Available online at www.ilcpa.pl

International Letters of Chemistry, Physics and Astronomy

8(1) (2013) 1-7 ISSN 2299-3843

Comparative study on cytotoxicity activity of N-α-acylarginine ethyl ester

Jyoti D. Mhatre[1],*, Pravin U. Singare[2]

[1]Department of Chemistry, Shri. Jagdishprasad Jhabarmal Tibrewala University, Jhunjhunu, Rajasthan 333001, India

[2]Department of Chemistry, Bhavan's College, Munshi Nagar, Andheri (West), Mumbai 4000058, India

*E-mail address: pravinsingare@gmail.com

ABSTRACT

Amino-acid derived compounds, for example N-α-lauroylarginine ethyl ester (LAE), N-α-myristoylarginine ethyl ester (MAE) and a 1:1 mixture of N-α-myristoylarginine ethyl ester with monolaurin (MAE + MLN) are examined for their cytotoxicity towards L929-Mouse connective tissue to explore their use as microbicidal agent, in comparison to sodium dodecylsulfate (SDS) as an anionic control detergent. MTT (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide Cytotoxicity assay method was used to determine IC50 value. Cytotoxicity of MAE is found to be more toxic as compared to LAE, having IC50 value 0.052 mg/ml against 0.68 mg/ml of LAE. But MAE when mixed with monolaurin (1:1), showed less toxicity with IC50 0.89 mg/ml. These results suggest that a combination of MAE and Monolaurin can be a potential candidate for studying its microbicidal properties.

***Keywords*:** cytotoxicity, arginine, IC_{50}, microbicidal, monolaurin.

1. INTRODUCTION

Recently there has been an increasing necessity for preparedness against infectious diseases to a greater extent. Every year we encounter emerging and reemerging infectious diseases. The treatment and prevention of sexually transmitted diseases (STDs) is a growing challenge mainly due to multi-drug resistance tendency of pathogens and unavailability of effective vaccines for the majority of them and even if available, not accessible for every individual due to various reasons (e.g., cost, distribution, and politics). Therefore, there is an urgent need to develop effective microbicides against STDs.

Although there are large numbers of organic solvents and detergents which capable of inactivating viruses, but majority of them are having toxic effect on cells and tissues of the skin, particularly the mucosal surfaces, resulting in their limited *in vivo* applications. Previous studies have reported inactivation of various enveloped viruses by aqueous arginine

solution, although at high arginine concentration [1-6]. Application of such enhanced virus inactivation by arginine was made for clearance of contaminated viruses in biopharmaceutical products. Long-chain cationic surfactants from arginine amino acid are surfactants with a satisfactory toxicity profile, high biodegradability, and excellent antimicrobial properties against bacteria, fungi, and yeast [7]. Yamasaki H. et al. (2011) [8], in their research article had undertaken a systematic screening of amino acid derived compounds, such as N-α-cocoyl-L-arginine ethyl ester (CAE), alkyloxyhydroxypropyl arginine, arginine cocoate and cocoyl glycine potassium salt (Amilite) in search for effective, nontoxic virus-inactivating agents. The results are compared with those obtained for benzalkonium chloride (BKC) and sodium dodecylsulfate (SDS) as a cationic and anionic control detergent and also to the other commercially available disinfectants. It has been found that, against herpes simplex virus type 1 and 2 (HSV-1 and HSV-2), N-α-cocoyl-L-arginine ethyl ester (CAE) has notable activities to inactivate the infectivity of extracellular virus particles and to suppress the virus multiplication in the infected cells at the concentration with tolerable cytotoxic effect. The toxicity is basically correlated with their hydrophobicity and not with surfactant-specific parameters. The more hydrophobic the surfactant, the lower the cmc and the easier the aggregation in aqueous solutions with the result that it accumulates at interfaces exerting a toxic effect.

