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ASSESSMENT OF THE PHYSICOCHEMICAL AND ANTIMICROBIAL QUALITY OF SOME ANTIBIOTIC AND NON-ANTIBIOTIC EYE DROPS MARKETED IN DIFFERENT REGISTERED PHARMACIES IN PORT HARCOURT, RIVERS STATE

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ABSTRACT
Antibiotic eye drops are antibacterial compounds with some preservatives to strengthen their preservative efficiency, but non-antibiotics being non-infective require a good preservative system to maintain stability and sterility especially during in-use. We hereby undertake this research on the physicochemical properties, sterility, preservative efficiency, and pyrogen testing, of antibiotic and non-antibiotic eye drops used for different eye ailments. **Method:** Twelve different eye-drops products comprising antibiotics and non-antibiotic eye drops were randomly purchased from different registered Pharmacies in Port Harcourt in Rivers State. They were assessed for proper packaging (container and closure system), presence of particulate matter, physicochemical property (pH, clarity, and colour), microbiological analysis (sterility and preservative challenge test) and limit test for pyrogen (bacterial endotoxin). **Result:** All the eye drops had proper packaging and closure system. They all passed physicochemical assessment on clarity and particulate matter while the pH of the eye drops ranged from 5.40 – 7.26. The sterility testing showed that all the products were sterile on opening while only one product failed the preservative efficiency test. The pyrogen test showed that nine out of twelve eye drops had the endotoxin level above 0.25 EU/mL. **Conclusion:** Pharmaceutical industries should not only focus on sterility of eye drops in quality control but also on the endotoxin level which can also be regarded as the bane of the industry.

KEYWORDS: Eye drops are topical applications to the eye for the treatment of eye diseases especially.

INTRODUCTION
Eye drops are topical applications to the eye for the treatment of eye diseases especially diseases of the anterior segment of the eye.[1] Eye drops are preferred to other conventional dosage forms, because they are non-invasive, convenient, localized therapeutic effect and with less systemic side-effect[2] and they constitute more than 70% of the total eye formulations.[3] They are formulated to contain preservatives so as to prevent microbial contamination during in-use or to extend time of its usage[4] Preservative-free eye drops may be ideal in the following condition: i) when the solution of the eye-drop itself is self-sterilizing (antibiotics).[5] ii) when used for chronic eye ailments such as glaucoma or dry eyes as toxicity may develop from extended use[6] iii) In the case of pharmaceutical interaction between the preservative and other drug components; and iv) when eye drop is dispensed in sterile unit dose vials.[7]

Information from literature has revealed the possible presence of microbial contamination for both preservative-containing and preservative-free eye drops for both in-patient and out-patient clinical situations[7-9] and more so for multi-use eye drops.[4]

Antibiotic eye drops though they are antibacterial compounds are usually fortified with preservatives (benzalkonium chloride, thiomersal, phenyl mercuric nitrate etc.) for effective preservation of eye drop, while the non-antibiotic eye drops contain a bioactive substance and an efficient preservative system to resist microbial contamination. Microbial contamination of eye drops is an important risk factor for some eye diseases such as bacterial keratitis and endophthalmitis and other secondary eye infections[10] which can eventually lead to blindness if untreated. Other effects include degradation of the drug due to alteration of the pH from increased microbial growth and finally loss of activity of the drug.[5]

This study therefore investigates the sterility, preservative efficiency and the endotoxin level of some antibiotic and non-antibiotic eye drops of sold in some retail pharmacies in Port Harcourt.

MATERIALS AND METHODS

Sample collection

In the present study, 12 eye drops (6 antibiotic and 6 non-antibiotic) were purchased from different pharmacies in Port Harcourt, Rivers state. The antibiotic eye products (n = number of eye drop) consists of ciprofloxacin (2), ofloxacin (1), chloramphenicol (2) and gentamicin (1) while the non-antibiotic eye drop (n = number of eye drop) consists of betamethasone (2), dexamethasone (1), Timolol (2) and Tetrahydrozoline (1). The different brands were labelled Cipro A, Cipro B, Oflox, Chlorph A, Chlorph B, and Genta for the antibiotics while Betam A, Betam B, Dexam, Timol A, Timol B and THZ were for the non-antibiotics for the purpose of the study. Each brand of eye drop was physically inspected and found to be intact with the appropriate product information: brand name, strength of the active constituent and the preservative, country of manufacture, manufacturing and expiry dates, National Agency for Food and Drug Administration and control (NAFDAC) registration number were clearly stated.

