Cell Viability after 24 hr (mean)	
	DH	DH LNP
0.5	92.50	88.59
1	91.34	80.17
2	86.91	75.26
5	85.83	71.99
10	76.23	67.52
20	62.46	65.35
25	59.05	52.89
50	43.98	45.19
100	39.03	34.65

The DH was found to be non-toxic for macrophages up to a concentration of 5 µg / ml. At concentrations of 10 µg / ml and onwards, the DH proved cytotoxic for human macrophages. The toxicity of doxycycline a synthetic tetracycline has previously been reported in the literature. Sourdeval *et al.* studied the effect of doxycycline on human bronchial epithelial cell line and found a time and concentration dependent cell proliferation inhibition, decreased cell viability due to apoptosis and necrosis and cell detachment. The findings of the present investigations thus are in consistent with those of Sourdeval *et al*[17]. In contrast to DH, the DH LNP was found to be more cytotoxic. The DH LNP proved safe for macrophages only up to a concentration of 2 µg/ml whereas at concentrations of 5 µg/ml and onwards it was found to have cytotoxic effect on macrophages. The results clearly point out a greater cytotoxicity of doxycycline lipomer (DH LNP) for macrphages as compared to plain doxycycline (DH) which could possibly be due to synergistic toxic hydrophilic polymer-lipid combination (LIPOMER).

Cytotoxicity of RIF and NP RIF : The RIF proved non-cytotoxic for macrophages at concentrations ranging from 0.5 to 10 µg /mL (Table 2 & Fig. B). It was however, found to be cytotoxic at the concentration of 20 µg/mL & above. The NP RIF was found to be safe for macrophages up to the concentration of 5 µg/mL whereas, at concentration of 10 µg/mL and above it proved cytotoxic. The comparison of results of cytotoxicity assays of RIF and NP RIF suggest a greater cytotoxicity of NP RIF for macrophages than that of RIF.

Table-2: Assessment of cytotoxicity of RIF and NP RIF for U937 human macrophage cell line

Drug Concentration (µg/ml)	Per Cent Cell Viability after 24 hr (mean)	
	RIF	NP RIF
0.5	98.23	94.19
1	94.78	90.57
2	92.98	86.78
5	86.14	84.97
10	81.18	75.77
20	70.76	67.89
25	60.16	42.33
50	50.16	20.01
100	10.56	01.10

The RIF was non-cytotoxic for cells up to a concentration of 10 µg/ml whereas it proved cytotoxic at 20 µg/ml and onwards. These observations are concordant with those of Valderas *et al.*[14], who conducted an experiment to evaluate the efficacy of antimicrobials against intracellular *B. abortus* in J774 cell line. They observed no significant impairment in the viability of cells treated with rifampicin at a concentration that was eight times higher than the MIC of rifampicin. Similar findings have also been reported by Mhase *et al.*[10], while assessing the cytotoxicity of rifampicin for J774 murine macrophages.

The results of cytotoxicity of NP RIF for U937 macrophages however were contrary to those of RIF. The NP RIF was found to be safe and did not cause appreciable cell deaths up to a concentration of 5 µg/ml. However, at 10 µg/ml and onwards, it was found to exert cytotoxic effect on macrophages. It is obvious from the results of cytotoxicity assays that the NP RIF showed a greater cytotoxicity as compared to RIF and the finding was as per our expectation. The NP RIF i.e. rifampicin entrapped in polymer Gantrez AN 119 is a nanoparticulate formulation and nanoparticles have been known to trigger oxidative burst leading to release of large quantity of toxic free radicals that cause the cell death. The observations of the present investigation are also in agreement with those reported by

Mhase[10]. and Suryawanshi, who assessed the cytotoxicity of rifampicin entrapped in polymer Gantrez AN 119 and observed that the NP RIF at the concentration of 5 µg/ml and onwards was toxic for macrophages. Both RIF and NP RIF however were found non cytotoxic at their MIC levels.

