Original Article

Evaluation of *in vitro* toxicity of Rumalaya liniment using mouse embryonic fibroblasts and human keratinocytes

Sandeep Ravi Varma, Ashok Godavarthi, Satyakumar Vidyashankar, Krishna S. Nandakumar, Pralhad Sadashiv Patki
Department of Cell biology and Immunology, Research and Development, The Himalaya Drug Company, Bangalore, Karnataka, India

The skin irritation potential of topical formulations is investigated prior to human exposure to identify the chemicals which might induce adverse skin reactions. Rumalaya liniment (RL) a novel formulation using natural oils and plant extracts is used for reducing inflammations associated with musculoskeletal disorders. Preclinical studies on RL are needed prior to skin application. The aim of the study was to evaluate the possible cytotoxic effects of RL and a commercial sample (CS) on mouse embryo fibroblasts and human keratinocytes using neutral red uptake, (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and resazurin assay. The CTC_{50} values obtained for RL was significantly higher than that of CS, which revealed that RL is less toxic to CS. RL was less toxic (<17%) on both cell lines at 400 µg/ml and was nontoxic at further lower concentrations, whereas the toxicity of CS was above 59% even at 400 µg/ml. It was observed from the present study that by using three different assay methods and two different cell lines, the toxicity of RL was significantly lower than that observed with CS. From the study, it could be concluded that RL could be safer to skin due to their low cytotoxicity as compared with CS.

Key words: Cell based assay, cell lines, cytotoxicity, herbal formulation, skin irritation

INTRODUCTION

Rumalaya liniment (RL) is a poly herbal formulation used for management of pain and inflammation associated with the musculoskeletal inflammatory disorders. Skin irritation potential of formulations is investigated prior to human exposure, since application of several active ingredients and pharmaceutical additives have been reported to cause skin irritation.[1] The majority of adverse skin reactions are presumed to be caused by surfactants. It is mandatory to develop rapid assays to assess potentially damaging effects of these products.[2] Preclinical studies on the toxicity of RL are needed to ensure its safety prior to skin application.

Each ml of RL contains oil extracts of bakuchi (*Psoralea corylifolia*), maricha (*Piper nigrum*) at a concentration of 35 mg each, 365 mg of Tila oil (*Sesamum indicum*), 350 mg of Gandhapura taila (*Gaultheria fragrantissima*), 50 mg of sarala oil (*Pinus roxburghii*) and powders of karpura (*Cinnamomum camphora*), pudina satva (*Mentha arvensis*) and ajamoda satva (*Carum copticum*) at an amount of 90, 40 and 35 mg, respectively.

Access this article online	
Quick Response Code:	Website: www.greenpharmacy.info

No reports are available on the toxicity studies of Rumalaya till date. The only available report is on the reproductive toxicity of Rumalaya tablets and cream on white mongrel rats.[3] However, due to increasing concern over animal experimentation and its ethical implications, *in vitro* alternatives is being used for determining the cytotoxicity of the skin applications. Moreover, the *in vitro* models are safer, more efficient, cost effective and has generally been found to be a useful predictor of skin irritation potential.[4]

Skin irritation is a reversible inflammatory reaction produced by the arachidonic acid cascade and cytokines in the viable keratinocytes and fibroblasts of the skin.[5] In the present study, NIH3T3 and HaCaT cells were used as model cell systems to study the cytotoxicity of RL. A good correlation between *in vivo* results and that with NIH3T3 and HaCaT cells has been reported.[6,7] The main advantage of using these cell lines is that they are non-tumorigenic and unlimited supply of identical cells can be obtained with high reproducibility.[8] Moreover, these models were considered a helpful tool to predict irritancy, despite the lack of 100% accuracy.[9]

Address for correspondence: Dr. Sandeep R. Varma, Research and Development, The Himalaya Drug Company, Bangalore - 562 123, Karnataka India. E-mail: sandeepravivarma@indiatimes.com
Received: 01-11-2010; **Accepted:** 26-11-2010

The aim of the present study was to evaluate the possible cytotoxic effects of RL on mouse fibroblast cells (NIH3T3) and human keratinocyte cell lines (HaCaT) and compare with a competitive commercial sample (CS) using three different assay systems such as neutral red uptake (NRU), MTT and resazurin assay.