Organoleptic examination

The eye drops were examined for particulate matter, colour and clarity using a visual inspection board with a black and white background under sufficient illumination. The presence of black particles was seen using white background whereas any white particles are seen using black background.

Evaluation of the pH of eye drops

The pH of the different brands of the eye drops were determined using a properly calibrated pH meter (pH Universal meter. PEC medical, USA). The calibration of the meter was done using a buffered solution of known pH (4 and 8). Approximately, 10 mL of the particular eye drop was poured into a sterile 20 mL beaker and the sensitive bulb of the pH meter was dipped into the eye drops and allowed to stabilize for 20 seconds before taking the reading.

Sterility testing of the eye drops

This experiment was performed by direct inoculation method in a laminar air flow cabinet under an aseptic condition. This is to avoid accidental contamination of eye product during testing. Approximately, 1 mL of the different brands of eye drops was transferred aseptically into 20 mL of different media (fluid thioglycollate agar for anaerobic bacteria and Soyabean Casein Digest medium for aerobic bacteria) using a sterile pipette and then incubated at 37°C for 48 h. Approximately1 mL of the same eye drop was also transferred into Sabouraud Dextrose broth and incubated at 25 °C for 72 h for colony forming units of fungi. For positive controls: 20 mL of fluid thioglycollate media in a sterile universal bottle was inoculated aseptically with 0.1 mL of *Staphylococcus aureus* (adjusted using McFarland standard) to serve as a positive control for the anaerobic bacteria while 20 mL of Soyabean Casein Digest medium inoculated with 0.1 mL of *Pseudomonas aeruginosa* (adjusted using McFarland standard) served as positive control for aerobic bacteria. A 20 mL of Sabouraud dextrose agar inoculated with 0.1 mL of *Candida albicans* (adjusted using McFarland standard) also served as positive control for fungi. These were done in triplicates.

Microbial challenge test

The preservative efficiency of the eye drops to withstand microbial contamination during in-use by patients was determined by challenging the eye drops with four different microorganisms separately.[11] This involves a simulation of real use situation, and it involves an inoculation of the sample with a known CFU/mL of organisms.

Preparation of the inoculum for the challenge test

The strains of the organisms (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans*) were obtained from the culture collections maintained in Pharmaceutical Microbiology Laboratory, University of Port Harcourt. A loop full of each organism was sub-cultured into a sterile Peptone water contained in a sterile universal bottle. This was diluted further until it matches the McFarland standard.

Inoculation of the eye drop with the organisms

With the use of a sterile syringe, 0.06 mL of the challenging microorganism prepared to McFarland standard was transferred into 6 mL of each eye drop contained in sterile universal bottle. This was mixed thoroughly to determine the rate of microbial kill. Each organism was tested separately and the inoculated products was maintained at 20 - 25 °C throughout the test period. At days 1, 7, 14, 21 and 28 post inoculation, 1.0 mL of the mixture was withdrawn and diluted into three 10 fold serial dilutions. The last dilution was then plated out in duplicate and spread on the nutrient agar for bacteria and Sabouraud dextrose agar for fungi. This was incubated at 37 °C for 18-24 h for bacteria and 20 - 25 °C for 7 days for fungi.

Pyrogen test

Commercial reagent Kit (GenScript ToxinSensor™ Chromogenic LAL Endotoxin Assay Kits (32 r×ns) Cat. No.L00350C Lot No. C50091512) for the measurement of bacterial endotoxin concentration in samples was used without any modification. The different components of the reagent such as: lyophilized Limulus amebocyte lysate (LAL), chromogenic substrate, Stop solution, buffer S for colour stabilizer #1, colour stabilizer # 2 and colour stabilizer # 3 and standard endotoxin solution were all reconstituted using LAL reagent water according to the manufacturer's protocol in the user's manual.