Cytotoxicity of combinations: DH + RIF and DH LNP + NP RIF : The plain DH + RIF did not prove cytotoxic for U937 macrophages at concentrations ranging from 0.5 to 20 µg /ml. It was however, found to be cytotoxic at 25 µg/ml and above. Similarly, the combination of nanoparticulate drugs DH LNP + NP RIF was found to be safe for macrophages up to the concentration of 20 µg/ml while it proved cytotoxic at concentration of 25 µg/ml and above (Table 3 & Fig. C).

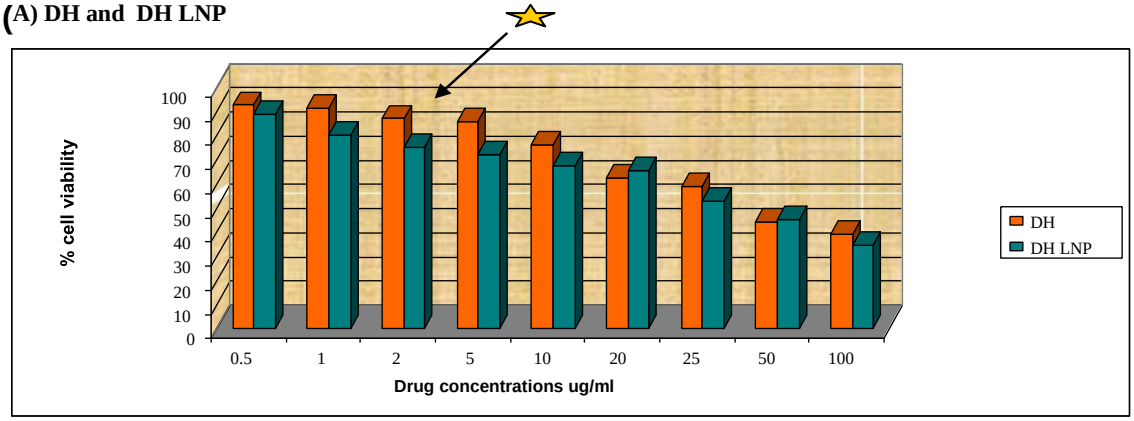
Table 3 : Assessment of cytotoxicity of DH + RIF and DH LNP + NP RIF for U937 human macrophage cell line

Drug Concentration (µg/ml)	Per Cent Cell Viability after 24 hr (mean)	
	DH + RIF	DHLNP + NP RIF
0.5	100	100
1	98.48	97.39
2	98.32	97.04
5	97.49	95.84
10	94.70	91.05
20	93.86	71.05
25	89.61	67.46
50	26.13	29.08
100	25.36	30.35

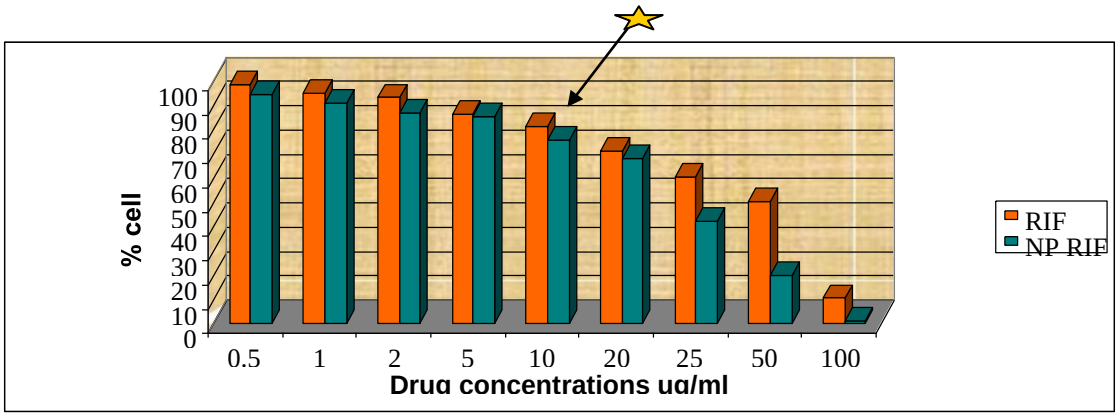
The cytotoxicity of combinations of drugs including DH + RIF and DH LNP + NP RIF was also assessed in a manner similar to that described earlier. The DH + RIF combination was found to be non toxic at concentration 20 µg/ml & below, while, DH LNP + NP RIF proved safe at the dose rate of 5 µg/ml and below. It is evident from the results that the combination nanoparticulate drug formulations proved to be more toxic than that of plain drugs, the possible reasons of which have been discussed earlier.

