

Microbial Contamination And Preservative Effectiveness Of Neem (*Azadirachta Indica*) Seed Oil Cream

By

Aremu, O.I. And M.N. Femi-Oyewo

Abstract

Although topical preparations need not be sterile but may contain low levels of microbial load during use. This study aimed at determining effectiveness of parabens used as preservative in neem oil cream formulations.

In the study, total counting of bacteria and fungal loads as well as types were carried out. Also preservative capacity was evaluated by challenging the formulations with commonly encountered contaminants of topicals during in-use such as *P. aeruginosa*, *S. aureus* and *C. albicans*. Viable counting was performed by the surface viable method.

In all, six samples of Neem oil cream were used namely NC0, NC1, NC2, NC3, NC4, NC5-NC0 (Neem cream without preservative) serving as control samples. Control sample was found to be contaminated with bacteria, *S. aureus* and mould, *A. fumigatus*. The rest of the samples (NC1-NC5) were mostly contaminated with *Bacillus* spp but with absence of fungal growth.

The result of the challenge test indicated that control formulation had uncontrollable growth throughout the entire period of study. The rest of the samples had an initial decline in the bacteria up to the 14th day of the study only to be followed by a resurgence of growth thereafter. The exception was sample NC5 (concentration 10.0%w/w) that was able to reduce growth of *S. aureus* consistently throughout the entire period of study. Samples NC1-NC5 were able to reduce the Log count of *C. albicans* till the end of the study. The inference therefore is that a co-preservative with parabens or completely an alternative with broad spectrum in activity may be necessary.

Keywords: Preservative, Contaminant, Samples, Neem

Introduction

Azadirachta indica, *A. Juss* synonymous with *Melia azadirachta* and *Melia indica* (A. Juss) belongs to the family *meliaceae*. This plant is indigenous to the Indo-Pakistan subcontinent (Vander et. al. 1987), although it is now widely distributed in many countries of the world, it is believed that Indian migrating to African countries introduced it into that continent. Recently, Aremu et. al. (2008) in their review article reported on earlier indications of neem seed oil medicinally proven severally in the literature. Repellent action against *Aedes aegypti* mosquitoes (Ilati et. al. 1995), anticandidal activity (Charmaine et. al. 2005), antipyretic effect (Okpanyi and Ezeukwu, 1981), etc. Aremu et. al. (2008) also revealed in their article the need for a delivery design of neem seed oil in a dermatological preparation targeted to be applied topically.

The warm and rather humid climatic conditions that prevail in most tropical countries including Nigeria, would tend to support the survival and growth of many microorganisms. In a situation whereby a nutritionally rich pharmaceutical/cosmetic product is severely contaminated, rapid growth and multiplication would be expected. This could lead to biodegradation of the product and hence the risk of infection to consumers of the product (Bos et. al. 1989). Product contamination may arise from raw materials or water used in formulation or accidentally during use. Studies carried out till date (Malcolm 1976; Becks and Lorenzoni, 1995) to assess the incidence and hygienic status of many tropical products, do not sufficiently simulate actual-use conditions of the products in the sense that laboratory-prepared sterile creams are frequently used in the challenge tests (Okeke and Lamikanra, 2001). Such studies do not, therefore, realistically monitor the true capacity of the cream to suppress accidental in-use contamination, a very important consideration for successful preservation.

This paper reports the results of evaluation of non-sterile five different concentrations (0%^{w/w}, 10.0%^{w/w}) of Neem oil cream and the sixth formulation without preservative serving as control. They were evaluated for the load and type of microbial contamination and also, the ability of such products to consistently suppress the multiplication of microbial contaminants with which they were challenged.

Materials And Method

Six formulations in all of neem oil cream using varnishing cream as base was prepared. Five of the formulations were preserved with conventional methyl propyl hydroxybenzoate. The sixth one, without preservative served as the control.