Preparation of standard endotoxin solution for calibration curve

The lyophilized endotoxin standard was dissolved using 2 mL of LAL reagent water in endotoxin free vials, vortexed for 15 minutes and incubated at 37 ± 1 °C for 12 min in a water bath. A 0.1 mL of reconstituted Chromogenic substrate solution was added to each vial and swirled gently. The contents in the vials were incubated for 6 min at 37 ± 1 °C, then was added 0.5 mL aliquot of reconstituted stop solution (colour stabilizer # 1) and vortexed gently. To the mixture again, 0.5 mL of reconstituted colour stabilizer # 2 was added and mixed. Finally, 0.5 mL of reconstituted colour stabilizer # 3 was added, swirled gently for 3sec to mix well.

To obtain the endotoxin stock solution for calibration, 1.0 EU/mL of the standard stock solution was further diluted to obtain: 0.5, 0.25, 0.125, and 0.05 EU/mL solutions. The absorbance reading of these concentrations were read using a UV/VIS spectrometer (Techmel & Techmel, USA) at 545nm wavelength. A standard calibration curve was obtained from a plot of absorbance against the corresponding concentration.

Test procedure for sample eye drops

The test procedure involves taking 0.1 mL of test samples of the eye drops and also preparing them according to the protocol stated above and their absorbance readings also taken at wavelength 545nm. The concentration of the bacterial endotoxin in the different samples of eye drops was obtained by extrapolation of the absorbance reading on the standard calibration curve taking note of the dilution factor.

RESULT

Properties of the eye drops

All the products had a plastic container built with a dropper and a closure that had an intact seal. The date of manufacture, expiry date, NAFDAC registration number, batch number and country of origin were clearly written on the package (Table 1). The eye drops were also within the expiry dates which ranged from 2- 5years. The volume of each eye drop was about 10.0 – 15.0 mL with an advice to use within a month after first opening. The (Table 2) shows more properties of the sample eye drops such as the percent drug content, pH, clarity and preservative %. Some products had the name of the preservative written but without the percent content (Chlorph A and B).

Table 1: Some relevant information on the package of the different brands of eye drops.

Sample code/ Batch	Country of manufacture	Manufacturing date	Expiry date	Batch number	NAFDAC status	Volume (mL)
Antibiotic						
Cipro A	India	06/2015	05/2018	Yes	Registered	10 mL
Cipro B	Nigeria	12/2015	11/2018	Yes	Registered	10 mL
Oflox	China	07/2014	06/2017	Yes	Registered	10 mL
Chlorph A	India	07/2015	06/2018	Yes	Registered	10 mL
Chlorph B	India	05/2015	04/2018	Yes	Registered	10 mL
Genta	Nigeria	10/2015	09/2018	Yes	Registered	15 mL
Non-Antibiotic						
Betam A	Nigeria	02/2015	02/2017	Yes	Registered	10 mL
Betam B	India	03/2016	03/2018	Yes	Registered	10 mL
Dexam	Nigeria	04/2015	03/2018	Yes	Registered	10 mL
Timol A	Nigeria	11/2015	10/2018	Yes	Registered	10 mL
Timol B	India	12/2015	11/18	Yes	Registered	10 mL
THZ	Indonesia	05/2015	06/2017	Yes	Registered	10 mL
Cipro- Ciprofloxacin, Oflox- Ofloxacin, Chlorph- Chloramphenicol, Genta- Gentamicin, Betam- Betamethasone, Dexam- Dexamethasone, Timol- Timolol, THZ- Tetrahydrozoline, NAFDAC- National Agency for Food and Drug Administration and Control)						

Table 2: Sample eye-drops with % Active drug, % Preservative, and Physicochemical properties.

Category	Sample code	Active drug	(% content)	Preservative	% Content	pH	Colour and clarity
	Cipro A	Ciprofloxacin	0.3% w/v	BAK	0.01%	6.97	Clear
	Cipro B	Ciprofloxacin	0.3% w/v	BAK	0.01%	7.21	Clear
Antibiotics	Oflox	Ofloxacin	0.3% w/v	BAK	0.02%	6.90	Clear
	Chlorph A	Chloramphenicol	0.5 %w/v	Thiomersal & Boric acid		6.97	Clear
	Chlorph B	Chloramphenicol	0.5 %w/v	PMN & Boric acid		6.02	Clear
	Genta	Gentamicin	0.3 %w/v	BAK	0.01%	5.40	Clear
	Betam A	Betamethasone sodium sulphate	0.1%w/v	BAK	0.01%	5.07	Clear
	Betam B	Betamethasone sodium sulphate	0.1%w/v	BAK	0.01%	7.13	Clear
Non-Antibiotics	Dexam	Dexamethasone	0.1%w/v	PMN	0.001%	7.26	Clear
	Timol A	Timolol	0.5%w/v	BAK	0.01%	7.23	Clear
	Timol B	Timolol	0.5%w/v	BAK	0.01%	6.45	Clear
	THZ	Tetrahydrozoline		BAK	0.01%	6.74	Clear
BAK – Benzalkonium chloride, PMN – Phenyl mercuric nitrate							

