THE ROLE OF PRIMARY AND SECONDARY PACKAGING ON THE ANTIBACTERIAL ACTIVITY OF SUNLIGHT EXPOSED SYNTHETIC AND HERBAL EYE DROPS

A DISSERTATION SUBMITTED TO THE DEPARTMENT OF MATHEMATICS AND NATURAL SCIENCES, BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF BACHELOR OF SCIENCE IN BIOTECHNOLOGY

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Dedicated to my beloved

Parents and Sister

DECLARATION

I hereby declare that the research work representing the results reported in this thesis entitled **"The role of primary and secondary packaging on the antibacterial activity of sunlight exposed synthetic and herbal eye drops"** submitted by the undersigned has been carried out under the supervision of Kashmery Khan, Lecturer, Biotechnology program, Department of Mathematics and Natural Sciences, BRAC University, Dhaka. I also declare that the research work presented here is original and any information or reference to research works performed by other researchers has been cited accordingly. This research paper has been submitted in the partial fulfillment for the degree of Bachelor of Science in Biotechnology, BRAC University, Dhaka and has not been submitted to any other institution for any degree or diploma.

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ACKNOWLEDGEMENT

In the outset, I am thankful to the Almighty Allah to have enabled me to perform this thesis work by giving me patience, protection and good health.

I express my sincere gratitude to Professor **A F M Yusuf Haider**, Chairperson, Department of Mathematics and Natural Sciences, BRAC University and Professor **A. A. Ziauddin Ahmad**, former Chairperson, Department of Mathematics and Natural Sciences, BRAC University, for allowing me to conduct this study.

My deepest regards, gratitude and appreciation goes to my supervisor **Kashmery Khan**, Lecturer, Department of Mathematics and Natural Sciences, BRAC University for her constant supervision, constructive criticism, expert guidance, devoted involvement and enthusiastic encouragement throughout my entire research work. Without her supervision, cooperation and valuable suggestions, it would not have been possible to complete the study.

I would like to extend deepest regards and my utmost appreciation to **S M Rakib-Uz-Zaman** Lecturer, Department of Mathematics and Natural Sciences, BRAC University for his constant guidance, care and enthusiastic encouragement and constant supervision.

A very special thanks to **Zubaida Marufee Islam**, Lecturer (On Leave), Department of Mathematics and Natural Sciences, BRAC University for her constant guidance, care and enthusiastic encouragement starting from my first semester at BRAC University. Life at BRAC University would not have been the same if it weren't for her constant supervision and motivation**.**

It is a great pleasure to express my sincere gratitude to all the faculty members of Department of Mathematics and Natural Sciences, BRAC University especially **Dr. M. Mahboob Hossain**, Professor; **Dr. Aparna Islam**, Professor; **Ms. Jebunnesa Chowdhury**, Assistant Professor; **Ms. Romana Siddique**, whose knowledge and experiences shared during the coursework of my undergraduate program have made me capable of performing this research work by myself.

I would like to extend my special thanks to **Asma Binte Afzal** and **Nahreen Mirza** a for their never -ending support, motivation and diligent guidance which helped pave my way

through this research much easier. In times when I felt completely helpless with my work, it was them of all people who came forward with a bright smile and helped me out in all situations.

My cordial thanks and best wishes go to my friend **Permita Mazumder** for constantly helping me out throughout my thesis with her valuable support and company.

Last but not the least, I would like to thank **Asif Hossain** for always being there for me and supporting me throughout my BRAC University life.

To all the people mentioned and also not-mentioned, “Thank You” is a very small word to show my gratitude but still a big Thank You to you all from the core of my heart.

Fariha Zerin Lamisha

December, 2017

ABSTRACT

Eye infections are considered as one of the most serious public health problem around the world, and are responsible for the blindness or visual impairment of about 1.9 million people every year. These Infection spreads through personal contact (via hands, clothes or bedding) and by flies that have been in contact with discharge from the eyes or nose of an infected person. As a protective measure the use of tropical ophthalmic solution is increasing. So, understanding the photo-stability and photosensitivity of these ophthalmic drugs are very important to treat eye diseases effectively. In the present study, the photo-degradation of sunlight treated and non- treated ciprofloxacin and herbal eye drops were compared in terms of their antibacterial activity against gram positive and gram negative bacteria. Ciprofloxacin and herbal eye drops manufactured by different companies were collected from local market and exposed to sunlight and their antibacterial activities were tested. Each eye drops was exposed to sunlight for ten hours in three different ways with secondary and primary packaging, primary packaging and transparent glass tubes. Antibacterial activity of synthetic and herbal eye drops was tested by well diffusion method against *Staphylococcus aureus, Bacillus cereus, Salmonella typhi, Escherichia coli* (STEC) and *Proteus vulgaris*. The result indicates a photo-degradation of ciprofloxacin eye drops which were turned into deep yellow color in primary and transparent glass packaging which might cause photo-toxicity, photo-allergenicity and other reaction of eyes. In contrast, the herbal eye drop did not show any color change. Significant differences in zone of inhibition were also observed among synthetic eye drops. However, herbal eye drops did not show any antibacterial activity against *Bacillus cereus, Salmonella typhi, Escherichia coli* (STEC) and *Proteus vulgaris*. The sunlight exposed eye drops showed decreasing photo-stability, so emphasis should be given to improve the manufacturing, packaging and storage systems of ophthalmic formulations.

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List of Abbreviations

Abbreviatons	Elaborations
WHO	**World Health Organisation**
CIPRO	**Ciprofloxacin**
DNA	**Deoxyribonucleic acid**
NA	**Nutrient Agar**
MHA	**Mueller-Hinton Agar**
ºC	**Degree Celsius**
et al.	**And others**
ml	**Milliliter**
Mm	**Millimeter**
ATN	**Anti-toxin**
No ZOI	**No zone of inhibition**

Chapter 1- Introduction

Introduction:

1.1 Background:

Eye infections can be vision-threatening and must be treated effectively by appropriate and safe use of topical ophthalmic anti-infectives for example; anti-toxins (ATN), antiseptics, antifungal, against helminthes or hostile to viral can be utilized relying upon the type of contamination (Gignac *et al*., 2011). Eye is a very sensitive organ of human body it might be infected by bacteria, fungi, parasites, or viruses the prevalence of these infections and the responsible bacterial organisms may vary with patient's age and geographic location (Reed, 2011). Antibiotics are broadly used to treat eye infection but prolong use of synthetic antibiotics are causing resistance of antibiotics against pathogenic bacterial and viral agents; it is also proven to have various kind of side effects (Dhamankar *et al*., 2014). The human eye is one of the most important and phenomenal sensory systems we assemble a large portion of information about our external environment through our eyes moreover we also depend on sight more than on some other senses. The surface of iris and the fundus blood vessel design are totally different in each individual, which gives appropriate features to biometric-recognition (Irsch & Guyton, 2009). The microbial flora of the external ocular surface during birth is formed by a number of microorganisms closely associated with the eye. There are several mechanisms that protect the extra-ocular surface of eyes and its surface epithelium keeping them sterile. Because of injury or decrease of local or systemic immunity we may incline the eye to diseases. Furthermore, the number and virulence of the invading pathogens play a vital part in initiating a disease, other than numerous ecological microbes, bacteria, protozoa and organisms can set up infection which can be treated by antibiotics (Sharma S, 2011).