Evaluation Of Microbial Quality Of Preparations

Microbial contamination of the formulations was determined by thinly spreading a loopful of material withdrawn from the depths of the bulky product on Nutrient Agar and Sabouraud Dextrose Agar and then incubated for 24-48 hours at 37°C and 5 days at room temperature for bacterial and fungi respectively.

In order to assess the degree of contamination, 1g of material was dispersed in 4ml Ringers solution containing 0.25% Tween 80. Appropriate dilutions were made in the same dispersing vehicle and 0.5ml was plated out on Nutrient agar and Sabouraud Dextrose Agar respectively. Emergent colonies were counted after the necessary incubation. Attempts were made using appropriate biochemical tests to carry out identifications of possible contaminants. All operations were carried out in duplicates.

Challenge Test For Preservative Capacity

Staphylococcus aureus, *Pseudomonas aeruginosa* and *Candida albicans* regarded as common contaminants were used as challenge inocula. An overnight broth culture of the three organisms were prepared using 0.1% peptone water as diluent, compared with 0.5MCFarland standard containing 1×10^8 organisms. The preparations above were diluted to achieve a population of 1×10^1 for bacteria and 1×10^2 for mould, the population that (on day 7, 14, 21 and 28) 1ml sample material was withdrawn for enumeration of bacterial and fungi survivors.

Results

Table 1: Microbial Counts (CFU/ml) and Types found in Neem Cream Preparations

Preparation	Bacteria		Fungi	
	Counts	Types	Counts	Types
NC0	1.25×10^4	<i>S.aureus</i>	3.0×10^3	Asp. Fumigants
NC1	1.25×10^2	<i>Bacillus spp.</i>	NIL	NIL
NC2	1.0×10^2	<i>Bacillus spp.</i>	NIL	NIL
NC3	1.0×10^2	<i>Bacillus spp.</i>	NIL	NIL
NC4	1.0×10^2	<i>Bacillus spp.</i>	NIL	NIL
NC5	1.5×10^2	<i>Bacillus spp.</i>	NIL	NIL

Key:

NC0 = Neem cream without preservative
 NC1 = Neem cream (0%^{w/w} with preservative
 NC2 = Neem cream (2.5%^{w/w})
 NC3 = Neem cream (5.0%^{w/w})
 NC4 = Neem cream (7.5%^{w/w})
 NC5 = Neem cream (10%^{w/w})

Table 2: Counts and Log survival of *S.aureus* (CFU/ml)

Samples	<i>S.aureus</i>	
	Counts	Log survival
7th Day		
NCO	3.0×10^3	3.48
NC 1	7.0×10^1	1.85
NC 2	5.0×10^1	1.69
NC 3	4.2×10^1	1.62
NC 4	4.7×10^1	1.67
NC 5	4.3×10^1	1.63
14th day		
NCO	3.5×10^3	3.54
NC 1	3.0×10^1	1.48
NC 2	4.2×10^1	1.62
NC 3	3.6×10^1	1.56
NC 4	4.0×10^1	1.60
NC 5	4.1×10^1	1.61
21st day		
NCO	5.8×10^3	3.76
NC 1	3.8×10^1	1.58
NC 2	4.9×10^1	1.69
NC 3	3.8×10^1	1.58
NC 4	4.8×10^1	1.68
NC5	3.9×10^1	1.59
28th day		
NCO	6.2×10^3	3.79
NC 1	4.3×10^1	1.63
NC 2	5.2×10^1	1.72
NC 3	4.5×10^1	1.65
NC 4	5.6×10^1	1.75
NC 5	2.9×10^1	1.46

Table 3: Counts and Log survival of *P.aeruginosa* (CFU/ml)