MATERIALS AND METHODS

Materials

Dulbecco's minimum essential media (DMEM), DMEM-Ham's F12 medium, Neutral red dye, (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium dodecyl sulphate (SDS), trypsin, cremafor and phosphate buffer solution (PBS) were obtained from Sigma Aldrich, USA. Foetal bovine serum (FBS) was from Gibco BRL, India. Sterile tissue culture plastic wares were purchased from Tarsons, India. Absolute ethanol and glacial acetic acid was obtained from Merck, India. Tissue culture grade streptomycin, penicillin and trypsin- ethylene diamine tetra acetic acid (EDTA) solutions were purchased from Hi-media, India. Resazurin assay was carried out using the kinetic blue solution supplied by Krishgen Biosystems, India.

Cell Culture

NIH3T3 fibroblasts cell line was grown in DMEM high glucose medium containing 10% FBS and antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml). HaCaT keratinocyte cell line was maintained in DMEM -HamF12 medium with 10% FBS and routinely cultured into 25 cm^2 plastic flasks and in a humidified incubator at 37°C with 5% CO_2 and 95% humidity. The cells were harvested with trypsin/EDTA and seeded 100 µl/well from a suspension of a density of 2×10^4 cells/ml into a 96-well plate and incubated overnight.

Drug Treatment

RL and CS was dissolved in 2% Cremafor solution in DMEM medium and later diluted using the appropriate cell culture medium with 2% FBS to obtain concentrations ranging from 1000 µg/ml to 6.25ug/ml. The cell culture medium from the 96-well plates was aspirated and 100 µl of each dilution was added to the plates in triplicates. The standard surfactant SDS was also added in different dilutions ranging from 100 to 6.25 µM and cell control/vehicle control was also maintained. The cultures were incubated for 24 hours and thereafter the cytotoxicity assay was carried out.

Cytotoxicity Evaluation

Cell viability was evaluated by NRU,[10,11] MTT[12] and resazurin assay.[13,14] For NRU assay, the medium was aspirated and replaced with 100 µl per well of NR solution (50 µg/ml in culture medium). After 3 hours incubation at 37°C, the medium was aspirated, cells were washed twice with PBS and a solution containing 50% absolute ethanol, 1% acetic acid in distilled water (100 µl/well) was added to extract the dye. After 10 minutes, the absorbance of the solutions was read at a wavelength of 540 nm in a microplate reader.

MTT assay was performed according to the method of Mosmann.[12] After treatment with the drugs for 24 hours, 10 µl of MTT solution was added and incubated for 3 hours at 37°C. The plates were washed once with PBS and 100 µl/well of dimethyl sulphoxide was added to dissolve the purple formazan product and the absorbance of the resulting solutions was read at a wavelength of 540 nm in a microplate reader.

Resazurin assay was carried out using the kinetic blue solution provided by Krishgen biosystems. After 24 hours incubation with the drugs, 10 µl of kinetic blue solution was added to the medium and incubated the cells at 37°C overnight. The absorbance was taken at 570 and 600 nm using a micro-titre plate reader.

Statistical Analysis

All the experiments were performed in triplicate and repeated at least three times. The cytotoxicity of the test drugs was expressed as the percentage viability compared with controls in terms of its CTC_{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. Resazurin assay results are expressed as the percentage of reduction of resazurin to resorufin.

RESULTS

RL and CS had cytotoxic effects in both cell lines, as shown by the decrease in NRU and reduction of resazurin and MTT salt. The curve profiles obtained by the three *in vitro* tests were similar [Figure 1]. However, the CTC_{50} values listed in the Table 1 revealed that RL is less toxic and thus predicted to be less irritant than CS.

The CTC_{50} value of RL and CS with MTT assay in NIH3T3 cells was 690.14±14.14 and 133.75±8.83 µg/ml, respectively. Similarly, with HaCaT cells, the CTC_{50} values were 697.5±10.60 and 286±8.48 µg/ml for RL and CS, respectively. The toxicity levels of RL and CS differed by 58.4 and 80.62% in HaCaT and NIH3T3 cells, respectively, as observed in MTT assay. NRU assay carried out with RL and CS in both cell lines gave similar results. High toxicity of CS as compared with RL was evident from the CTC_{50} values obtained

Table 1: Comparative cytotoxicity of RL with CS in NIH3T3 and HaCaT cells evaluated as CTC_{50} (Mean±SEM)

	NIH3T3 cells (CTC_{50} µg/ml)			HaCaT cells (CTC50 µg/ml)		
Sample	**NRU test**	**MTT test**	**Resazurin test**	**NRU test**	**MTT test**	**Resazurin test**
RL	740±14.14	690±14.14	647.5±3.53	752.5±10.60	697.5±10.60	745±7.07
CS	282.5±10.60	133.75±8.83	197.5±3.53	492.25±14.49	286.0±8.48	307.5±17.32
SDS	62±5.5	58.25±4.57	67.5±2.0	72.5±1.55	68.5±4.55	75±2.75