Figure 1.1: Human eye

(Retrieved from: https://www.tes.com/lessons/IxYOmgZW9gwJxg/eyes-3)

According to the World Health Organization (WHO) blindness as a visual acuteness of 3/60 or less, it is evaluated that presently there are 45 million people worldwide who are bilaterally blind and another 135 million that have seriously debilitated vision in both eyes on the other hand 180 million individuals in the world today are suffering with visual impairment, it is very unfortunate circumstance in both social and monetary terms, however this number is not enough to address the extra millions who are incapacitated by monocular visual loss (Gignac *et al.,* 2011). Significant reasons for visual impairment worldwide are the infection in cornea. The study of disease transmission of corneal blindness is complicated and includes a wide range of infectious and inflammatory eye diseases that cause corneal scarring, which eventually prompts blindness (Whitcher *et al.,* 2001). The most common bacterial eye infections is conjuctivities there are some other bacterial infections also such as keratitis, blehpharitis, endophthalmitis, cellulitiseic which are caused by *Staphylococcous spp., Streptococcus spp., Haemophilus influenza, Pseudomonas*, enteric gram negative *bacilli* and some anaerobic bacteria (Gignac *et al.*, 2011). The predominance of corneal infections varies changes from nation to nation while cataract causes almost 20 million of the 45 million blind individuals on the world the following significant reason is trachoma which blinds 4.9 million people due to corneal scarring and vascularization (WHO, 2001). Infections of the eye can spread rapidly damaging important functional structures and lead to permanent vision loss or blindness. Broad-spectrum antibiotic eye drops are administered at the affected region as soon as a diagnosis is made based on

treatment for eye disease; fortified eye drops are often used (Snyder & Glassier, 1994). Antibiotic treatment may bring about executing the microorganism or restraining bacterial development when bacteriostatic drugs are used to ocular infection, the host defense mechanisms are ultimately eradicates the infective organism (Lakey, 2017). The penicillin, cephalosporin, amino glycosides, and fluoroquinolones are bactericidal drugs that are used for treatment ocular infections. Antimicrobial resistance is a crucial concern for physicians, resistance is found to ampicillin, gentamycin, tobramycin, more up to date cephalosporin, and fluoroquinolones (Snyder & Glassier, 1994). Generally, it is considered that regular and prolong use of given antibiotic ophthalmic solutions increases the rate of resistance of drug against bacterial strains on the contrary, there is not much studies available about increased resistance of bacteria following the treatment of acute bacterial conjunctivitis or other ocular infection. It can be an assumption that increased antibiotic resistance for a prescribed drug is the systemic concentrations of the drug. Thus, antibiotics administered topically to the eyes may not affect bacterial resistance to a substantial level (Chandra *et al*., 2017).

1.2 Ciprofloxacin antibiotic eye drops:

Ciprofloxacin (CIPRO) is a broad- spectrum antibiotic of synthetic fluoroquinolone series. The IUPAC name of ciprofloxacin is 1-cyclopropyl-6-fluoro-4-oxo-7-piperazin-1-yl-quinoline-3-carboxylic acid (Gignac *et al*., 2011). It is a second-generation fluoroquinolone antibacterial agent which eliminates bacteria by hindering the enzymes that cause DNA to rewind after being copied and stops synthesis of DNA and of protein. Ciprofloxacin ophthalmic solution USP, 0.3% is a synthetic, sterile, multiple dose, and antimicrobial drug for topical use (Horspool & Armesto, 1992).

Ciprofloxacin is marketed worldwide with over three hundred different brand names. In the United States, Canada, and the UK, it is marketed as Baycip, Ciloxan, Ciflox, Cipro,CiproXR, Cipro XL, Ciproxin, Prociflor, and most recently, Proquin. In Bangladesh a wide range of ciprofloxacin eye drops are marketed as Cipcin, Ciprocin, Aprocin, Ciprozid and Bactin etc.

1.3 Chemistry of ciprofloxacin:

Ciprofloxacin is a fluorinated quinolone which structurally resembles to nalidixic acid, which is the prototype 4- quinolone antibiotic it has a fluorine atom at the 6-position, a piperazine moiety at the 7-position, and a cyclopropyl ring at the 1 position. The gram-negative in vitro activity is related with the presence of the piperazine group at the 7-position; the of this group may improve entrance of the quinolones into bacteria and may increase action against *P. aeruginosa*. A fluorine attached at the 6-position broaden the spectrum and increases antimicrobial activity upto 30 times; A 2-carbon group at the 1-position is fundamental for antimicrobial activity. Larger or smaller group for the most part prompt decreased activity; it also has a cyclopropyl attachment, which might be spatially equal to a 2-carbon gathering and is thought to enhance general antibacterial movement (Al-Omar, 2004).

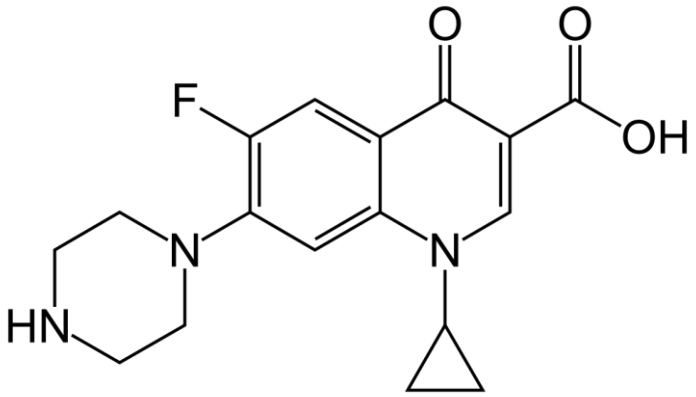


Figure 1.2: Molecular structure of Ciprofloxacin (retrieved from: https://en.wikipedia.org/wiki/Ciprofloxacin)

It is commercially available as the mono-hydrochloride monohydrate salt of 1-cyclopropyl-6-fluoro-1, 4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline-carboxylic acid it has a faint to light yellow crystalline powder component. Its empirical formula is C17H18FN3O3• HCl•H2O (Torniainen *et al*., 1996).

1.4 Mechanism of action of ciprofloxacin eye drops:

The fundamental mechanism of ciprofloxacin is inhibition of the action of the subunit of DNA gyrase. Hindrance of bacterial gyrase causes unwinding of the supercoiled DNA. These progressions lead to termination of chromosomal replication and to interference with cell division and gene expression. Secondary mechanisms inhibit the action of topoisomerase IV, leads to partition of two joined DNA molecules and consequent obstruction with cellular replication (Heydari and Samarai, 2011).

Ciprofloxacin has been appeared to eliminate microorganisms in both growth and stationary phases. This might give benefits over different classes of antibiotics, for example, b-lactams, which are not bactericidal when bacteria are in the stationary period of development or developing gradually (Reed, 2011).

1.5 Therapeutic uses of ciprofloxacin eye drops:

Ciprofloxacin is highly active *in vitro* against a wide range of gram-negative and gram-positive organisms. Ciprofloxacin ophthalmic solution is used for treatment of different kind of eye disease such as - corneal ulcers caused by susceptible isolates, bacterial conjunctivitis, external eye infection and keratitis etc. It is also indicated in the treatment of kerato- conjunctivitis, bhepharo- conjunctivitis, acute meibomianitis, dacryocystitis and prohylaxis caused due to *Neisseria gonorrhea* or *Chlamydia trachomatis* (Modarrea *et al*., 1998). *Chlamydia trachomatis* and *Mycobacterium tuberculosis* are only moderately susceptible. Most strains of *Pseudomonas* such as *Pseudomonas cepacia* and *Pseudomonas maltophiliaare* resistant to ciprofloxacin also anaerobic bacteria such as *Bacteroides fragilis* and *Clostridium difficileare* resistant to ciprofloxacin (J Hong *et al*., 2012).

