Assessment of the potential skin irritation of lysine-derivative anionic surfactants using mouse fibroblasts and human keratinocytes as an alternative to animal testing

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ABSTRACT

Purpose. The aim of this study was to identify new surfactants with low skin irritant properties for use in pharmaceutical and cosmetic formulations, employing cell culture as an alternative method to in vivo testing. In addition, we sought to establish whether potential cytotoxic properties were related to the size of the counterions bound to the surfactants.

Methods. Cytotoxicity was assessed in the mouse fibroblast cell line 3T6, and the human keratinocyte cell line NCTC 2544, using the MTT assay and uptake of the vital dye neutral red 24 h after dosing (NRU).

Results. Lysine-derivative surfactants showed higher IC_{50}s than did commercial anionic irritant compounds such as sodium dodecyl sulphate, proving to be no more harmful than amphoteric betaines. The aggressiveness of the surfactants depended upon the size of their constituent counterions: surfactants associated with lighter counterions showed a proportionally higher aggressivity than those with heavier ones.

Conclusions Synthetic lysine-derivative anionic surfactants are less irritant than commercial surfactants such as sodium dodecyl sulphate and Hexadecyltrimethylammonium bromide and are similar to Betaines. These surfactants may offer promising applications in pharmaceutical and cosmetic preparations, representing a potential alternative to commercial anionic surfactants as a result of their low irritancy potential.

Key words: skin irritation, lysine-derivative surfactants, cytotoxicity, fibroblast, keratinocyte

INTRODUCTION

Surfactants are common constituents in many topical drugs and cosmetics. They are often used as additives in pharmaceutical and dermatological preparations, cleansers, soaps and shampoos, due to their surface and interface properties (1).

Application of active ingredients and pharmaceutical additives may cause skin irritation; the majority of adverse skin reactions to personal-care products are presumed to be caused by surfactants (2). As a result, it is of great interest to identify surfactants with low irritant properties (3), and it is necessary to develop rapid assays to assess potentially damaging effects.

Evaluation of the potential for an ingredient or product to cause skin irritation is one of the various studies undertaken in the overall safety assessment process. Testing for skin corrosion or irritation has traditionally been conducted in animals, particularly in rabbits via the Draize test method (4). However, due to increasing concern over animal experimentation and its potential prohibition in the near future (5), alongside the obvious ethical implications of the use of human subjects, *in vitro* alternatives must now be developed.

In vitro toxicity testing systems also offer several advantages over *in vivo* systems, an obvious advantage being their immediate availability and reproducibility (6). Cell culture has been gradually introduced as an *in vitro* technique for the assessment of skin irritancy (7,8). Skin cultures are useful for the design of safer, more efficient and cost effective human skin irritation tests, in certain cases, eliminating the need for human or animal skin and *In vitro* cytotoxicity has generally been found to be a useful predictor of skin irritation potential (5).

Skin irritation is a reversible inflammatory reaction produced by the arachidonic acid cascade and cytokines in the viable keratinocytes and fibroblasts of the skin (9). Because of the increasing appreciation of the complex and dynamic regulatory role played by these cells in terms of the inflammatory responses to irritants and sensitizers, we chose to use both 3T6 and NCTC 2544 cells as model cell systems: the use of keratinocyte and fibroblast cultures offering an appropriate in vitro model for skin irritation (10-12).

Previous studies have suggested that cultured normal human keratinocytes may be predictive of irritancy caused by various surfactants in human subjects (13) and these

monolayer cultures have been compared with *in vivo* skin irritation data (14,15). A good correlation with in vivo human skin data has been demonstrated for surfactants of different chemical types and irritation potential (16). However, in spite of the advantages of *in vitro* models, cell culture lacks some of the properties of intact skin, such as its selective barrier role or the interaction between different cell types. Thus, although irritation potential may be overestimated**,** it can nevertheless function as a useful pre-screening tool (11).

Cell cytotoxicity assays are amongst the most common *in vitro* bioassay methods used to predict the toxicity of substances in various tissues and the potential of a chemical to elicit a corrosive response can be successfully predicted using appropriate endpoints because they demonstrate the degree of damage caused by the chemical. Thus,to assess the *in vitro* cytotoxic effects of surfactants, we measured cell viability through neutral red uptake and the MTT assay.