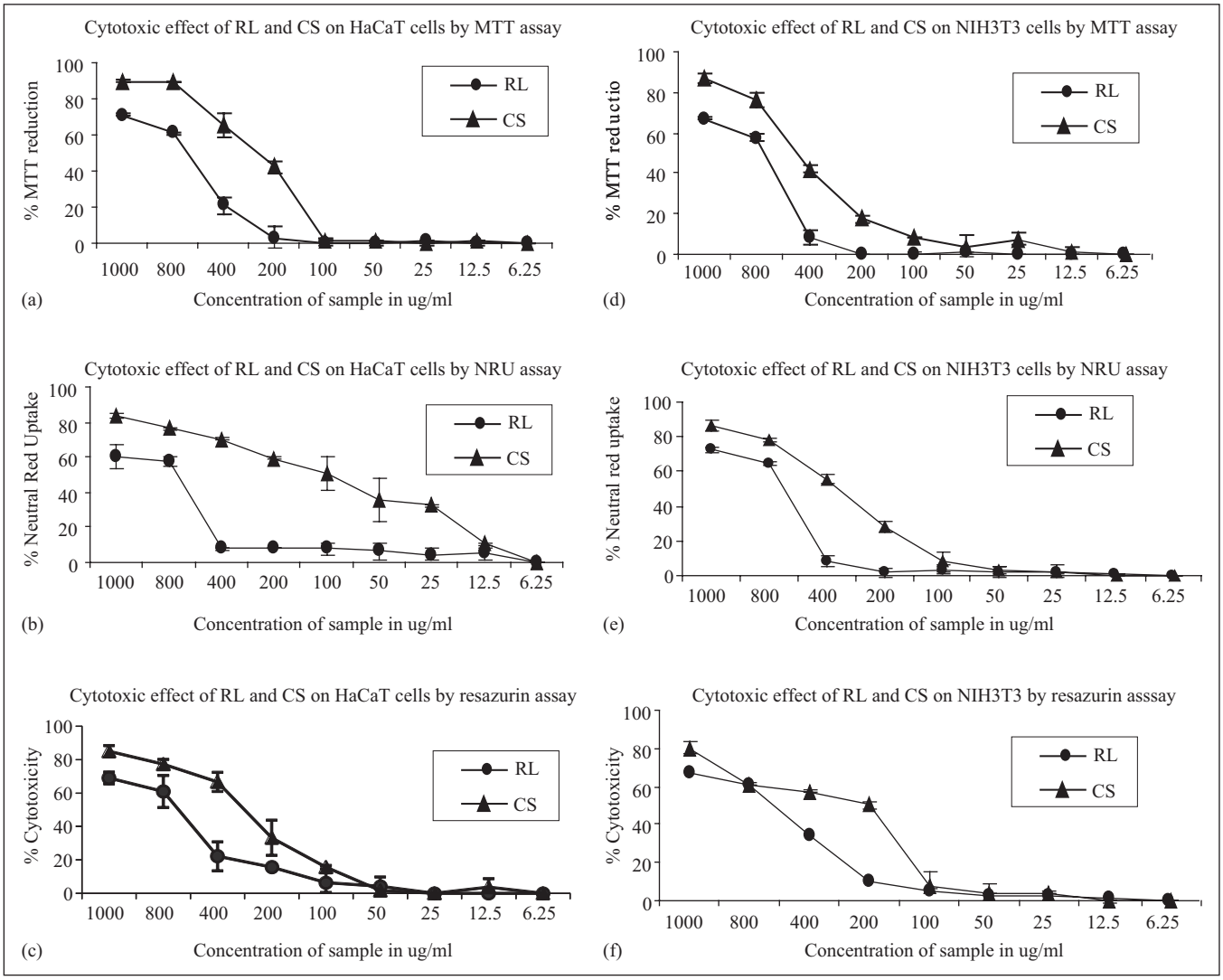

Figure 1: Dose response curves of the cytotoxicity of RL and CS in HaCaT and NIH3T3 cells. (a and d) represents the % MTT reduction by the test drugs in HaCaT and NIH3T3 cells, respectively. (b and e) indicates the % Neutral red uptake by RL and CS in HaCaT and NIH3T3 cells, respectively. (c and f) represents the percentage of resazurin reduction by RL and CS in NIH3T3 and HaCaT cells, respectively