Ciprofloxacin ophthalmic solution is additionally used to treat bacterial diseases of the eye including conjunctivitis, pinkeye; infection of the membrane that covers the outside of the eyeball and within the eyelid and corneal ulcers infection and loss of tissue free front piece of the eye. Ciprofloxacin ophthalmic ointment is utilized to treat conjunctivitis (Soreth, 1998). It is effective against a wide range of ocular pathogens, except fungi and viruses. Because of the drug's broad-spectrum coverage of ocular pathogens and the ease of using a single, commercially available ophthalmic antibiotic solution compared with a pharmacy compound, fortified

antibiotic solution, and ciprofloxacin has increasingly been used to treat infectious keratitis (John, 2001).

1.6 Adverse effect of ciprofloxacin eye drops:

Serious adverse effects occur more commonly with fluoroquinolones than with any other antibiotic drug classes. In most cases, adverse reactions are mild to moderate; however, occasionally serious adverse effects occur (Jose & Kuttan, 1995). Prolong use of CIPRO may result in overgrowth of non-susceptible organisms including fungi; it should always be discontinued if skin rash or any other sign of hypersensitive reaction occurs. During pregnancy CIPRO can be used only if there is no potential risk to the fetus (Sharma S, 2011).

All ophthalmic antibiotics, 1.5%, are approved by the Food and Drug Administration to treat bacterial conjunctivitis (Modarrea *et al*., 1998). Resistance to ciprofloxacin *in vitro* usually develops slowly. The adverse effect of ocular ciprofloxacin is mild. Potential side-effects are the formation of a white crystalline precipitate on the superficial portion of the treated cornea, lashes, discomfort, foreign-body sensation, chemosis, punctate epithelialerosion, eyelid oedema, conjunctival hyperaemia, tearing, itching and superficial punctate keratitis (John, 2001)

Table 1.1: Composition of ciprofloxacin ophthalmic formulation used in this study

Product name	Composition	Preservative	Vehicle
Ciprofloxacin A	Ciprofloxacin Hydrochloride BP equivalent to Ciprofloxacin 3 mg	Benzalkonium Chloride 0.06 mg	-
Ciprofloxacin B	Ciprofloxacin Hydrochloride BP equivalent to Ciprofloxacin 3 mg	Benzalkonium Chloride 0.01 %	-
Ciprofloxacin C	Ciprofloxacin Hydrochloride BP equivalent to Ciprofloxacin 3 mg	Benzalkonium Chloride 0.06 %	Polyvinyl Alcohol USP 1.4 %
Ciprofloxacin D	Ciprofloxacin USP 3.00 mg	-	-
Ciprofloxacin E	Ciprofloxacin USP 3.00 mg as Ciprofloxacin Hydrochloride	Benzalkonium Chloride	

1.7 Sunlight induced photochemical degradation of ciprofloxacin ophthalmic solution (0.3%):

Photo-stability studies of drug are an integral part of drug advancement process in pharmaceuticals industry. Drugs are sensitive to sunlight that degrades the formulated product during manufacturing, storage, and administration. This may bring about loss of potency, changed adequacy, and unfavorable biological effects (Ahmad *et al.*, 2016). The photo sensitivity of drug to a specific spectral region of light varies with its chemical structure, photo-reactivity, and nature of the dosage (Horspool and Armesto, 1992). The rate of a photo-chemical response is influenced by wavelength of the radiation source and transmission attributes of the container (Ahmad *et al.*, 2016). The investigation of the photo-chemical responses gives information on the form of standardized active ingredients in a product and in which reactions are often complex and involve free radical species forming photo-products (Turro *et al.*, 2010). The photo products of a drug are destructive and can cause photo-toxic, photo-allergic or photo-sensitization responses upon administration (Kullavanijaya and Lim, 2005). According to *European pharmacopeia* they prescribe more than 250 drugs and adjuvant, photochemical reactions may lead to the degradation of the drug substances by one or more pathways to form different products. This elucidation mechanism of these pathways requires a thorough understanding of the nature and type of the photochemical reactions involved in ciprofloxacin eye drops formulation. These would largely depend on the presence of certain functional groups, physical characteristics (light absorption, p*K*as, solubility, etc.), and the degradation mode of the compound. The knowledge of photochemical behavior of drugs can provide guidance for handling, packaging, and labeling of drug products. The use of the appropriate containers and packaging material can protect the products from the deleterious effects of sunlight or light (Sun *et al.*, 2008).

Ciprofloxacin is perceived as a safe to utilize tropical solution, yet there have been reports of skin reddening and rashes in patients taking these antibiotics after sun-exposure, this symptom is photosensitivity. The loss of antibiotic efficiency happens when ciprofloxacin is irradiated in sunlight and afterward tested against *Escherichia coli* (Tobin *et al.*, 1990). Fluoroquinolones are photodegradable compounds that deliver different photo-degrades items after exposure to natural sunlight of ciprofloxacin in eye drops preparation forms an ethylenediamine derivatives of

ciprofloxacin that opens the piperzinyl ring (Tiefenbacher *et al*., 1994). The structure of degraded ciprofloxacin expose to sunlight was also affirmed by electro spray mass spectrometric analysis which contains a piperazine aggregate that is the site of photodegradation and the breakdown of ciprofloxacin ophthalmic solution happened quickly after sunlight presentation (Jiben *et al*., 2005). The solid inductive impact of ortho-fluorine in ciprofloxacin improved the light-catalyzed cleavage of the piperazine ring (Das and Subodh, 2005). Daylight uncovered examples of ciprofloxacin eye drops demonstrated 20-30% loss of power of ciprofloxacin and marginally lesser antimicrobial movement (Jiben *et al*., 2005). The photosensitivity of the medication substances the solid and fluid dose frames makes it important to embrace proper measures for their adjustment during assembling, manufacturing, compounding, and storage. The normal way to deal with the cost of photo-stabilization of pharmaceuticals is to shield them from light by utilizing reasonable essential suitable primary and secondary packaging (Ahmad *et al*., 2016).

1.8 Herbal eye drops

Herbal preparations are gaining popularity worldwide over past few years. Due to immense side effects of synthetic drug and increasing antibiotic resistance, people are now using traditional or alternative medicine all over the world. Herbal formulations are made up of medicinal herbs, minerals and metals or any combination herbal medicines, which consist of biologically active compounds from plant materials, or whole plants (WHO, 2002). The use of medicinal plants in modern medicine suffers from the fact that although hundreds of plants are used in the world to prevent or to cure diseases, scientific evidence in terms of modern medicine is lacking in most cases. Now a day, it has turned out to be exceptionally hard to treat bacterial disease because of ability of microbial pathogens to develop resistance against antimicrobial agents. Antimicrobial agents are ordered by their system of activity, for example, interference with cell wall synthesis, DNA and RNA synthesis, lysis of the bacterial membrane, inhibition of protein synthesis and inhibition of metabolic pathways (Chandra *et al*., 2017). As of now, the clinically accessible treatment isn't compelling against the antibiotic resistance created by some bacterial species. But, plant metabolites incorporate quinines, alkaloids, lectins, polypeptides, flavones, flavonoids, flavonols, coumarin, terpenoids, basic oils and tannins have demonstrated tremendous potential

to fight against bacterial, contagious, protozoal and viral maladies with no known symptoms (Iwu *et al*., 1999). Even though there are efficacies of various medicinal plants available for ophthalmic disorder, no herbal preparation is available for clinical use. Moreover, the plants used in the traditional system of medicine have not been studied extensively using experimental models (Srinivas and Prabhakaran, 1989).

An herbal ophthalmic preparation is evaluated to investigate their phytodegradation capability. The preparation contains the following components:

Table 1.2: Composition of herbal ophthalmic preparation

Product name	**Extracts**	**Quantity**	**Preservative**
Herbal eye drop	Honey	37% w/v	Phenyl mercuric nitrate -0.002 % w/v
	Emblica officinalis	1.3 % w/v	-
	Curcuma longa	1.3 % w/v	-
	Ocimum sanctum	1.3 % w/v	-
	Rosa damascene	1.1 % w/v	-
	Cinnamomum camphora	0.05 % w/v	-
	Carum copticum	6.00 % w/v	-
	Terminalia belerica	6.50 % w/v	-

In this herbal eye drops formulation each ingredient are known to possess antimicrobial, anti-inflammatory, antioxidant and soothing properties and are used in traditional medicine for the treatment of a variety of ocular disorders (Biswas *et al*., 2001).