We have investigated the cytotoxic effects of five anionic lysine-derivative surfactants on human keratinocyte NCTC 2544 and Swiss albino 3T6 mouse fibroblast cultures in order to predict their skin irritation potential. We have also evaluated the relationship between potential cytotoxic properties and the size of the counterions bound to the surfactants.

MATERIALS AND METHODS

Chemicals

L-lysine monohydrochloride, L-Lysine, Tris and the bases NaOH, LiOH, KOH and Sodium dodecyl sulphate (SDS) were purchased from Merck (Darmstadt, Germany), TEGO® Betaine T-50 (TGB) was obtained from Goldschmidt Ltd (Essen, Germany). RPMI 1640 medium without glutamine, L-Glutamine, Phosphate Buffered Saline (PBS) and Foetal Calf Serum (FCS) were supplied by Reactiva (Beit Haemek, Israel). Neutral red (NR) dye, MTT salt, Dimethylsulphoxide (DMSO) and Hexadecyltrimethylammonium bromide (HTAB) were supplied by Sigma-Aldrich (St Louis, MO, USA).

Penicillin (10000U/mL) Streptomycin (10000 μg/mL) mixture and Foetal Bovine Serum (FBS) were purchased from Bio-Whittaker (Verviers, Belgium).

Surfactants

Five types of salts were tested in this study: lysine salt (77KK); tris(hydroxymethyl)amminomethane salt (77KT); sodium salt (77KS); lithium salt (77KL) and potassium salt (77KP) (Fig. 1). Anionic surfactants of the salts derived from N^{α},N^{ε}-dioctanoyl lysine were synthesized in our laboratory according to the procedure described previously (18). The potassium salt is a new lysine-derivative surfactant synthesized for first time to perform this study according to the same procedure described previously.

Cell cultures

The normal human keratinocyte cell line, NCTC 2544, (provided by Interlab Cell Line Collection of Genoa, Italy) and the mouse cell line, 3T6, were cultured in RPMI 1640 medium supplemented with 10% Foetal calf serum (FCS), , 2mM L-glutamine and 1% Penicillin (10000U/mL)/Streptomycin (10000 µg/mL) mixture at 37ºC, 5%CO_2.

When 75 cm^2 culture flasks were approximately 80% confluent, the cells were seeded ($5x10^4$ cells/mLl for NCTC 2544 and $4x10^4$ cells/mL for 3T6) into the central 60 wells of 96 well plates and then incubated at 37ºC, 5%CO_2 for 24 hours.

Experimental treatments

24 hours after seeding in 96 well plates, cultures were exposed to several concentrations (from 7µg/mL to 500µg/mlL of the surfactants (sterilized by filtration) dissolved in RPMI medium supplemented with 5%FCS, 2mM glutamine and 1% antibiotic mixture. Controls, containing culture medium only, were included in each plate. Plates were then incubated at 37ºC, 5% CO_2 for 24 hours.

NRU assay

The NRU assay was performed according to the method described previously (17). After treatment with the surfactants, medium was aspirated and replaced with 100µl per well of NR solution (50 µg/mlL in culture medium). After 3 hours incubation at 37ºC, 5% CO_2, medium was aspirated, cells were washed twice with PBS and a solution containing 50% absolute ethanol, 1% acetic acid in distilled water (100µl/well) was added to extract the dye.

After 10 minutes shaking on a microtitre-plate shaker, the absorbance of the solutions was read at a wavelength of 550 nm in a Bio-Rad 550 microplate reader.

MTT assay

The MTT assay was performed according to the method of Mosmann (18). After treatment with the surfactants, medium was aspirated and replaced with 100µl per well of the MTT solution (dissolved at 5mg/mL in PBS and diluted 1:10 in cell culture medium without phenol red). At the end of the 3 hour incubation, cultures were washed once with PBS and 100µl/well of dimethylsulphoxide (DMSO) was added to dissolve the purple formazan product while shaking for 10 minutes at room temperature. The absorbance of the resulting solutions was read at a wavelength of 550nm in a Bio-Rad 550 microplate reader.

Statistical analysis

Both NRU and MTT experiments were performed at least 3 times using three wells for each concentration of the surfactant.

The cytotoxicity of each surfactant was expressed as the percentage viability compared to controls in terms of its IC_{50} (concentration of surfactant that causes 50% inhibition of growth), calculated from the dose-response curves by linear regression analysis. The NRU assay is expressed as percentage uptake of neutral red dye by the lysosomes and the MTT assay as percentage reduction of tetrazolium salt by the mitochondrial enzyme.