In our search of microbicidal agent, we studied first the antimicrobial properties of N-α-acylarginine ethyl esters synthesized in our lab, of which N-α-myristoylarginine ethyl ester (MAE) was found to possess very good antimicrobial activity against *Staphylococcus Aureus* and *Candida albicans*. In the study of preclinical microbicide development it is equally important to understand the biocompatibility of the material [9]. The failure of nonoxynol-9 (N-9) containing microbicides in phase III trials [10] was mainly due to lack of biocompatibility resulting in irritation of genital tissue thereby promoting events of early infection.

The chemical/enzymatic hydrolysis of N-α-acylarginine alkyl ester salts is one of the shortcomings due to which these salts losses their anti-microbial activity and water solubility. Such chemical hydrolysis is found to be rapid at about pH 4.0 or below or at about pH 8.0 or above. Since in most of the applications the pH is in these critical ranges, it is desirable that the alkyl ester salts of N-α-acylarginine with other bioactive substances should retain the activity even after the hydrolysis. Isaacs *et al* [11], reported excellent antimicrobial activity of fatty acyl glycerides, a chelating acid, and a surfactant when used in combination for preserving processed meats and for disinfecting poultry carcasses. However the antimicrobial activity was reduced considerably when only one of these three agents was used. A spermicidal, antimicrobial and cytocidal activity of glycerol monofatty acid esters is also disclosed. Monolaurin, also known as glycerol monolaurate, glyceryl laurate or 1-lauroyl-rac-glycerol, is a monoglyceride. It is the mono-ester formed from glycerol and lauric acid.

Monolaurin has antibacterial and other antimicrobial effects *in vitro* [12]. One of the purposes of this invention is to formulate a synergistic N-α-acylarginine alkyl ester salt type biocide mixture that will overcome a significant shortcoming found in the sole use of N-α-acylarginine alkyl ester salts. Hence by combining monolaurin with MAE, significant broadening of cidal activity is desired.

The present study aimed to evaluate the possible cytotoxic activity of MAE and MAE in combination with monolaurin (1:1, w/w). We determined the cytotoxicity of these compounds towards L929-Mouse connective tissues using MTT assay method and compared IC_{50} values against LAE, a known Antimicrobial Food grade cationic surfactant. Alone MAE is found to

be cytotoxic than LAE, but composition of MAE and MLN to the ratio 1: 1 (w/w) has shown reduction in cytotoxicity to greater extent.

2. MATERIAL AND METHODS

MAE and LAE are synthesized in our lab [9], monolaurin is received from one of the Local suppliers for research purpose. A 1:1 (w/w) mixture of MAE and monolaurin is prepared by grinding equal parts of both the materials till it becomes homogeneous. A white paste obtained was used for cytotoxicity test.

Sample-test concentration preparation: Samples were at first diluted to 10 %. This stock was filter sterilized and the working stock of 0.01 % in case of MAE and 0.1 % in case of LAE and a mixture of MAE with MLN.

Cell Line: L929-Mouse Connective tissue Cell line was used for assay, due to following reasons:

- Low maintenance
- High Correlation with specific animal assay
- First cell types that attach to implanted medical
- Better reproducibility and accuracy of the cytotoxic response.

2. 1. Invitro Cytotoxicity test

Test for cytotoxicity are designed to determine the biological response of mammalian cells to the test material/ Extract of test material. At the end of the exposure time, the evaluation of the presence and the extent of cytotoxic effect is assessed. It signifies Biological compatibility of the test material and its potential to cause cell damage.

2. 2. Assay Principle

MTT (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide Cytotoxicity assay. Test procedure is based on measurement of viability metabolic activity. Yellow water soluble MTT is metabolically reduced in viable cells to a blue insoluble formazan. The number of viable cells co-relates to the colour intensity determined by photometric measurement after dissolving the formazan in DMSO.

The following parameters are used during performing analysis:

Cell Culture Medium: Complete MEM medium with 10 % FBS,
Positive control: 0.001 % SDS (sodium dodecyl sulfate) solution,
Medium Control/ Blank: Complete MEM medium with 10 % FBS,
Diluent: Complete MEM medium
Incubation Conditions: 37 °C with 5 % CO_2 atmosphere.