1.9 Effects and functions of components of herbal eye drops:

One of the main components of herbal eye drop is honey. Honey is generally reported to use for sore eyes. Honey has ingredients similar to antibiotics, it is reported to prevent infection and

promote healing (Mitra *et al*., 2000). The exudates obtained from the incisions of *Emblica oficinalis* fruits are used as an external application for eye inflammation (Nadkarni, 1976). An aqueous extract of *Emblica oficinalis* was found to be a potent inhibitor of lipid peroxide formation as radicals of hydroxyl and superoxide *in vitro* (Jose & Kuttan, 1995). Extracts of *Curcuma longa* possesses anti-inflammatory, antioxidant and antimicrobial properties. Extracts of *Curcuma longa* exhibited anti-inflammatory, antioxidant and antimicrobial properties (Amon and Wahl, 1991). The volatile and fixed oils of *Ocimum sanctum* are known for their anti-inflammatory activity (Singh and Agrawal, 1991). The essential oil of *Ocimum sanctum* possesses bactericidal activity against gram-positive and gram-negative bacteria (Prasad and Rao, 1987). Rose water obtained from petals of *Rosa damascena* is known for its soothing effect and is also found to be beneficial in ophthalmopathy (Kirkitikar and Basu, 1987). Fruits of *Terminalia belerica* were found to be useful in many ocular diseases. Fully ripe or dried fruit, mixed with honey is used as an external application in ocular diseases (Nadakarni, 1976). It also possesses significant antimicrobial activity against both gram-positive and gram-negative organisms (Valsaraj *et al*., 1994). *Carum copticum* has been shown to possess antibacterial activity against *Salmonella typhora*, *Micrococcus pyogenes* and *Escherichia coli*. It is also recommended as a potential source of natural antioxidant (Mehta *et al*., 1994). The extract of *Cinnamomum camphora* showed antibacterial activity against gram-positive and gram-negative organisms (Naqvi *et al*., 1985).

The positive role of herbal eye drop formulation has been proved by the above mentioned experimental and limited clinical studies (Reddy *et al*., 1998). However, further clinical evaluation of herbal eye drops is necessary to estimate the clinical potency. More study needs conducted in order to evaluate the effectiveness of herbal eye drop in various ocular disorders (Mitra *et al*., 2000).

1.10 Mechanism of action of herbal eye drops:

Ingredients in herbal eye drop have antimicrobial, anti-inflammatory, antihistaminic, analgesic and antioxidant properties, which make it effective and useful in cases infective eye diseases and inflammatory ophthalmic disorders. *Cinnamomum Camphora* and *Emblica Officinalis* in it provide cooling sensation and help to reduce irritation, redness and burning sensation. Other

ingredients provide relief from eye strain and eye congestion. Herbal eye drop is effective in the management of infective and inflammatory eye disorders due to its potent pharmacological activities. It also has eye cooling activity; relieves eye congestion and is beneficial in eye strain (Singh, 2017).

1.11 Antibacterial activity of herbal eye drops:

Some components of have antibacterial properties such as *Carum copticum* have been shown to possess antibacterial activity against *Salmonella typhora*, *Micrococcus pyogenes* and *Escherichia coli.* The essential oil of *Ocimum sanctum* and *Cinnamomum camphora* possesses bactericidal activity against gram-positive and gram-negative bacteria (Kumari, 1991).

1.12 Therapeutic application of herbal eye drops:

Herbal eye drops is indicated in all types of eye infections and inflammatory disorders. Some of its important indications are allergic acute conjunctivitis, allergic chronic conjunctivitis, infective acute conjunctivitis, infective chronic conjunctivitis, inflammatory ophthalmic disorders, eyestrain excessive lacrimation, dry eyes, and computer vision syndrome (Reddy *et al*., 1998).

1.13 Bacterial strains sensitive to eye drops:

Bactericidal activities of ciprofloxacin ophthalmic solution (0.3%) and herbal eye drops were observed against the following gram positive and gram negative bacterial species isolated from clinical samples:

Staphylococcus spp:

Staphylococci are Gram-positive bacteria, with diameters of 0.5 – 1.5 μm and characterized by individual *cocci*, which are divided in more than one category to form grape-like clusters. *Stapylococcus aureus* is a gram positive bacterium and a major human pathogen that causes a wide range of clinical infections. It is the leading cause of bacteremia and infective endocarditis as well as osteoarticular, skin and soft tissue, pleuropulmonary, and device-related infections (Tong *et al*., 2015).

Bacillus spp:

Members of the bacterial genus *Bacillus*, which are ubiquitous in the environment, are aerobic or facultative anaerobic gram positive organisms, variable spore forming rods. The vegetative cells range from 0.5 by 1.2 to 2.5 by 10µm in diameter (Tajkarimi, 2007). And can grow at optimal temperatures ranging from 25 to 37°C. *Bacillus cereus* is a gram-positive, spore-forming microorganism capable of causing food borne disease (Drobniewski, 1993).

Salmonella spp:

Salmonella typhi is a strain of bacteria that lives only in humans. It causes a bacterial infection of the intestinal tract and occasionally the bloodstream which is called typhoid fever. *Salmonella typhi* is a gram-negative bacterium. A very similar but often less severe disease is caused by *Salmonella* serotype paratyphoid A (WHO, 2007).

Escherichia coli:

The gram negative organisms are commonly gut bacteria, but some pathogen strains can sometime produce enterotoxin that can cause food borne diseases and gastrointestinal infections. These organisms can cause urinary tract infection (Tortora *et al*., 2010).

Proteus vulgaris:

Proteus vulgaris is a species of gram-negative and facultative anaerobic bacteria that shows swarming motility and urease activity. *Proteus* ranked third as the cause of hospital-acquired infections. The organism is rod-shaped and motile bacterium with diverse mode of transmission. *P. vulgaris* is a common causative organism of urinary tract infection (UTI) in the complicated urinary tract, most frequently in patients with the indwelling catheters or structural abnormalities of the urinary tract (Trivedi *et al*., 2016).

1.14 Objectives:

The present study is carried out to analyze the sunlight sensitivity and antibacterial activity of the formulated products. The objectives of this study are given below:

- To investigate the role of primary and secondary packaging on the photo degradation of ciprofloxacin and herbal eye drops
- To understand and compare the photo-degradation of ciprofloxacin and herbal eye drops
- To reveal the antibacterial activity of sunlight-treated and non- treated eye drops against gram positive and gram negative bacteria
- A comparative study to evaluate the efficacy of synthetic and herbal eye drops.

Chapter 2 - Materials and Methods

2.1 Research Laboratory

This research work is laboratory based so it was carried out in the Microbiology and Biotechnology Laboratories of the Department of Mathematics and Natural Sciences at BRAC University, Dhaka, Bangladesh. In this laboratory, BSL-2 (Bio-safety level 2) facility is followed and the entire microbiological works were done within laminar flow cabinet. In this research, the experiment was done to evaluate the antimicrobial activity of ciprofloxacin eye drops after exposure to sunlight and also was compared with the antibacterial activity of degraded herbal eye drops on some gram-positive and gram-negative bacteria.