Samples	<i>P.aeruginosa</i>	
	Counts	Log survival
7th Day		
NCO	2.5×10^3	3.39
NC 1	6.0×10^1	1.78
NC 2	4.8×10^1	1.68
NC 3	5.2×10^1	1.72
NC 4	6.4×10^1	1.81
NC 5	4.3×10^1	1.63
14th day		
NCO	3.9×10^3	3.59
NC 1	4.5×10^1	1.65
NC 2	4.8×10^1	1.68
NC 3	3.5×10^1	1.54
NC 4	3.6×10^1	1.56
NC 5	4.0×10^1	1.60
21st day		
NCO	4.6×10^3	3.66
NC 1	5.2×10^1	1.72
NC 2	5.1×10^1	1.71
NC 3	4.2×10^1	1.62
NC 4	3.9×10^1	1.59
NC5	4.7×10^1	1.671
28th day		
NCO	5.3×10^3	3.72
NC 1	5.7×10^1	1.76
NC 2	5.9×10^1	1.77
NC 3	4.9×10^1	1.69
NC 4	4.5×10^1	1.65
NC 5	5.3×10^1	1.72

Table 4: Counts and Log survival of *C.albicans* (CFU/ml)

Samples	<i>C.albicans</i>	
	Counts	Log survival
7th day		
NCO	2.1×10^2	2.32
NC 1	2.5×10^1	1.39
NC 2	2.8×10^1	1.45
NC 3	1.9×10^1	1.28
NC 4	1.6×10^1	1.21
NC 5	1.8×10^1	1.26
14th day		
NCO	3.6×10^1	2.56
NC 1	3.3×10^1	1.52
NC 2	2.4×10^1	1.38
NC 3	1.2×10^1	1.08
NC 4	$1.2.0 \times 10^1$	1.08
NC 5	1.0×10^1	1.
21st day		
NCO	4.8×10^2	2.68
NC 1	3.7×10^1	1.57
NC 2	0.8×10^1	0.9
NC 3	0.8×10^1	0.9
NC 4	0.6×10^1	0.7
NC5	1.2×10^1	1.08
28th day		
NCO	5.2×10^2	2.72
NC 1	4.2×10^1	1.62
NC 2	0.9×10^1	0.95
NC 3	0.7×10^1	0.85
NC 4	0.6×10^1	0.78
NC 5	0.6×10^1	0.78

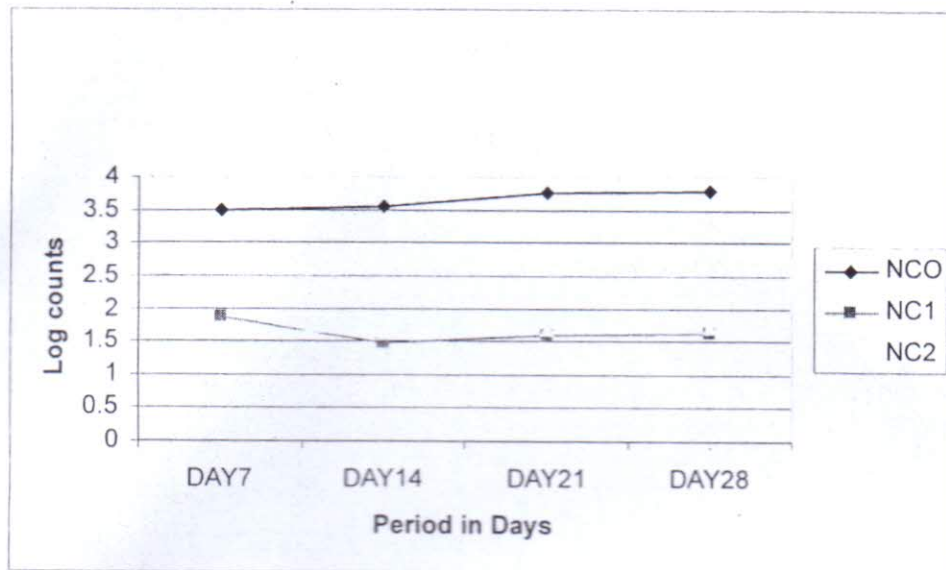


Fig: 2.1. Log survival of *S.aureus* in NCO-NC2 (CFU/ml)