2. 3. Assay Procedure

L929 cells seeded in 96 well plates at a concentration of 10,000 cells per 100 µl of MEM culture medium per well were maintained in culture for 24 hours to form a semi confluent layer and were exposed to the test material over a range of concentration. After 24 hours exposure, formazan formation is determined for treatment concentration and compared to that determined in growth control. For each treatment the percentage inhibition of growth is calculated by Viability of cells as per formula-

Viability Percentage = 100 x (OD_{ex} / OD_{bl})
Where OD_{ex} and OD_{bl} are optical densities of extract and the blank at 570 nm
Evaluation Criteria: The lower the viability percentage value, the higher the cytotoxic potential. The percentage viability of 100 % test sample is ≥ 70 %, it is non cytotoxic.

Table 1. Cytotoxicity activity data of arginine esters on L929 cell line.

% conc. of MAE	% Cell viability of MAE	% conc. of MAE + MLN (1:1)	% Cell viability MAE + MLN (1:1)	% conc. of LAE	% Cell viability of LAE
0.01	13.8	0.1	46.82	0.1	22.21
0.009	25.4	0.09	54.07	0.09	33.09
0.008	27.99	0.08	52.66	0.08	48.57
0.007	49.83	0.07	58.13	0.07	62.09
0.006	51.13	0.06	64.78	0.06	63.13
0.005	59.33	0.05	82.46	0.05	68.88
0.004	65.67	0.04	86.28	0.04	72.7
0.003	66.04	0.03	89.39	0.03	79.92
0.002	72.39	0.02	98.02	0.02	102.4
0.001	86.1	0.01	94.	0.01	107.55

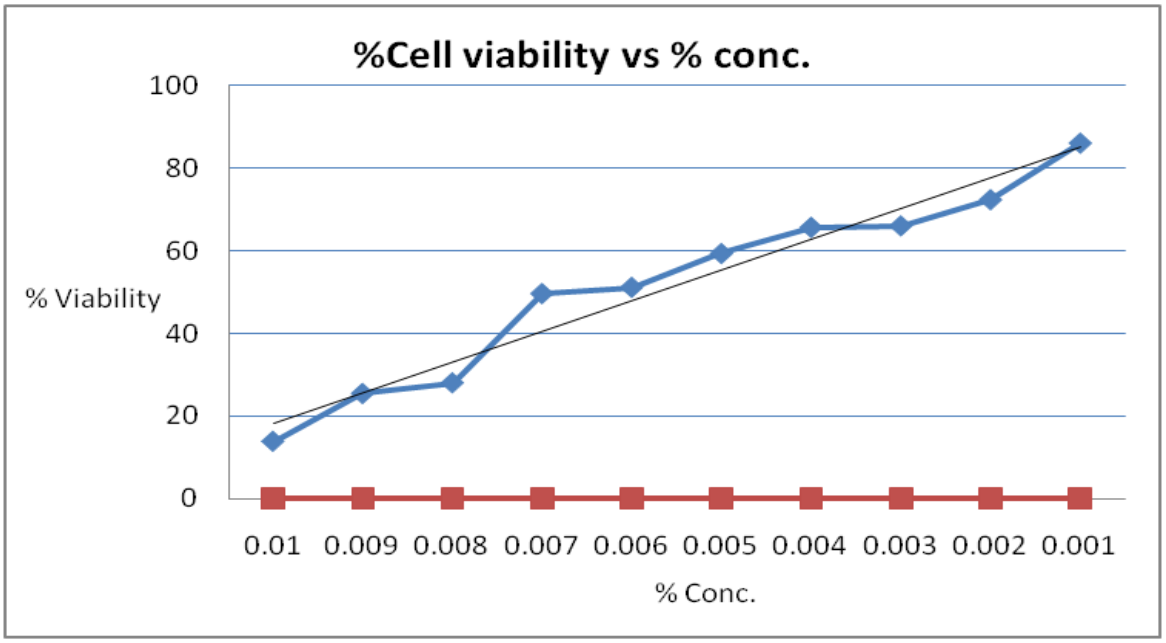

Fig. 1. Cytotoxicity of MAE against l929-cell line.