Sterility testing result

The sterility testing for the different brands of eye drops cultured in three different nutrient media that support and encourage the growth of aerobic, anaerobic bacteria and fungi respectively (Table 3), showed no growth.

Table 3: Evaluation of the microbiological quality of the different samples of freshly opened antibiotic and non-antibiotics containing eye drops.

Category	Sample code	Liquid thioglycollate medium	Soyabean Casein Digest medium	Sabouraud Dextrose broth
	Cipro A	NG	NG	NG
	Cipro B	NG	NG	NG
Antibiotics	Oflox	NG	NG	NG
	Chlorph A	NG	NG	NG
	Chlorph B	NG	NG	NG
	Genta A	NG	NG	NG
	Betam A	NG	NG	NG
	Betam B	NG	NG	NG
Non-Antibiotics	Dexam	NG	NG	NG
	Timol A	NG	NG	NG
	Timol B	NG	NG	NG
	THZ	NG	NG	NG
NG = No colour change and no growth of organism in the medium				

Microbial challenge test

The antimicrobial efficiency of the preservatives in the eye drops was examined by a preservative assay using four different test microbial strains as challenging organisms (Table 4 and 5). The antibiotic- containing eye drops all had no growth of micro-organism for the 28 days experiment (Table 4) while the other eye drops had growth in a brand of Timolol eye drop (Table 5).

Table 4: Antimicrobial preservative efficacy for the antibiotic eye drops challenged with *E.coli, P. aeruginosa, S. aureus, and C. albicans.*

Sample code	Organism	Sampling time/viable load ($CFUmL^{-1}$)				
		Day1	Day 7	Day 14	Day 21	Day 28
	E.coli	92×10^3	68×10^3	22×10^3	0	0
Cipro A	*P.aeruginosa*	88×10^3	60×10^3	28×10^3	0	0
	S. aureus	79×10^3	50×10^3	16×10^3	0	0
	C. albicans	72×10^3	50×10^3	50×10^3	0	0
	E.coli	104×10^3	82×10^3	38×10^3	0	0
Cipro B	*P.aeruginosa*	77×10^3	50×10^3	26×10^3	0	0
	S. aureus	92×10^3	88×10^3	14×10^3	0	0
	C. albicans	92×10^3	80×10^3	30×10^3	0	0
	E.coli	113×10^3	82×10^3	28×10^3	0	0
Oflox	*P.aeruginosa*	47×10^3	20×10^3	8×10^3	0	0
	S. aureus	109×10^3	88×10^3	30×10^3	0	0
	C. albicans	122×10^3	92×10^3	22×10^3	0	0
	E.coli	41×10^3	22×10^3	9×10^3	0	0
Chlorph A	*P.aeruginosa*	72×10^3	53×10^3	22×10^3	0	0
	S. aureus	70×10^3	50×10^3	16×10^3	0	0
	C. albicans	68×10^3	40×10^3	16×10^3	0	0
	E.coli	87×10^3	60×10^3	20×10^3	0	0
Chlorph B	*P.aeruginosa*	99×10^3	70×10^3	12×10^3	0	0
	S. aureus	68×10^3	50×10^3	12×10^3	0	0
	C. albicans	120×10^3	44×10^3	40×10^3	0	0
	E.coli	98×10^3	50×10^3	38×10^3	0	0
Genta	*P.aeruginosa*	92×10^3	70×10^3	28×10^3	0	0
	S. aureus	123×10^3	70×10^3	22×10^3	0	0
	C. albicans	144×10^3	80×10^3	40×10^3	0	0

Table 5: Antimicrobial preservative efficacy for the non-antibiotic eye drops challenged with *E.coli, P. aeruginosa, S. aureus, and C. albicans.*