RESULTS AND DISCUSSION

In the present work, we studied the cytotoxicity of five anionic lysine-derivative surfactants, compared with three commercial surfactants: an irritant anionic surfactant, sodium dodecyl sulphate (SDS); a slight irritant amphoteric surfactant, TEGO® Betaine (TGB); and a highly irritant cationic surfactant, Hexadecyltrimethylammonium bromide (HTAB).

The effects of the surfactants on cell membrane integrity were measured using two different endpoint assays for *in vitro* cytotoxicity: measurement of mitochondrial dehydrogenase activity (MTT) and a colorimetric assay of the ability of live cells to take up neutral red (NRU).

Results of NRU and MTT tests, carried out in keratinocytes and fibroblasts, were obtained at different concentrations for the surfactants tested and are presented as dose-response curves (Fig. 2 and Fig. 3). Uptake of neutral red dye by lysosomes and mitochondrial viability decreased with increasing surfactant concentration. Although similar curve profiles were obtained in both tests, with the exception of HTAB, in which the results were similar, the IC_{50} values obtained by NRU analysis were higher than those with MTT (Table 1). A possible explanation for this is that the cytotoxic effect of the majority of compounds was greater in the MTT reduction than in the neutral red uptake because mitochondria might generally be a more prominent site of surfactant cytotoxicity in the cells while high NRU values show an increase of the NR dye into the cells and thus a decrease in lysosomal damage.

Both the NRU and MTT methods have been used as indirect measures of cell vialbility (19,20) The IC_{50} values of the surfactants studied (Table 1) reveal that the lysine-derivative compounds are less cytotoxic, and thus predicted to be less irritant, than the commercial surfactants HTAB and SDS. The lysine-derivative surfactants showed higher IC_{50}s than SDS, one of the most widely used surfactants, which has been shown to damage barrier function by denaturation of the corneocytes and alteration intercellular lipids (19), whilst the commercial cationic surfactant HTAB was the most cytotoxic compound in all experiments, its IC_{50} value being two hundred orders of magnitude lower than that of the other compounds tested. Although the nature of TEGO® Betaine is not the same as that of the five anionic surfactants tested here, the results suggest that these compounds are as harmless as amphoteric betaines. In agreement with this, a number of studies have shown that betaines cause less irritation both in skin and oral personal products than SDS (2).

No significant differences in the cytoxicicty, and thus the predicted dermal irritation of the lysine-derivative surfactants were observed in our study. Nevertheless, there was a clear trend towards the surfactants bound to the heavy counterions, Lysine (77KK) and Tris (77KT), being less irritant than those bound to light counterions. Neither surfactants associated with heavy counterions nor surfactants associated with light counterions were found significant differences in the cytotoxicity. In agreement with these data, we may conclude that there was a relationship between the size of the counterion and the cytotoxic properties of these surfactants: the heavy counterion they are bound to, the lower

cytotoxicity they induce. In light of this observation, it is interesting to note that similar results have been reported for the induction of necrosis or apoptosis in a mammalian cell line: of the surfactants studied, the 77KT surfactant showed significantly lower apoptotic and necrotic activity (20). However, we have not found significant differences between both surfactants associated with heavy counterions (77KT and 77KK. The physico-chemical properties of anionic surfactants depend on the counterion they were bound to. To further research of the irritation potential toxicity of the lysine-derived surfactants (21), the results obtained in this study can be related to previous works performed in our laboratory to test these compounds on human and rat erythrocytes (22,23).

By comparing the IC_{50}s (concentration killing 50% of the cell population), it is clear that**,** with the exception of SDS, the cytotoxicity of the compounds in the 3T6 fibroblasts was greater than in the NCTC 2544 keratinocytes, as evaluated by both NRU and MTT methods. The irritancy classification of the compounds was different in both cellular models, due to morphological and physiological differences between the cell lines. These results indicate that keratinocytes might be more resistant to surfactant exposure because of their ultrastructure. Keratinocytes have been described as epithelial-like cells (24), whilst the fibroblasts have a dermal origin (25). Thus, the use of human keratinocytes may be of greater human relevance in the prediction of skin irritation.

CONCLUSIONS

According to the results of the present work we conclude that the synthetic lysine-derived anionic surfactants are less irritant than the commercial surfactants tested (HTAB and SDS) and similar to Betaines.