Comparative cytotoxicity of different drug formulations : Overall comparison of the results of cytotoxicity tests carried out with different drug formulations revealed that the plain drugs including DH and RIF were relatively less cytotoxic than their nanoparticulate formulations (DH LNP and NP RIF) (Fig. A.- C.).

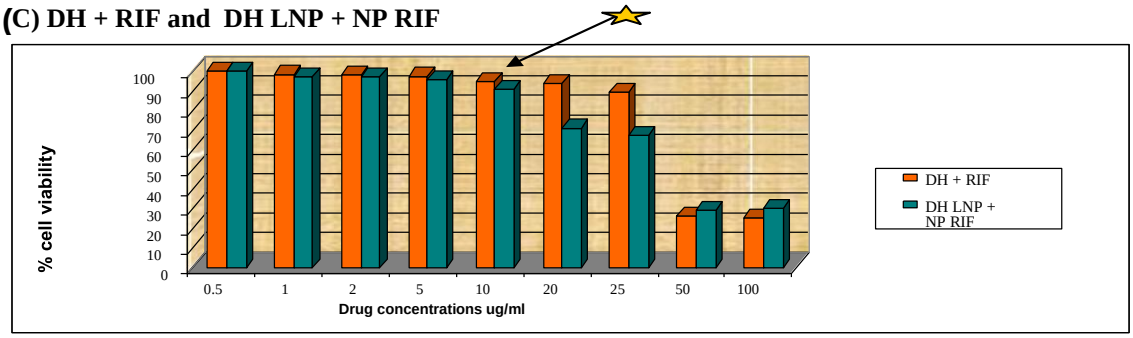
Cytotoxicity of plain and nanoparticulate drugs for U937 human macrophage cell line



(B) RIF and NP RIF



(C) DH + RIF and DH LNP + NP RIF



Maximum non toxic dose

Plate 1 : Assessment of cell viability by trypan blue dye exclusion Techniqe (400X)

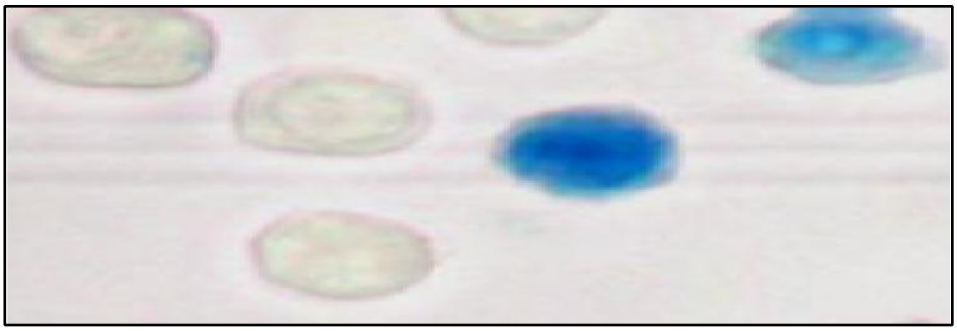


Plate 2A: Assessment of cytotoxicity by trypan blue dye exclusion technique (400X)