2.2 Materials:

2.2.1 Equipments:

- Laminar airflow cabinet (Model-SLF-V, vertical, SAARC group Bangladesh)
- Incubator (Model-0SI-500D, Digi system Laboratory Instruments Inc. Taiwan)
- Vortex machine (Digi system Taiwan, VM-2000)
- Autoclave machine (Model: WIS 20R Daihan Scientific Co. ltd, Korea)
- Refrigerator
- Glass wares, laboratory distillation apparatus – fractional distillatory set up, petri-dishes, micro-pipettes, Bunsen burner, test tube, vial, inoculating loops, cotton swabs, cork borer, conical flask, beaker, and spatula, measuring tube.
- McFarland turbidity standards no: (1, 2 and 3)

2.2.2 Reagents:

- 0.9 % NaCl (Sodium chloride) – normal saline
- Distilled water
- 70% ethanol

2.2.3 Media:

Different types of media were used for selective growth and to determine the zone of inhibition.

2.3 Nutrient agar (NA):

Nutrient Agar is a general purpose, nutrient medium used for the cultivation of microbes supporting growth of a wide range of non-fastidious organisms. Nutrient agar is popular because it can grow a variety of types of bacteria and fungi, and contains many nutrients needed for the bacterial growth.

Composition of Nutrient Agar:

- 0.5% Peptone: It is an enzymatic digest of animal protein. Peptone is the principal source of organic nitrogen for the growing bacteria.
- 0.3% beef extract/yeast extract: It is the water-soluble substances which aid in bacterial growth, such as vitamins, carbohydrates, organic nitrogen compounds and salts.
- 1.5% agar: It is the solidifying agent.
- 0.5% NaCl: The presence of sodium chloride in nutrient agar maintains a salt concentration in the medium that is similar to the cytoplasm of the microorganisms.

Distilled water: Water is essential for the growth of and reproduction of micro-organisms and also provides the medium through which various nutrients can be transported.

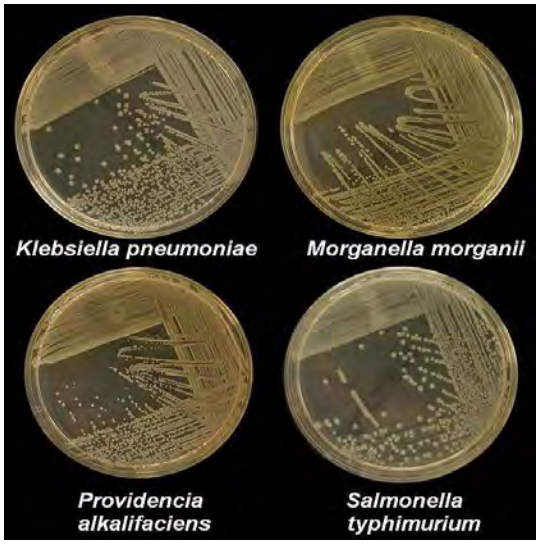


Figure 2.1: Nutrient Agar (Retrieved from: https://microbiologyinfo.com/nutrient-agar-composition-preparation-and-uses/)

2.4 Muller Hinton Agar (MHA):

Mueller and Hinton developed Mueller Hinton Agar (MHA) in 1941 for the isolation of pathogenic *Neisseria* species. Nowadays, it is more commonly used for the routine susceptibility testing of non-fastidious microorganism by the Kirby-Bauer disk diffusion technique.

A growth medium, usually Mueller-Hinton agar, is first evenly seeded throughout the plate with the isolate of interest that has been diluted at a standard concentration (approximately 1 to 2 $x10^8$ colony forming units per ml). Commercially prepared disks, each of which is pre impregnated with a standard concentration of a particular antibiotic, are then evenly dispensed and lightly pressed onto the agar surface. The test antibiotic immediately begins to diffuse outward from the disks, creating a gradient of antibiotic concentration in the agar such that the highest concentration is found close to the disk with decreasing concentrations further away from the disk. After an overnight incubation, the bacterial growth around each disc is observed. If the test isolate is susceptible to a particular antibiotic, a clear area of "no growth" will be observed around that particular disk.

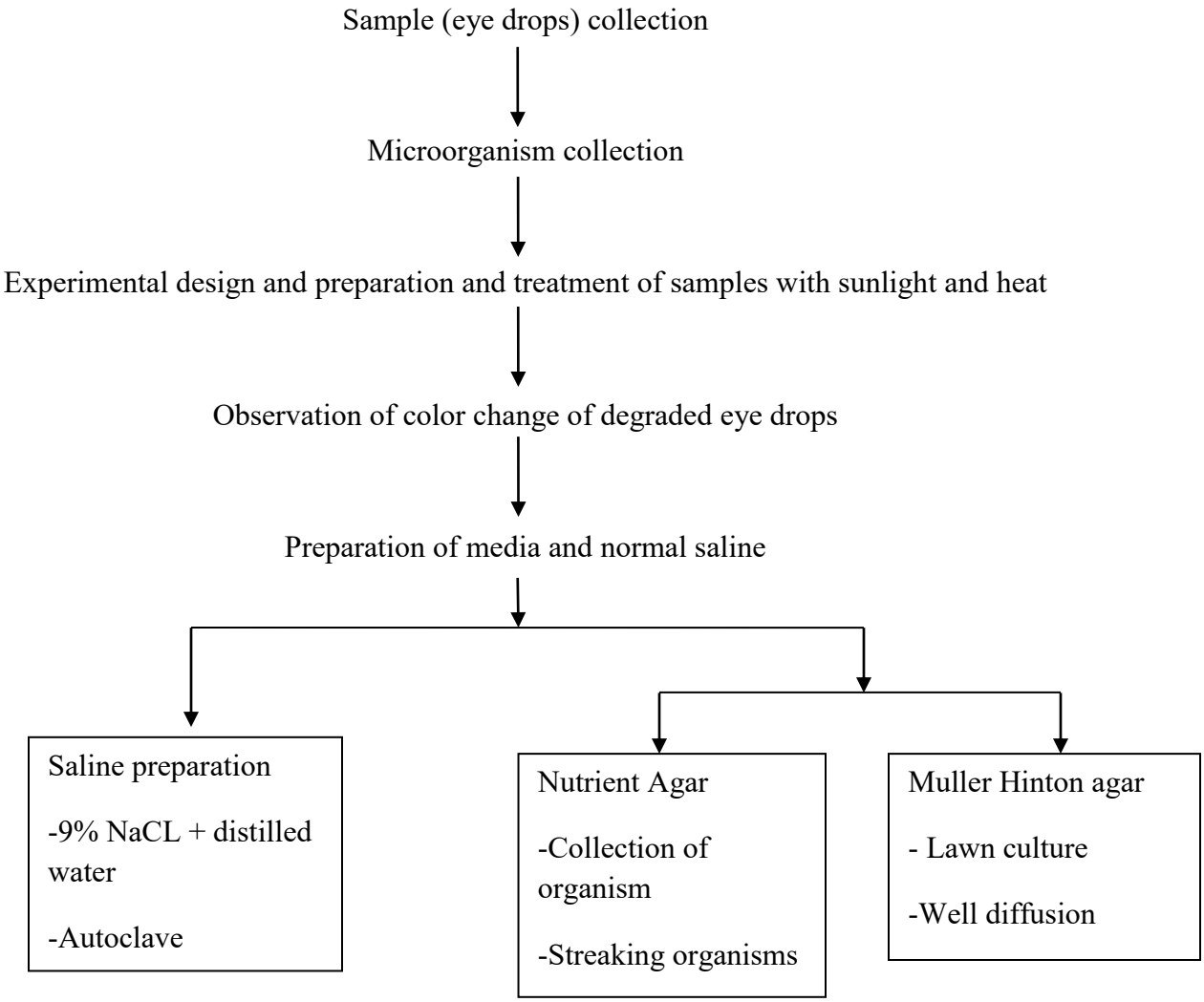
Mueller Hinton Media contains beef extract, acid hydrolysate of casein, starch and agar. Beef extract and acid hydrolysate of casein provide nitrogen, vitamins, carbon, amino acids, sulfur and other essential nutrients. Starch is added to absorb any toxic metabolites produced. Starch hydrolysis yields dextrose, which serves as a source of energy**.** Agar is the solidifying agent.