[Table 1]. CS was found to be 34.59 and 61.83% more toxic than RL in NRU assay carried out in HaCaT and NIH3T3cells, respectively. The results of resazurin assay also clearly established the low toxicity of RL as compared with CS. In NIH3T3 cells, the CTC_{50} values obtained by RL and CS was 647.5±3.53 and 197.5±3.53 µg/ml, respectively, showing that CS is 69.5% more toxic than RL. The CTC_{50} values of RL and CS was found to be 745±7.07 and 307.5±17.32 µg/ml in HaCaT cells, respectively, which accounts to 58.73% more toxicity than RL.

Microscopic observation of the cells treated with the test drugs showed that the cells were degenerated at 1000 and 800 µg/ml concentrations in both RL- and CS-treated plates, whereas at 400 µg/ml, RL-treated cells were intact without apparent degradation and was comparable to cell control [Figures 2a and b]. At the same concentration (400 µg/ml), CS was toxic to both cell lines and degradation of the cells was evident [Figures 2a and b].

The three different cytotoxic assays employed in the

present study revealed that RL is less toxic compared with CS in HaCaT and NIH3T3 cells. The toxicity of RL was higher at 1000 and 800 µg/ml and the toxicity substantially reduced below 400 µg/ml and further lower concentrations were nontoxic to both cell lines as observed by NRU, MTT and resazurin assay systems. The toxicity of RL in HaCaT cells was below 20% at 400 µg/ml and the toxicity gradually decreased at lower concentrations, whereas CS exhibited toxicity above 30% even at 100 µg/ml, which clearly showed the low toxicity of RL over CS. In NIH3T3 cells, RL exhibited 8% toxicity at 400 µg/ml, whereas CS showed 42% toxicity at the same concentration as observed by MTT assay. NRU assay results of RL and CS in NIH3T3 cells showed 8.27 and 55.64 % toxicity at 400 µg/ml respectively. The toxicity of RL and CS at 200 µg/ml concentration in NIH3T3 as observed by resazurin assay was 9.94 and 50.64%, respectively. The results obtained by the three assay systems employed in the present study clearly showed that RL is less toxic to both cell lines at concentrations below 400 µg/ml.

DISCUSSION

The cytotoxic effect of RL and CS were evaluated *in vitro* on mouse fibroblasts and human keratinocyte cells by NRU, MTT and resazurin assay. NIH3T3 and HaCaT cells were employed for assessing the skin irritation potentials of RL and CS. Previous studies have suggested that cultured human keratinocytes and mouse embryo fibroblasts may be predictive of surfactant irritancy[6], with a high correlation demonstrated with the *in vivo* human skin irritation potential data.[15]

Cell cytotoxicity assays are commonly used *in vitro* bioassay methods to predict the toxicity of substances in various tissues and the ability of a chemical to elicit a corrosive response is easily predicted using appropriate end points because they demonstrate the degree of damage caused by the chemical.[4] In the present study, we used MTT assay, NRU and resazurin assay to determine the cytotoxic effect of RL with CS. MTT assay is widely used to determine the cytotoxicity of test materials on various cell lines and it is based on the principle of measuring mitochondrial dehydrogenase activity.[16] The 3T3 neutral uptake phototoxicity assay has been validated and accepted to test for regulatory purposes and 95 to 100% correlation between *in vivo* and *in vitro* results has been reported.[8] The resazurin assay is based on the reduction of resazurin to resorufin by cellular reductases, which is a parameter to determine the cell viability by studying their mitochondrial integrity.[13] The resazurin assay does not injure the cells and it is possible to use the culture in repeat assays.[17] This assay also has a good correlation with the human patch test.[18]

In the present study, RL showed significantly lower toxicity as compared with CS, as indicated by the CTC_{50} values derived from MTT, NRU and resazurin assays on NIH3T3 and HaCaT cell lines [Table 1]. The toxicity of SDS, used as reference substance was also assessed on both cell lines by all assays. SDS, an anionic detergent, has been reported to be toxic to NIH3T3 and HaCaT cells by earlier workers.[4]

It was evident from the present study that the CTC_{50} values obtained by NRU assay were higher than that of resazurin and MTT assays. The possible explanation for this result