Sample code	Organism	Sampling time/viable load ($CFUmL^{-1}$)				
		Day1	Day 7	Day 14	Day 21	Day 28
	E.coli	127×10^3	70×10^3	40×10^3	0	0
Betam A	*P.aeruginosa*	37×10^3	20×10^3	8×10^3	0	0
	S. aureus	90×10^3	67×10^3	23×10^3	0	0
	C. albicans	77×10^3	20×10^3	5×10^3	0	0
	E.coli	88×10^3	62×10^3	23×10^3	0	0
Betam B	*P.aeruginosa*	50×10^3	25×10^3	12×10^3	0	0
	S. aureus	61×10^3	35×10^3	8×10^3	0	0
	C. albicans	73×10^3	51×10^3	6×10^3	0	0
	E.coli	50×10^3	30×10^3	16×10^3	0	0
Dexam	*P.aeruginosa*	60×10^3	32×10^3	15×10^3	0	0
	S. aureus	90×10^3	60×10^3	16×10^3	0	0
	C. albicans	98×10^3	62×10^3	16×10^3	0	0
	E.coli	67×10^3	40×10^3	20×10^3	10×10^3	5×10^3
Timol A	*P.aeruginosa*	50×10^3	30×10^3	15×10^3	8×10^3	3×10^3
	S. aureus	100×10^3	80×10^3	60×10^3	55×10^3	45×10^3
	C. albicans	37×10^3	21×10^3	13×10^3	13×10^3	2×10^3
	E.coli	128×10^3	83×10^3	38×10^3	0	0
Timol B	*P.aeruginosa*	72×10^3	45×10^3	21×10^3	0	0
	S. aureus	88×10^3	65×10^3	30×10^3	0	0
	C. albicans	80×10^3	80×10^3	80×10^3	0	0
	E.coli	95×10^3	67×10^3	44×10^3	0	0
THZ	*P.aeruginosa*	57×10^3	30×10^3	12×10^3	0	0
	S. aureus	120×10^3	90×10^3	32×10^3	0	0
	C. albicans	150×10^3	78×10^3	40×10^3	0	0

Pyrogen testing using Limulus Amebocyte Lysate (LAL) protocol

The standard calibration curve (Figure 1) shows the equation of the straight line as y = 0.1232x, and the linear regression coefficient of 0.9462. This graph shows a direct linear relationship between concentration of standard bacterial endotoxin and absorbance according to Beer- Lamberts law. The concentrations or the levels of bacterial endotoxin for each eye drop sample was calculated from this regression equation and displayed as bar charts (Figure 2).

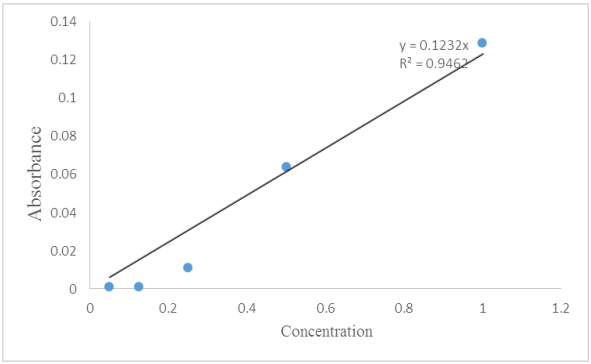

Fig. 1: Standard calibration curve for the bacteria endotoxin test.

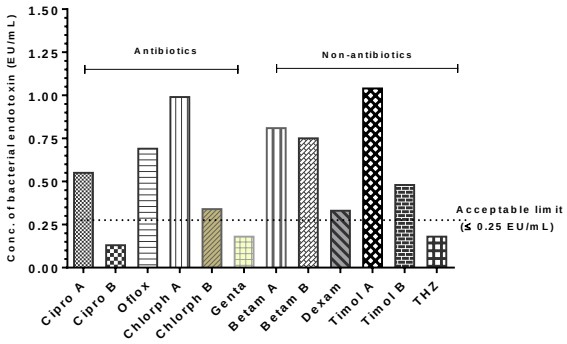

Figure 2. Bar charts representing the concentration of bacteria endotoxin in the eye drop samples

DISCUSSION

The present study assessed the physicochemical property, sterility, preservative efficiency and pyrogen (endotoxin) concentration of some antibiotic and non-antibiotic containing eye drops sold in some retail pharmacies in Port Harcourt, Rivers state, Nigeria. The eye drops used in the study is proliferated both in Nigeria and other neighboring countries due to the globalization of drug distribution.[12] The same types of eye drops are prescribed and dispensed in hospitals and are also available for sale from retail pharmacies in Nigeria. They include products manufactured both locally and internationally and therefore they are a true representation of the commonly used eye drops.