The use of a suitable medium for testing the susceptibility of microorganisms to sulfonamides and trimethoprim is essential. Antagonism to sulfonamide activity is demonstrated by para-aminobenzoic acid (PABA) and its analogs. Reduced activity of trimethoprim, resulting in smaller inhibition zones and inner zonal colonies, is demonstrated on unsuitable Mueller Hinton medium possessing high levels of thymidine**.** Both the PABA and thymine/thymidine content in Mueller Hinton Agar are reduced to a minimum, thus markedly reducing the inactivation of sulfonamides and trimethoprim when the media is used for testing the susceptibility of bacterial isolates to these anti-microbes.

Table 2.1 Muller Hinton Agar (MHA) composition

Ingredients	In Gram/Litre
Beef Extract	2.00 gm
Acid Hydrolysate of Casein	17.50 gm
Starch	1.50 gm
Agar	17.00 gm
Distilled Water	1000 ml

2.5 Flow chart and study design:



2.6 Methods:

2.6.1 Sample collection of ciprofloxacin eye drops:

Eye drops were collected from different locations of Dhaka city. Total five ciprofloxacin synthetic eye drops samples were collected from different local pharmaceuticals. Five ciprofloxacin synthetic eye drops are:

Eye drop samples	Collection Area
Ciprofloxacin A	Mohakhali
Ciprofloxacin B	Mirpur
Ciprofloxacin C	Mohakhali
Ciprofloxacin D	Mohakhali
Ciprofloxacin E	Mohakhali

2.6.2 Sample collection of herbal eye drops:

Herbal Eye drops were collected from India.

Eye drop samples	Collection Area
Herbal eye drops	India

2.6.3 Experimental design, preparation and treatment of ciprofloxacin synthetic and herbal eye drops with sunlight

An 8 ml aliquot of the five different commercially prepared samples of USP 0.3% ciprofloxacin synthetic and herbal eye drops of set 1, 2, 3 and 4 were used in this study.

- **Set-1**: Ciprofloxacin synthetic and herbal eye drops samples containing 8 ml aliquot were stored in the refrigerator with their containers which is considered as controls for the experiment (control)

- **Set-2**: Ciprofloxacin synthetic and herbal eye drops samples containing 8 ml aliquot were exposed to sunlight along with their primary and secondary packaging (container and carton).

- **Set-3**: Ciprofloxacin synthetic and herbal eye drops samples containing 8 ml aliquot were exposed to sunlight with their primary packaging (container only).

- **Set-4**: Ciprofloxacin synthetic and herbal eye drops samples containing 8 ml aliquot were transfer into clear transparent vial tubes which were directly exposed to sunlight.

2.6.4 Nutrient agar preparation:

In this research experiment nutrient agar is used to grow specific gram positive bacteria (*Staphylococcus aureus*, *Bacillus cereus*) and gram negative (*Escherichia coli (STEC), Salmonella typhi*, *Proteus vulgaris)*. It is the most efficient way to use this growth media as it is close as possible to the natural environment. The overall purpose of the agar is to customize the media for the specific bacteria. Nutrient agar is commercially available in powdered (free-flowing, homogeneous) form.

Dissolve the dehydrated medium in the appropriate volume of distilled water i.e., 28 gm dehydrated nutrient agar in 1000 ml distilled water. The required amount of agar was prepared in

a conical flask, and put onto a Bunsen burner and stirred with a glass rod until the boiling point was reached. At this point, visible small bubbles formed at the bottom of the conical flask which rose up and the solution gradually turned clear. The flask was then removed from the heat and let to cool down for some time. Then the mouth of the flask was covered with aluminum foil and autoclaved at 121°C for 120 minutes. In the laminar air flow chamber, the autoclaved nutrient agar solution was quickly but cautiously poured into previously labeled petri-dishes about 20 ml per medium sized plates or 30 ml per large sized plates. This was then kept in the refrigerator to solidify. The petri-dishes were labeled with the name of the agar, the name or initials of the person who made the agar, and the date when it was made.

2.6.5 Microorganisms collection:

The bacterial strains used in this study were collected from ICDDR'B lab. Microorganism isolates are clinical samples of ICDDR'B

Serial Number	Name of Microorganisms
1	*Staphylococcus aureus*
2	*Bacillus cereus*
3	*Salmonella typhi*
4	*Escherichia coli (STEC)*
5	*Proteus vulgaris*

The microorganisms (*Escherichia coli (STEC), Proteus vulgaris, Staphylococcus aureus, Salmonella typhi, Bacillus cereus*) were streaked on freshly prepared nutrient agar plates and incubated for 24 hours. After growth was clearly visible, the plates were wrapped with parafilm and stored at 4°C until further use. Before each respective experiment, the organisms were freshly subcultured and the 24-hour cultures were used. Purity of the cultures was maintained by regular subculturing.

2.6.6 Sub-culturing of microbes:

The stock cultures of the five microorganisms were taken. To subculture, these were streaked on to the NA plates inside a laminar air flow chamber. For each organism, the plates were taken inside the chamber and then a loop was burned till red hot over a sprit lamp flame. After cooling the loop, a loopful of microbes were taken from the stock culture and streaked onto a properly labeled NA plate. This was then incubated at 37°C temperature for 24 hours before use.

Figure 2.2: Bacterial inoculated agar plates

2.6.7 Preparation of saline solution:

To make 0.9% saline solution, 0.9 gm of sodium chloride (NaCl) was taken into 100 ml of distilled water. About 10 ml of the saline solution were put in each test tube. Several such test tubes were prepared and autoclaved, with the screw cap opened through 1.5 turns. When taken out of the autoclave machine, the screw caps were turned fully to close the mouth of the tube so that the saline does not get contaminated.

2.6.8 Preparation of Muller Hinton Agar plates:

To make Muller Hinton Agar 38 gm of Muller Hinton Agar Powder was dissolved in 1000 ml of distilled water. For each large sized plate 30 ml agar is needed.

Heating the media with frequent agitation and boiled for several minutes to completely dissolve the medium. The media was autoclaved at 121°C for 120 minutes. In the laminar air flow chamber autoclaved Muller Hinton Agar was poured into 30 ml large sized petri dishes. Then these plates were kept in the refrigerator to solidify the media.

2.6.9 Preparation of inoculums and serial dilution:

Using a sterile inoculating loop, four or five isolated colonies of the organism were taken from the NA plates. Organism was suspended in 9 ml of sterile saline solution. The saline solution was vortex to create smooth suspension. Then serial dilution is done initially, 1 ml of suspension was mixed with 9 ml of saline water in a test tube in order to dilution 10^{-1} and mixed with 9 ml of saline in it by repeated pipetting in order to make tenfold dilution. Again, 1 ml from the 10^{-1} test tube was transferred to 10^{-2} labeled test tube and mixed with 9 ml saline solution in it by repeated pipetting. This action was repeated for the test tubes labeled as 10^{-3} and 10^{-4}.