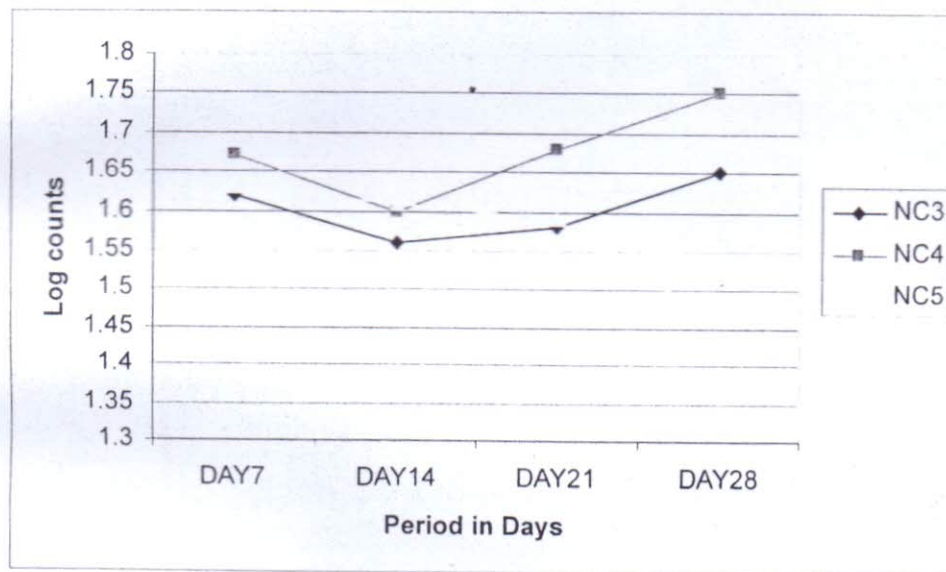


Fig2.2: Log survival of *S. aureus* in NC3-NC5 (CFU/ml)

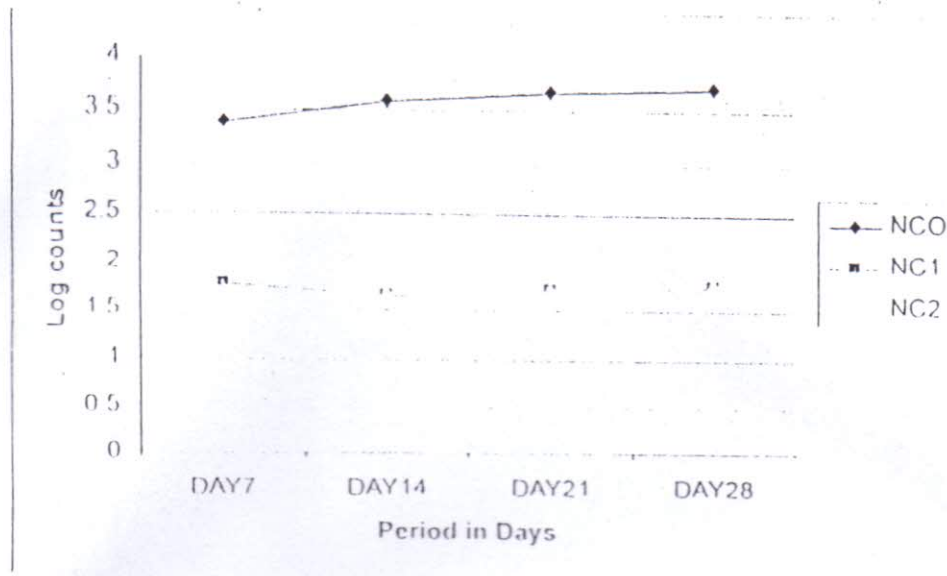


Fig 3.1: Log survival of *Paeruginosa* in NCO-NC2 (CFU/ml)