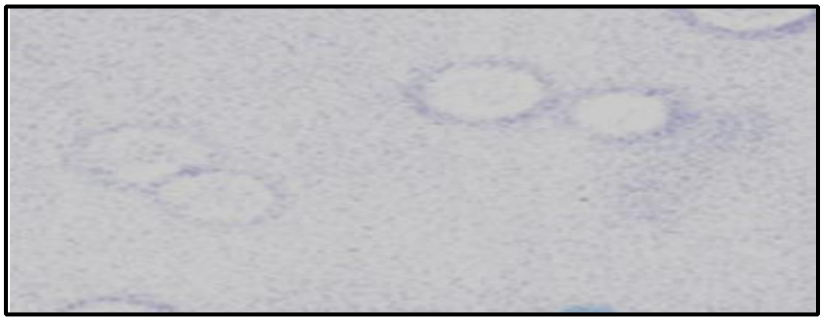


Plate 2(B): Assessment of cytotoxicity by trypan blue dye exclusion technique (400X)

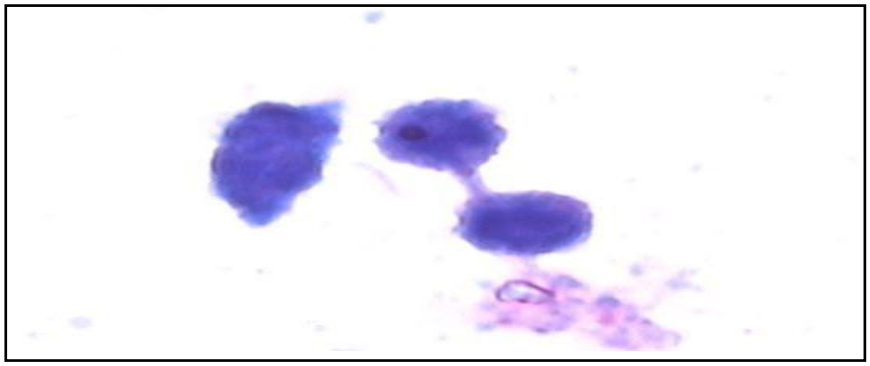
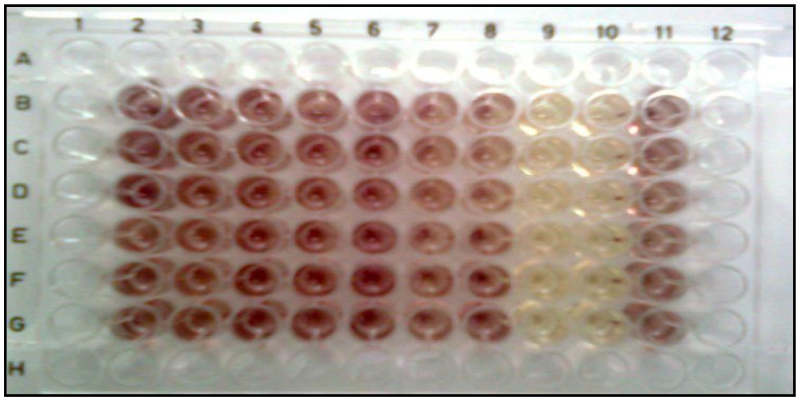


Plate 2 C.: Assesment of cytotoxicity by MTT assay



CONCLUSIONS

1. The maximum non-toxic dose of DH for macrophages was 5 µg /ml whereas it proved cytotoxic at concentration of 10 µg/ml and onwards. Whereas the maximum non-toxic dose of DH LNP for macrophages was 2 µg /ml whereas it was found cytotoxic at concentration of 10 µg/ml and onwards.
2. The maximum non-toxic dose of RIF for macrophages was 10 µg /ml whereas it was found cytotoxic at concentration of 20 µg/ml and onwards. Whereas the maximum non-toxic dose of NP RIF for macrophages was 5 µg /ml whereas it was found cytotoxic at concentration of 10 µg/ml and onwards.
3. The maximum non-toxic dose of combination DH+RIF for macrophages was 20 µg/ml while the combination proved cytotoxic at 25 µg/ml and onwards. Whereas the maximum non-toxic dose of combination DH LNP + NP RIF was 20 µg/ ml whereas the combination proved cytotoxic at 25 µg/ml and onwards.

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