All the eye drops had proper packaging in multi-dose plastic containers and each had good container-closure system that could hinder microbial contamination and ensure sterility of product especially during their shelf-life. The Physical examination of the products (Table 1 and 2) showed the presence of the trade name and active drug substance, manufacturing and expiry dates, batch number and NAFDAC (National Agency for Food and Drug Administration and Control in Nigeria) registration number, which is the drug regulatory agency in Nigeria. The pH analysis for the different eye drops showed a range from 5.07 - 7.26. Studies has shown that pH between 6- 9 is not irritant to the eyes but outside this range may cause increased production of lacrimal fluid to assuage the discomfort and give relief to the eyes thereby reducing the drugs bioavailability[13] In this study only two products, Genta and Betam A had pH of 5.40 and 5.07 respectively. The pHs of an eye drop solution is an indication of its acidity or basicity, and are usually buffered with a suitable buffer system to resist changes in the pH. A minute alteration in the pH of an eye drop caused by microbial contamination could lead to precipitation of a drug, out of the solution leading to decreased stability and degradation of the product. Also the bioavailability of the drug into the tissues are also affected by the percentage of the drug in the ionized and nonionized form which is also pH-dependent. The pH changes of a formulation therefore affects the lipophilicity of the drug and hence its absorption and distribution into the eye tissues. All the eye drops showed clear and colourless solution indicating absence of particulate matter (Table 2). This shows excellent quality control and process capability in filtration process of the eye drops.

The goal of preservation of eye drops was to maintain the stability of the product and reduce the risk of further eye infection.[5] The most common (66.66%) preservative used in different brands of eye drops from our study was benzalkonium chloride (BAK) at a percentage of 0.01% though Ofloxacin eye drop had a concentration of 0.02% BAK. The good and bad aspect of BAK regarding its corneal toxicity has been widely documented in literature[11,13,14] therefore regulatory bodies should regulate and harmonize its usage in eye drops. Other preservatives used in some of the eye drops were phenyl mercuric nitrate and thiomersal combined with boric acid, but their % content was not indicated by the manufacturers. All the eye drops in the study had an efficient preservative system (Table 3) since there was absence of cloudiness or growth indicating no microbial (fungi and bacteria) contamination and hence sterility of products was assured on purchase of product and during its shelf life.

The microbial challenge test or preservative efficiency test is a test that simulates the in-use situation of multi-dose eye drops after breaking the seal and covering. The result of the challenge test (Table 4 and 5) show that all the antibiotic eye drops resisted the microbial challenge while the eye drop (Timol A), a non-antibiotic continued to have microbial growth after inoculation which showed a failed preservative system. The drug Timolol (Timol A) is a β-blocker and is used for long-term management of glaucoma. Much concern has been raised clinically

over the use of preservatives in glaucoma medications in multi-dose containers because BAK which is used in over 70% of topical eye formulations.[11] tend to accumulate on the eye over a long use and cause apoptosis and ocular surface diseas[15] there is the need to develop preservative-free eye drops, in unit-dose containers or develop less toxic (antimicrobial) preservative. Inefficient preservative system could result in contamination of product during usage and therefore lead to other secondary infections that could worsen the chronic ocular disease and compound the situation.

The pyrogen test (Figure 2) reveals that only two antibiotic eye drops (Cipro B and Genta) and a non-antibiotic (THZ) eye drop were within the acceptable limit which is ≤ 0.25 EU/mL while the rest of the nine eye drops failed the test. This may mean that most pharmaceutical companies producing sterile eye drops only emphasize on sterility and its preservation without caring about the endotoxin level (pyrogen test). This could be attributed to the fact that the eye drops are applied topically to the eye surface and do not have direct contact with the blood like parenteral products and medical devices.

CONCLUSION

The assessment of the eye drops sold in Port Harcourt found all of the products of good quality in terms of sterility and organoleptic analysis. Studies to ascertain the percent content of the preservative in the eye products should be carried out, as toxicity to their extended use has been implicated clinically. Pharmaceutical companies producing eye products should also check their endotoxin limit to secure their safety.

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