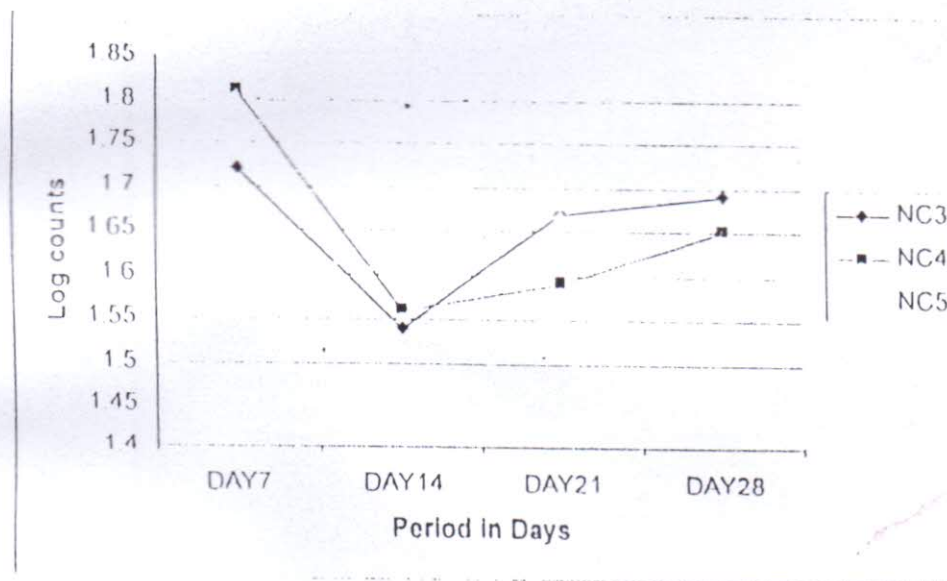


Fig 3.2: Log survival of *Paeruginosa* in NC3-NC5 (CFU/ml)

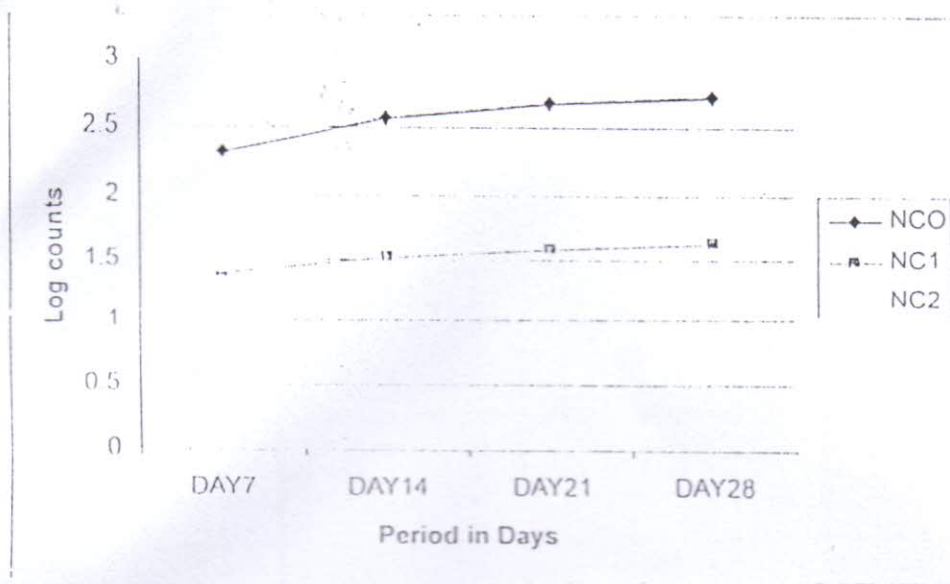


Fig4.1: Log survival of *C.albicans* in NCO-NC2 (CFU/ml)