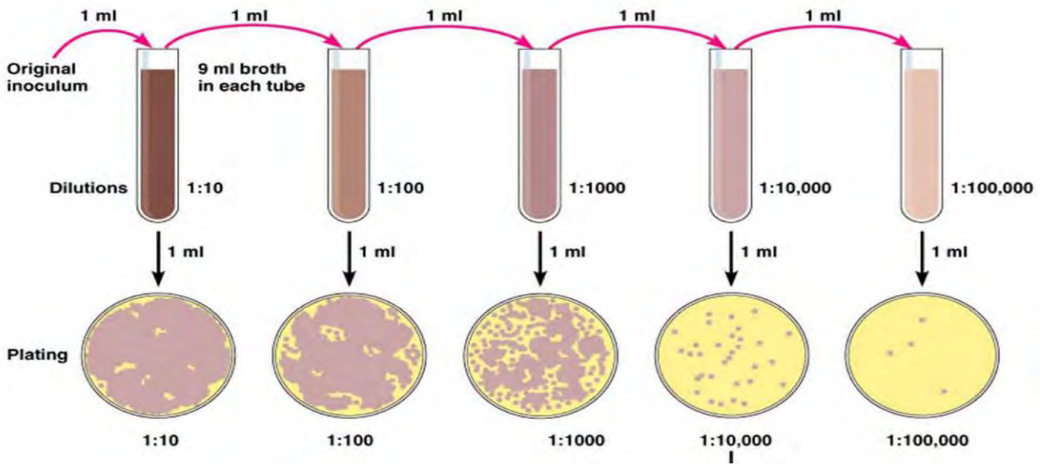


Figure 2.3: Serial dilution (Retrieved from https://www.slideshare.net/sarah_jumali/serial-dilution)

The turbidity of the suspension was adjusted to McFarland standard 1, 2 and 3. And by adding more organism if organism if the suspension is too light or diluting with sterile saline if the suspension is too heavy.

2.6.10 Inoculation of the MHA plate and lawn culture:

Using sterile cotton swab into the inoculums tube the suspension were taken in the loop for further lawn culture. Inoculate the dried surface of a MH agar plate by streaking the swab three times over the entire agar surface; rotate the plate approximately 60 degrees each time to ensure an even distribution of the inoculums.

2.7 Antibiotic assay by well diffusion test:

Well diffusion test is a test of the antibiotic sensitivity of bacteria. It uses antibiotic disc or antibiotic liquor drugs in wells to test the extent to which bacteria are affected by those antibiotics. The well diffusion susceptibility method is simple and practical and has been well-standardized. The test was performed by applying bacterial inoculums are applied from 9 ml bacterial culture suspension by sterile cotton swab to the surface of a large (150 mm diameter) Mueller-Hinton agar plate. Antibiotics eye drop samples with, fixed concentrations (2, 6 and 10 µl), were inoculated agar wells. Plates were incubated for 24 hours at 37°C prior to determination of results. The zones of growth inhibition around each of the antibiotic disks were measured to the nearest millimeter. The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium. The results of the disk diffusion test were "qualitative," in that a category of susceptibility (i.e. susceptible, intermediate, or resistant) was derived from the test rather than an MIC.

If the agar plate has been inoculated with a suspension of the pathogen to be tested prior to the placing eye drop samples in the agar well, simultaneous growth of the bacteria and diffusion of the antimicrobial compounds occurs. Growth occurs in the presence of an antimicrobial compound when the bacteria reach a critical mass and can overpower the inhibitory effects of the antimicrobial compound. The estimated time of a bacterial suspension to reach critical mass is 4 to 10 hours for most commonly recovered pathogens, but is characteristic of each species, and influenced by the media and incubation temperature. The size of the zone of inhibition of growth

is influenced by the depth of the agar, since the antimicrobial diffuses in three dimensions, thus a shallow layer of agar will produce a larger zone of inhibition than a deeper layer.

2.7.1 Measurement of zone of inhibition:

Presence of a clear area on the MHA plate around any agar well containing eye solutions represents the zone of inhibition which signifies the antibacterial activity of the ciprofloxacin synthetic and herbal eye drops. The diameter of the clear zone was measured three times in millimeter (mm) with a scale/ruler and the average value of zone of inhibition for each eye drops were calculated and recorded.

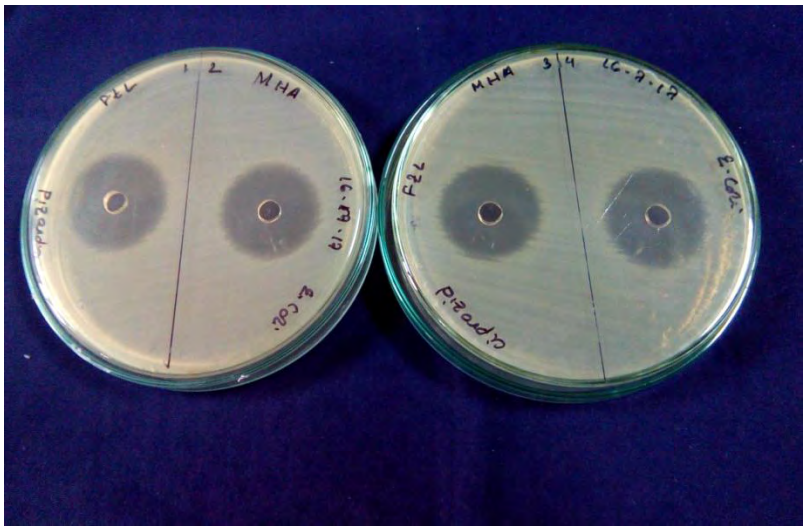


Figure 2.4: MHA agar plate with well diffusion

Chapter 3 - Results

3.1 Investigating the changes in color of sunlight treated ciprofloxacin mediated eye drops:

The change in the appearance after sunlight-induced degradation of five ciprofloxacin ophthalmic solution such as ciprofloxacin A, ciprofloxacin B, ciprofloxacin C, ciprofloxacin n D and ciprofloxacin E was noted before and after of exposure to sunlight. They were divided in four sets such as- set-1 was the control and it was kept in refrigerator at 4°C, set-2 was secondary and primary packaging i.e. the container and carton, set-3 was only the primary packaging and in set-4 the eye drops were transferred in sterile transparent glass tubes. These samples were exposed to sunlight and significant color change was observed, the change in color change was recorded firstly after 2 hours interval of sunlight exposure secondly after 10 hours of sunlight exposure. The change appearances after exposure to sunlight are given below:

Table 3.1.1: Change of appearance after exposure to sunlight of ciprofloxacin A

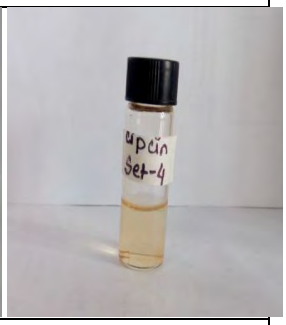
Time interval	Set - 1(control)	Set -2 (container + carton)	Set -3(primary packaging)	Set -4 (glass tube direct exposure)
Initial condition	-	Clear , colorless	Clear , colorless	Clear , colorless
2 hours sunlight exposure	-	Colorless, opaque	Colorless, opaque	Slight Yellowish
				
10 hours sunlight exposure	-	Colorless, opaque	Slight Yellowish	Yellowish
				

Table 3.1.2: Change of appearance after exposure to sunlight of ciprofloxacin B

Time interval	Set - 1(control)	Set -2 (container + carton)	Set -3(primary packaging)	Set -4 (glass tube direct exposure)
Initial condition	-	Clear , colorless	Clear , colorless	Clear , colorless
2 hours sunlight exposure	-	Colorless, opaque	Colorless, opaque	Yellowish
				
10 hours sunlight exposure	-	Colorless, opaque	Yellowish	Yellowish
				

Table 3.1.3: Change of appearance after exposure to sunlight of ciprofloxacin C

Time interval	Set -1(control)	Set -2 (container + carton)	Set -3(primary packaging)	Set -4 (glass tube direct exposure)
Initial condition	-	Clear , colorless	Clear , colorless	Clear , colorless
2 hours sunlight exposure	-	Colorless, opaque	Colorless, opaque	Slight Yellowish
				