Our results also show that the aggressiveness of the surfactants depends on the size of their constituent counterions: higher in surfactants associated with light counterions than in those carrying heavier ones.

In conclusion, these surfactants may be of interest for use in pharmaceutical and cosmetic preparations and represent an alternative to commercial anionic surfactants as a result of their low irritancy potential.

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Table 1. Cytotoxicity of surfactants and three commercial surfactants in NCTC 2544 human keratinocytes and 3T6 mouse fibroblasts evaluated as IC_{50} (the dose to inhibiting viability to 50%). Mean ± SEM

	3T6 Fibroblasts (IC_{50} μg/ml)		**NCTC 2544 Keratinocytes** (IC_{50} μg/ml)	
Surfactant	**NRU test**	**MTT test**	**NRU test**	**MTT test**
77KK	206.1 ± 4.25	129.07± 14.09	184.2 ± 9.99	166.45 ± 5.75
77KT	172.53 ± 17.57	137.68 ± 11.21	191.4 ± 10.63	175.63 ± 9.77
77KP	149.65 ± 6.85	114.92 ± 13.17	159.5 ± 10.45	133.63 ± 7.39
77KS	159.63 ± 14.21	116.12 ± 8.35	182.7 ± 7.95	138.14 ± 8.39
77KL	143.72 ± 2.21	104.4 ± 2.2	149.2 ± 16.9	108.47 ± 6.29
SDS	71.11 ± 5.11	63.86 ± 4.57	53.53 ± 0.69	44.67 ± 1.71
HTAB	0.46 ± 0.22	0.78 ± 0.13	2.07 ± 0.18	2.17 ± 0.15
TGB	165.66 ± 19.75	102.6 ± 3.96	203.23 ± 16.23	117.87 ± 13.70

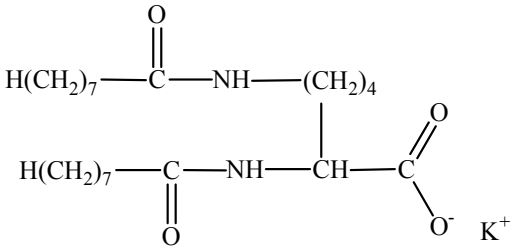
Legends to figures

Figure 1. Chemical structure and code of the lysine-derivative surfactants. K represents lysine in the international abbreviation.

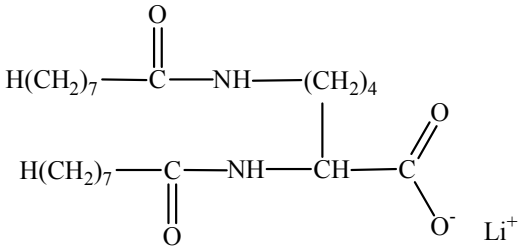
Figure 2. Comparative cytotoxicity of compounds 77KK (◆) and 77KT (□) (a,b); 77KS (△), 77KP (-) and 77KL (●) (c,d) in NCTC 2544 human keratinocytes as detected with: NRU and MTT assays. Results are expressed as mean ± SEM of three experiments.

Figure 3. Comparative cytotoxicity of compounds 77KK (◆) and 77KT (□) (a,b); 77KS (△), 77KP (-) and 77KL (●) (c,d) in Swiss Albino 3T6 mouse fibroblasts as detected with: NRU and MTT assays. Results are expressed as mean ± SEM of three experiments