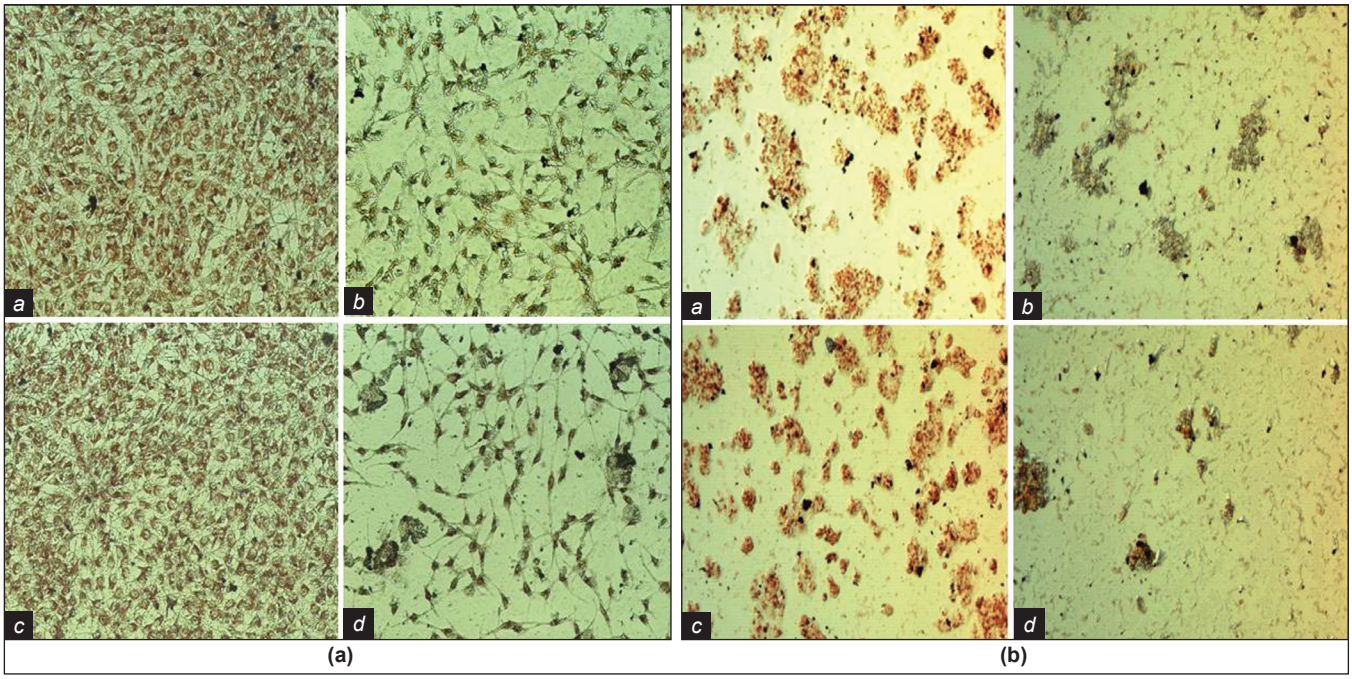

Figure 2: Photomicrograph of NIH3T3 (a) and HaCaT (b) cells treated with test drugs and stained with Neutral Red. (*a*) shows the control cells, (*b*) shows the cells treated with SDS (25 µM). (*c*) and (*d*) the cells treated with RL and CS at 400 µg/ml, respectively

is that the components present in RL and CS might have more toxicity toward MTT and resazurin reduction than in NRU assays since mitochondria is generally considered to be prominent site of cytotoxicity in the cells.[4] It was also concluded from the present study that the CTC_{50} values of RL and CS were higher in HaCaT cells when compared with NIH3T3 cells, showing that HaCaT cells is less sensitive to the test samples. The lower sensitivity of HaCaT compared with NIH3T3 may be due to their more differentiated phenotype.[19]

The ingredients of RL and CS are of herbal origin and the plants used in the formulations are reported to have anti-inflammatory properties. The high toxicity of CS to the cell lines could be attributed to the difference in the formulations or proportions of the plant extracts or the herbs not used in RL formulations. The difference in the cytotoxicity of emulsions containing the same components in different proportions has been reported earlier.[2] The toxicity of each ingredient of RL and CS when analysed individually on these cell lines might give a complete picture of the difference in the toxicities of both samples since a possible interaction between the components in the mixture promotes a synergistic effect on the cytotoxicity to the cells in total.[2]

CONCLUSION

The skin irritation predicted from the cytotoxic effects on NIH3T3 and HaCaT cells demonstrated that RL is less toxic than CS. In conclusion, RL may be a promising alternative to the CS given their low dermal irritancy. The present study involving the use of human keratinocytes and mouse embryo fibroblasts and assessing the toxicity using NRU, MTT and resazurin assay systems may be a strong and necessary tool to evaluate the irritation potential of new products prior to human exposure.