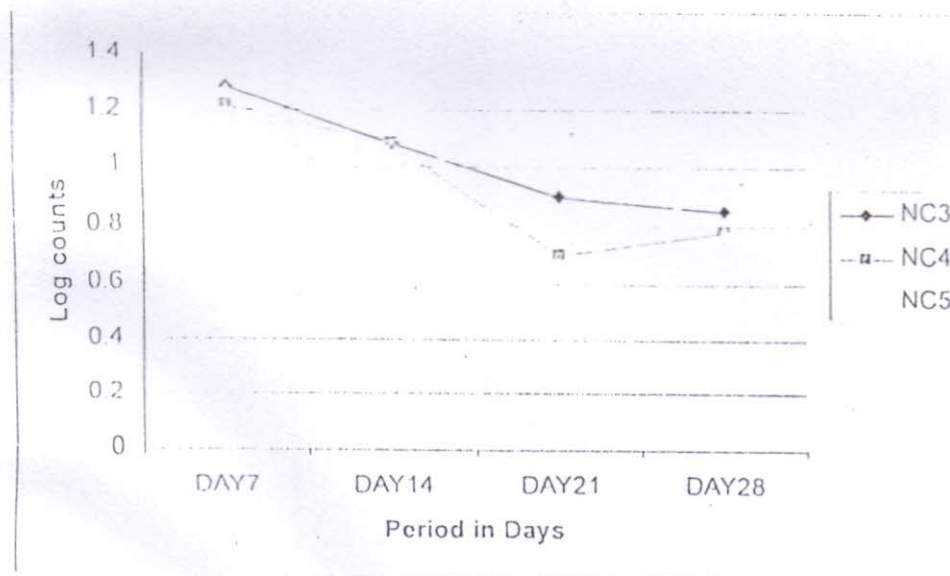


Fig 4.2: Log survival of *C.albicans* in NC3-NC5 (CFU/ml)

Discussions

Table 1 shows the result of qualitative microbial screening of neem oil preparations. Qualitative tests showed that the formulation without preservative (NC0) had contaminant gram positive *Staph aureus* bacteria and mould (*Aspergillus fumigatus*) in high load beyond the official limit allowed for topical preparations. This is probably not unexpected since in the absence of preservatives in preparations, bacteria and moulds are able to thrive.

In the other formulations (NC1-NC5), they were also contaminated to varying degrees though very low count in the range (1.0×10^3 - 1.5×10^3). The major isolate was *Bacillus spp.* and no moulds were isolated from the preparations. On McConkey agar, no bacteria were recovered, indicating the absence of Enterobacteriaceae. This perhaps suggest reliable preparation procedure.

The bacterial count for the challenged organisms over a period of 28 days result is presented in Table 2 to 4 and plotted as Figures 2.1-2.2, 3.1-3.2 and 4.1-4.2 respectively. As can be seen from the table, the log survival of organisms inoculated in formulation without preservative (NC0) was on steady increase throughout the entire 28 days of study. The matrix complex arrangement of the cream could not even retard the growth of the organisms.

On the other hand, for the formulations (NC1-NC4) with *S. aureus* as contaminant there was initial decrease in the bacterial population for the first 14 days but was subsequently followed by increase in bacteria and fungi growth till 28 days. However the observation was different for sample (NC5) that had decline in bacteria growth till 28 days of study. In the case of *P. aeruginosa* all formulations (NC1-NC5) had decline in bacteria population up to 14 days only to be followed by resurgence in growth thereafter till 28 days of investigation. For the *C. albicans* all the formulations (NC2-NC5) had consistent reduction in the mould growth throughout the period of 28 days incubation. It appears that parabens used as preservative, though commonly employed in topical preparations seem not to have broad spectrum activity (Lynn and Hugo, 1981). Even though the sample with the highest concentration of neem oil (NC5) was able to maintain a reduction in *S. aureus* growth which was an exception.

It is probably thought that the high concentration of azadirachtin and other biological components content of neem oil reported in literature to possess antimicrobial property (Rao *et al.*, 1986) might be acting as synergy in this sample with parabens. However, the case is not the same with *P. aeruginosa* as can be seen in Table 3 and Fig 3.1-3.2. Formulation NC5, was not able to retard the growth of this organism throughout the entire period of study as it did with *S. aureus*. Mechanism of action of azadirachtin as an antimicrobial agent may be responsible for this observed trend. Generally, antimicrobial agents that are bacteriostatic in action tend to be more active against gram positive organisms than gram negative ones. Azadirachtin may thus be acting as a bacteriostatic agent. On the other hand the samples with lower concentrations of neem oil (NC1-NC4) that were not able to exhibit similar trend with (NC5) against *S. aureus* may probably be due to minimum inhibition concentrations issues.