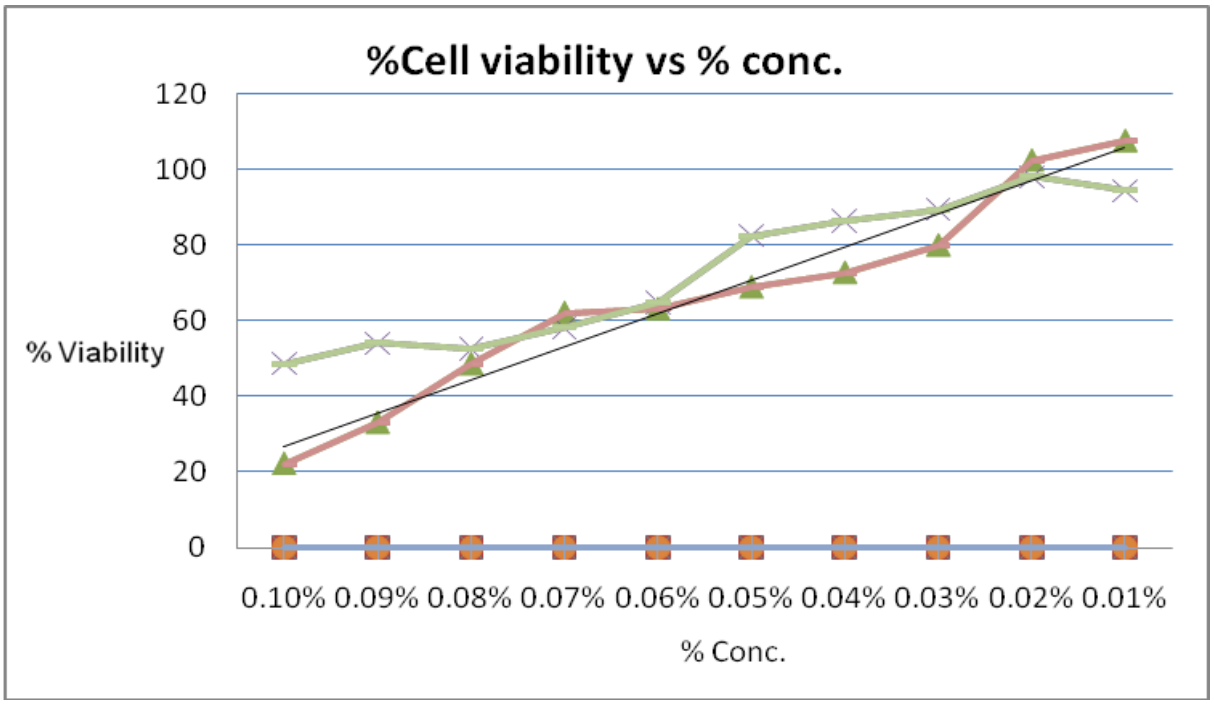

Fig. 2. Cytotoxicity of LAE & MAE+MLN against l929-cell line.

3. RESULTS AND DISCUSSIONS

The *in vitro* screening of N-α-lauroylarginine ethyl ester and N-α-myristoylarginine ethyl ester keeping SDS as positive control was done using MTT assay. The results obtained are shown in Table 1.

For the assay, a concentration range from 0.01%-0.001 % was maintained in case of MAE and a concentration range of 0.1%-0.01 % was maintained in case of LAE and MAE + MLN mixture. Positive control, SDS showing only 8.4-9.4 % as percent cell viability at a concentration of 0.001 %. Whereas, MAE showed about 86.1 % cell viability at the same concentration, with IC_{50} value 0.052 mg/mL, indicating that MAE is less cytotoxic than SDS.

The following observations can be noted while comparing the all three samples tested:

1. After applying evaluation criteria, it is clear that MAE can be said to non-cytotoxic upto concentration 0.002 % (72.39 % cell viability). But its mixture with Monolaurin has increased this concentration level upto 0.05% (82.46 % cell viability), which is 25 times more than MAE alone and found to be comparable to commercial known antimicrobial LAE.