REFERENCES

1. Vinardell MP, Benavides T, Mitjans M, Infante MR, Clapes P, Clothier R. Comparative evaluation of cytotoxicity and phototoxicity of mono and diacylglycerol amino acid-based surfactants. Food Chem Toxicol 2008;46:3837-41.
2. Zanatta CF, Ugartondo V, Mitjans M, Rocha-Filho P, Vinardell MP. Low cytotoxicity of creams and lotions formulated with Buriti oil (*Mauritia flexuosa*) assessed by the neutral release test. Food Chem Toxicol 2008;46:2776-81.
3. Nemova EP, Smolnikova NM, Tanirbergenov TB, Durnev AD. Studies on reproductive toxicity of Rumalaya. Pharm Chem J 2007;41:399-402.
4. Sanchez L, Mitjans M, Infante MR, Vinardell MP. Assessment of the potential skin irritation of lysine-derivative anionic surfactants using mouse fibroblasts and human keratinocytes as an alternative to animal testing. Pharm Res 2004;21:1637-41.
5. Moreno JJ. Arachidonic acid release and prostaglandin E-2 synthesis as irritant index of surfactants in 3T6 fibroblast cultures. Toxicology 2000;143:275-82.
6. Korting HC, Herzinger T, Hartinger A, Kerscher M, Angerpointner T, Maibach HI. Discrimination of the irritancy potential of surfactants *in vitro* by two cytotoxicity assays using normal human keratinocytes, HaCaT cells and 3T3 mouse fibroblast: Correlation with *in vivo* data from a soap chamber assay. J Dermatol Sci 1994;7:119-29.
7. Zuang V, Balls M, Botham PA, Coquette A, Corsini E, Curren RD, *et al*. Follow-up to the ECVAM prevalidation study on *in vitro* tests for acute skin irritation: The European centre for the validation of alternative methods skin irritation task force report 2. Altern Lab Anim 2002;30:109-29.
8. Spielmann H, Balls M, Dupuis J, Pape WJ, Pechovitch G, de Silva O, *et al*. The international EU/COLIPA *in vitro* phototoxicity validation study results of phase II (blind trial), Part 1: The 3T3 NRU phototoxicity test. Toxicol *In Vitro* 1998;12:305-27.
9. Wilhelm KP, Boettjer B, Siegers CP. Quantitative assessment of primary skin irritants *in vitro* in a cytotoxicity model: Comparison with *in vivo* human irritation tests. Br J Dermatol 2001;145:709-15.
10. Borenfreund E, Puerner JA. Toxicity determined *in vitro* by morphological alterations and neutral red absorption. Toxicol Lett 1985;24:119-24.
11. Ridell RJ, Clothier RH, Ball M. An evaluation of three *in vitro* cytotoxicity assays. Food Chem Toxicol 1986;24:469-71.
12. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Met 1983;65:55-63.
13. O'Brien J, Wilson I, Orton T, Pognan F. Investigation of the alamar blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur J Biochem 2000;267:5421-6.
14. Clothier R, Starzec G, Pradel L, Baxter V, Jones M, Cox H, *et al*. The prediction of human skin responses by using the combined *in vitro* fluorescein leakage/Alamar Blue (resazurin) assay. ATLA Altern Lab Anim 2002;30:493-504.
15. Osborne R, Perkins MA. An approach for development of alternative test methods based on mechanisms of skin irritation. Food Chem Toxicol 1994;32:133-42.
16. Suman G, Jamil K. Application of human lymphocytes for evaluating toxicity of anti-cancer drugs. Int J Pharm 2006;2:374-81.
17. Clothier RH, Beed M, Samson R, Ward R. An *in vitro* approach to the evaluation of repeat exposure in the prediction of toxicity. Toxicol *In Vitro* 1997;11:679-82.
18. Lee JK, Kim DB, Kim JI, Kim PY. *In vitro* cytotoxicity tests on cultured human skin fibroblasts to predict skin irritation potential of surfactants. Toxicol *In Vitro* 2000;14:345-9.
19. Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE. Normal keratinisation in a spontaneously immortalized aneuploid human keratinocyte cell line. J Cell Biol 1988;106:761-71.

Source of Support: The Himalaya Drug Company, **Conflict of Interest:** None declared.