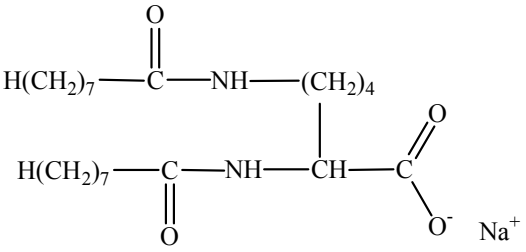
77KP



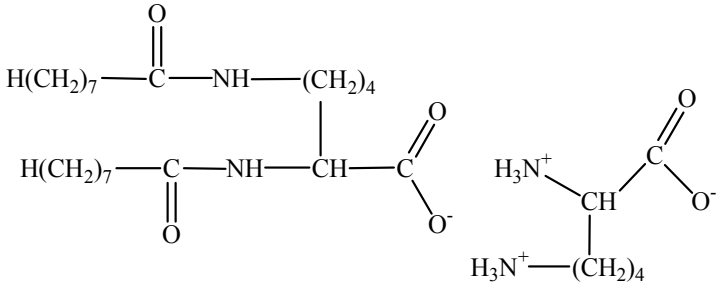
77KL



77KS



77KK



77KT

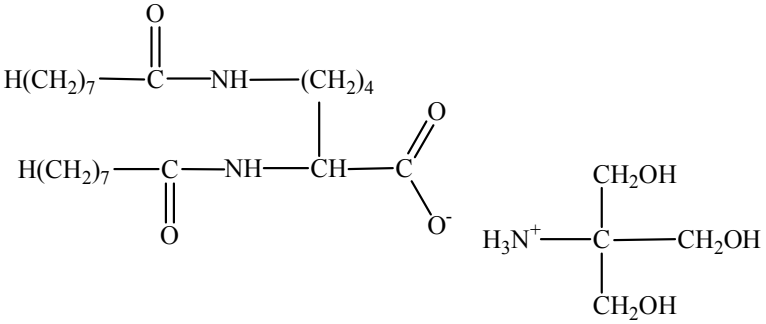


Figure 1

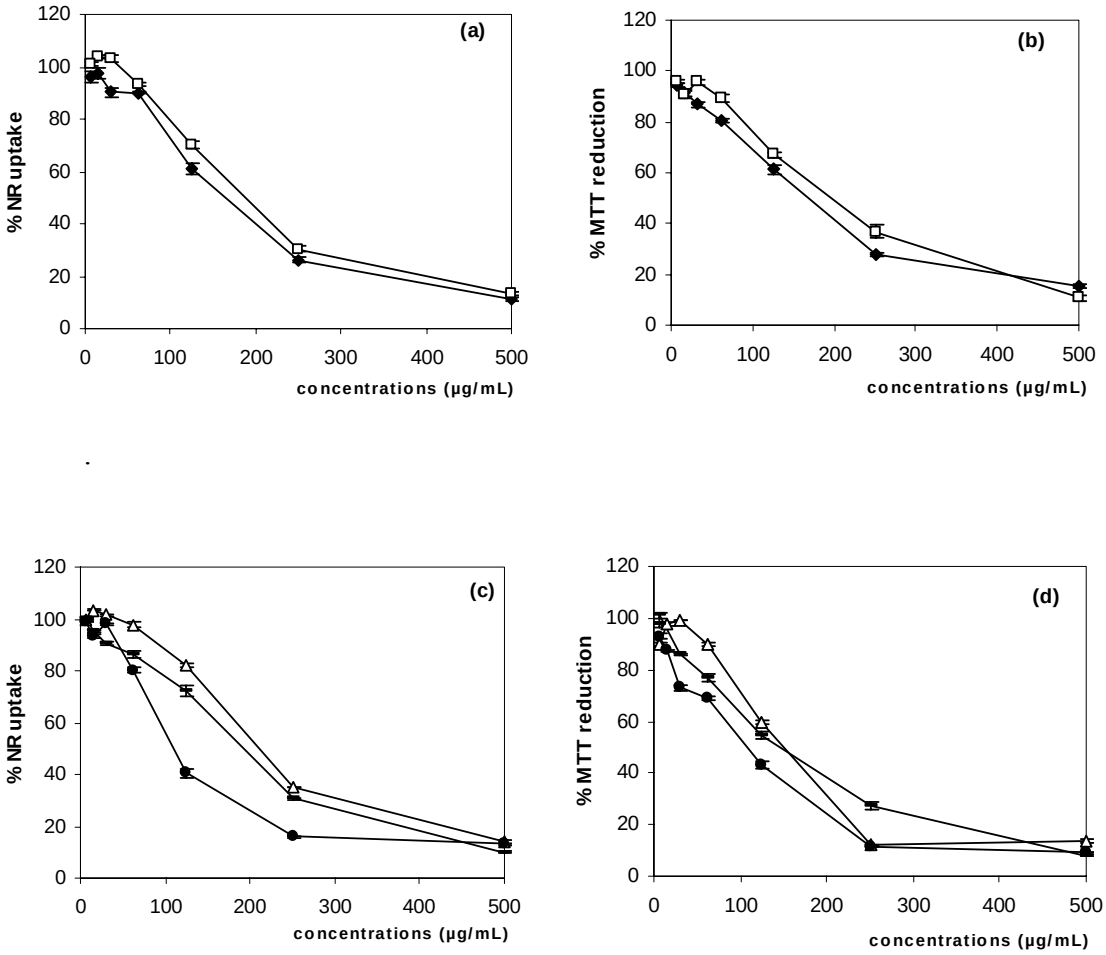

(a)
% NR uptake
concentrations (µg/mL)
(b)
% MTT reduction
concentrations (µg/mL)
(c)
% NR uptake
concentrations (µg/mL)
(d)
% MTT reduction
concentrations (µg/mL)