The formulations that had reduction in candida growth till the end is also likely to be a co-activity of parabens and azadirachtin since the latter has anticandidal property (Charmaine *et al.*, 2005). This is in view of the fact that formulation without neem oil (NC1) was unable to inhibit the increase in growth till the end of 28 days period of study. Parabens possess fairly high dilution coefficients (Lynn and Hugo, 1981), and hence are not likely to exhibit persistent activity in presence of extraneous matter or upon dilution. The inoculum introduced into the formulations definitely would have given rise to a large fall in residual concentration of the parabens used as preservative. Apart from this, parabens are even being reported of having potential allergy risks (Jocelyne *et al.*, 2001). Though this report is yet to be fully established, but the foregoing suggests that parabens need to be used with caution and perhaps in conjunction with any other compatible preservative to confer synergy against possible contaminants during-in-use.

Conclusion

The results presented in this result suggests that there is need for additional preservative to confer synergy on the use of parabens as preservative in Neem cream formulations or better still a completely alternative that will be able to inhibit immediate post-production contaminants as well as maintain subsequent

low inocula of in-use contaminants, and thereby maintain acceptable low levels of microorganisms in the preparation.

References

- Aremu, O. I., Femi-Oyewo, M. N., Adeyemi, A. A. (2008). Medicinal Relevance of *Azadirachta indica*-A Review. *Journal of Medical and Pharmaceutical Sciences*. (4), 2, 95-105.
- Bos, C. E., Van Doorne, H., Olerk, C. F. (1989). Microbiological stability of tablets stored under tropical conditions. *Inter. J. Pharm.* 55, 175-183.
- Becks, V., Lorenzoni, N. (1995). *Pseudomonas aeruginosa* outbreak in a neonatal intensive care unit: A possible link to contaminated hand lotion. *Amer. J. Inf. control.* 23, 396-398.
- British Pharmacopoeia Appendix xvIB. Tests for microbial contamination. The pharmaceutical Press, London, 1993.
- Charmaine Lioyd, A. C., Menon, T., Umamaheshwari, K. (2005). Anticandidal activity of *Azadirachta indica*. *Indian journal of Dermatology*. (37), 386-389.
- Hati, A. K., Bhowmik, T., Barnerjee, A., Poddar, G., Basu, D., Dhara, K. P. (1995). Repellent action of *Neem* (*Azadirachta indica*) seed oil against *Aedes aegypti* mosquitoes. *Indian journal of Dermatology*. (40), 150-158.
- Jocelyne, F., Marie-Louise, C., Eric, D. (2001). Comparative efficacy of antimicrobial agents in zinc gelatin preparation. *European Journal of Pharmaceutics and Biopharmaceutics*. 52, 255-259.
- Lynn, B., Hugo, W. B. (1981). Chemical disinfectants antiseptics and preservatives. In Hugo, W. B. and Russell, A. D. *Pharmaceutical Microbiology*. 3rd Ed. Blackwell Scientific Publications, UK.
- Malcolm, S. A. (1976). The survival of bacteria in toiletries. In: Skinner, F. and Hugo, W. B. (ed). *Inhibition and inactivation of vegetative microbes*. London Acad Press. pp 305-314
- Okpanyi, S. N., Ezeukwu, G. C. (1981). Antiinflammatory and antipyretic activities of *Azadirachta indica*. *Plant Medica*. (41), 34
- Okeke, I. N., Lamikanra, A. (2001). Bacteriological quality of skin moisturizing creams and lotions distributed in a developing country. *J. Appl Microbiology*: 91, 922-928.
- Vander, C. J., Tempesta, K., VanderSluis, W. G., DeSilvakt, T., Labadie, R. P. (1987). Immunomodulatory activity of an aqueous extract of *Azadirachta indica* stem bark. *J. Ethnopharmacology*. (19), 125-131.