2. As MAE+MLN is a 1:1 (w/w) mixture of MAE and MLN, 0.01 % concentration of MAE from Fig. 1 will correspond to 0.02 % of mixture from Fig. 2. But there is much difference in % cell viabilities of both the samples. Only 13.8 % cell viability is seen at 0.01 % concentration of MAE sample and almost 98 % cell viability is represented at 0.02 % concentration of MAE + MLN sample.
This can be attributed to antimicrobial activity of Monolaurin [13], which has decreased the cytotoxicity of MAE to greater extent in 1:1 formulation.

3. IC_{50} values of MAE, LAE and MAE+MLN are found to be 0.052 mg/ml, 0.68 mg/ml and 0.89 mg/ml respectively, after 24 hrs exposure. IC_{50} values were derived by graphical

extrapolation method Fig. 1 and Fig. 2. The lower the IC_{50} value, the higher the toxicity of the compound. The cytoxic activity is presumably related to the HLB (Hydrophilic Lypophillic balance), with a lower HLB (longer carbon chain length) giving rise to increased cytotoxicity.

4. With decreasing concentration of the sample, an increase in percentage viability was seen. These results suggest that though LAE exhibited lower cytotoxicity than MAE, with combination of monolaurin, cytotoxicity of MAE can be lowered. Concerning the effect of the alkyl chain length, higher toxicity would be expected with greater hydrophobicity in the MAE molecule. Furthermore, this synergistic composition allows the use of lower levels of MAE, while maintaining biocidal efficacy and thereby reducing cost.

4. CONCLUSION

In vitro cytotoxic activities against L929 cell line at different concentrations were evaluated. This test signifies biological compatibility of the test material and its potential to cause cell damage. The Test was considered to be important step towards developing microbicidal agent. The results obtained from the present study provide insights on the applicability and the safety of using cationic surfactants derived from arginine in combination with monolaurin as microbicidal agent. Different formulations of both MAE and LAE with monolaurin can be possible agents for studying the Cytotoxicity with respect to mammalian cells.

REFERENCES

[1] Yamasaki H., Tsujimoto K., Koyama H., Ejima D., Arakawa T., *Journal of Pharmaceutical Sciences* 97 (2008) 3067-3073.

[2] Katsuyama Y., Yamasaki H., Tsujimoto K., Koyama H., Ejima D., Arakawa T., *International Journal of Pharmaceutics* 361(1-2) (2008) 92-98.

[3] Utsunomiya H., Ichinose M., Tsujimoto K. et al, *International Journal of Pharmaceutics* 366(1-2) (2009) 99-102.

[4] Naito T., Irie H., Tsujimoto K., Ikeda K., Arakawa T., Koyama H., *International Journal of Molecular Medicine* 23(4) (2009) 495-499.

[5] Arakawa T., Kita Y., Koyama H., *Biotechnology Journal* 4(2) (2009) 174-178.

[6] Tsujimoto K., Uozaki M., Ikeda K. et al, *International Journal of Molecular Medicine* 25(3) (2010) 433-437.

[7] Moran C., Pinazo A., Clapes P., Perez L., Vinardell M. P., Infante M. R., *Green Chem.* 6 (2004) 233.

[8] Yamasaki H., Tsujimoto K., Ikeda K., Suzuki Y., Arakawa T., Koyama A. H., *Advances in Virology* 2011 (2011) 572868.

[9] Singare P., Mhatre J., *American Journal of Chemistry* 2(4) (2012) 186-190.

[10] Gupta N., Gupta S. K., *Indian J. Med. Res.* 134 (2011) 939-949.

[11] Isaacs Charles E., Kim Kwang S., Thormar Halldor Heird, William C., Wisniewski Henryk M., *Antibacterial fatty acid compositions*, US 5,434,182. (1995).

[12] Preuss H. G., Echard B., Enig M., Brook I., Elliott T. B., *Molecular and cellular biochemistry* 272(1-2) (2005) 29-34.

[13] Fu X., Zhang M., Huang B., Liu J., Hu H., Feng F., *Journal of Food Process Engineering* 32 (2008) 104-111.