Fig.2

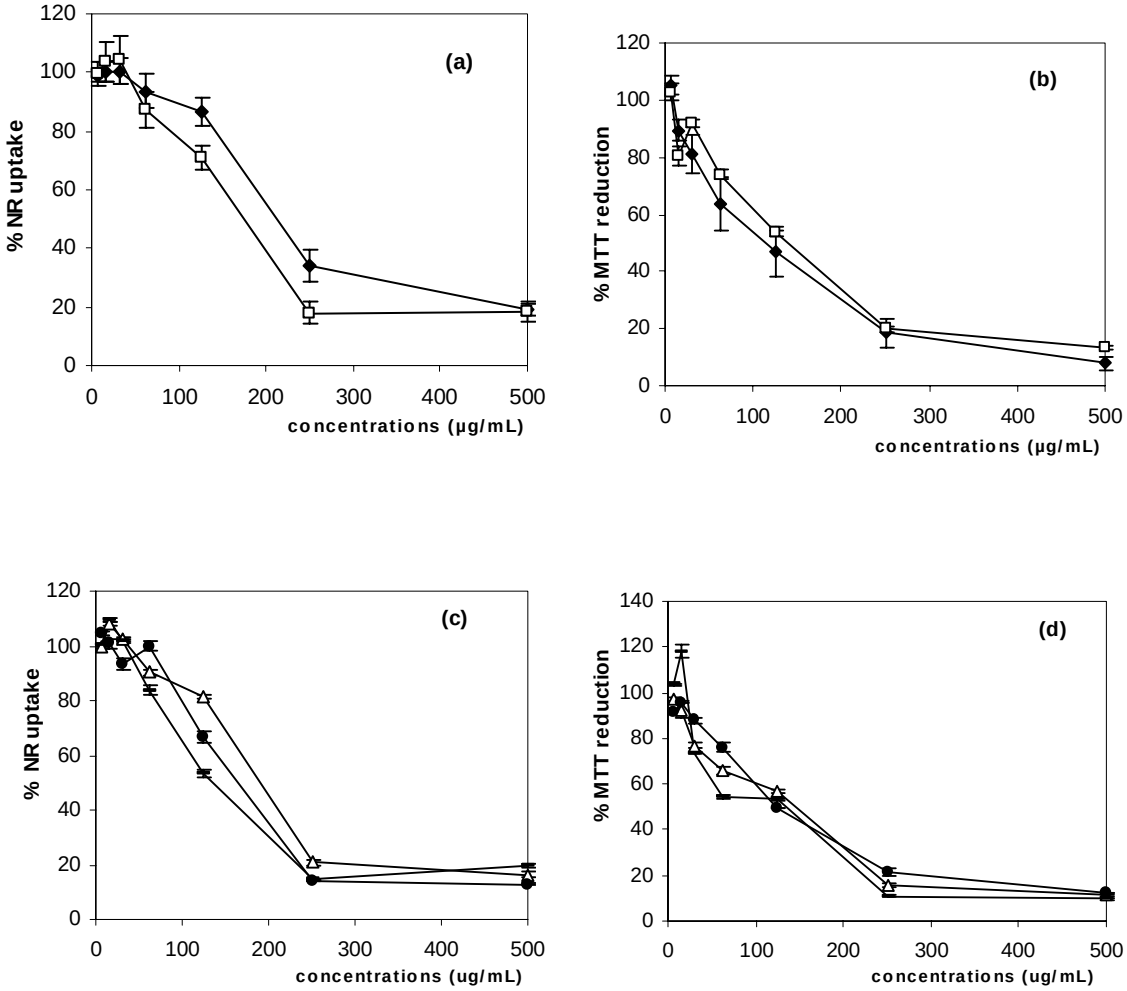

(a)
% NR uptake
concentrations (µg/mL)
(b)
% MTT reduction
concentrations (µg/mL)
(c)
% NR uptake
concentrations (ug/mL)
(d)
% MTT reduction
concentrations (ug/mL)

Fig.3