10 hours sunlight exposure	-	Colorless, opaque	Slight Yellowish	Yellowish
				

Table 3.1.4: Change of appearance after exposure to sunlight of ciprofloxacin D

Time interval	Set - 1(control)	Set -2 (container + carton)	Set -3(primary packaging)	Set -4 (glass tube direct exposure)
Initial condition	-	Clear , colorless	Clear , colorless	Clear , colorless
2 hours sunlight exposure	-	Colorless, opaque	Colorless, opaque	Slight Yellowish
				
10 hours sunlight exposure	-	Colorless, opaque	Slight Yellowish	Yellowish
				

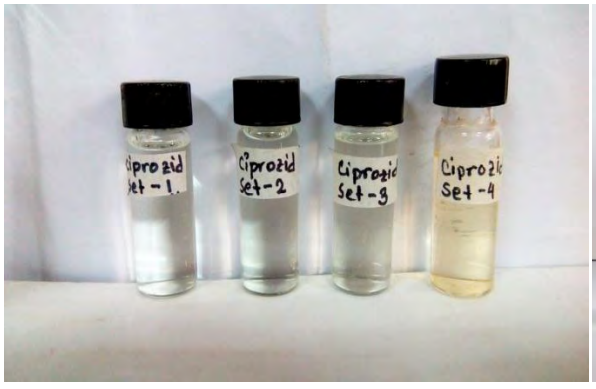
Table 3.1.5: Change of appearance after exposure to sunlight of ciprofloxacin E

Time interval	Set - 1(control)	Set -2 (container + carton)	Set -3(primary packaging)	Set -4 (glass tube direct exposure)
Initial condition	-	Clear , colorless	Clear , colorless	Clear , colorless
2 hours sunlight exposure	-	Colorless, opaque	Colorless, opaque	Slight Yellowish
				
10 hours sunlight exposure	-	Colorless, opaque	Slight Yellowish	Yellowish
				

a) Cipofloxacin A (set-1,2,3,and 4)

b) Ciprofloxacin B (set- 1,2,3 and 4)

c) Ciprofloxacin C (set- 1,2,3 and 4)



d) Ciprofloxacin D (set- 1, 2, 3 and 4)

e) Ciprofloxacin E (set- 1, 2, 3 and 4)

Figure 3.1.1: Comparison of the color changes ciprofloxacin of eye drops after sunlight treatment

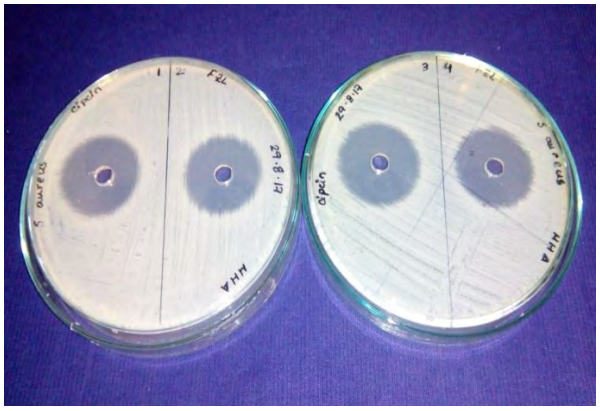
3.2: Comparison of antibacterial activity test ciprofloxacin eye drops after sunlight treatment

Antibacterial activity of five different companies of ciprofloxacin eye drops such as ciprofloxacin A, ciprofloxacin B, ciprofloxacin C, ciprofloxacin D and ciprofloxacin E were tested against five microorganisms such *Staphylococcus aureus, Bacillus cereus, Salmonella typhi, Escherichia coli* (STEC) and *Proteus vulgaris*.

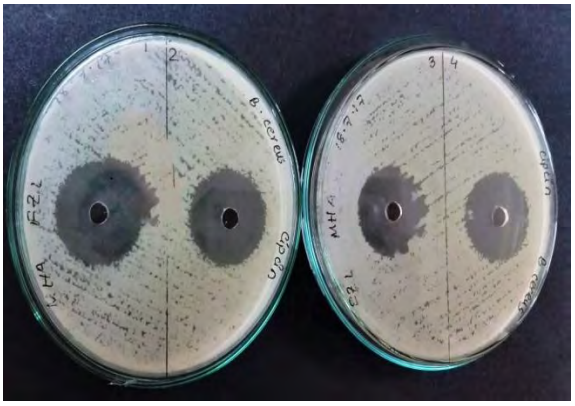
Chronological decreases in zone of inhibition of ciprofloxacin eye drops from set-1 to set-4 were observed. Ciprofloxacin A was most effective against *Bacillus cereus* in table 3.2.1 the highest zone of inhibition was observed against *Bacillus cereus* and it was least effective against *Proteus vulgaris.*

Table 3.2.1: Antibacterial activity of ciprofloxacin A eye drops against gram positive and gram negative bacteria

Bacterial isolates	**Concentration µl**	**Diameter of zone of inhibition (mm)**			
		Set-1 (Control)	Set-2 (container + carton)	Set-3 (primary packaging)	Set-4 (glass tube direct exposure)
Staphylococcus aureus	2	29	28.66	26	27.67
Bacillus cereus	2	32.33	30.67	27.67	29
Salmonella typhi	2	29	28.3	36	37.6
Escherichia coli (STEC)	2	24.3	23.5	21.66	21.87
Proteus vulgaris	2	24	22.33	21.67	22

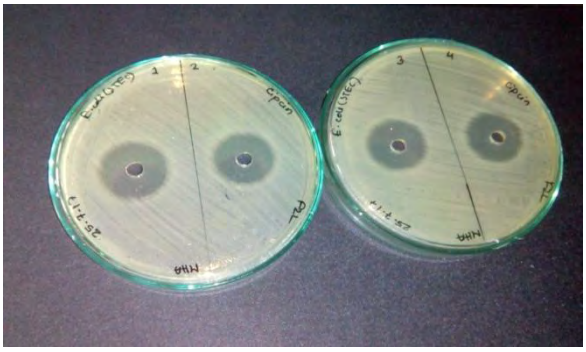


Staphylococcus aureus

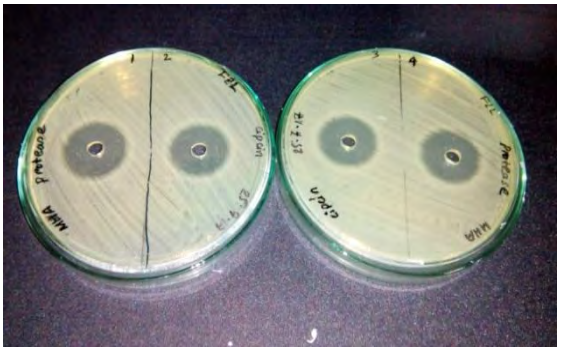


Bacillus cereus

Salmonella typhi



Escherichia coli (STEC)



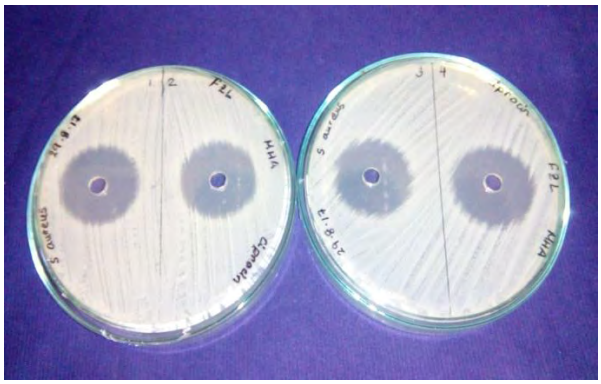
Proteus vulgaris

Figure 3.2.1: Antibacterial activity test ciprofloxacin A eye drops after sunlight treatment

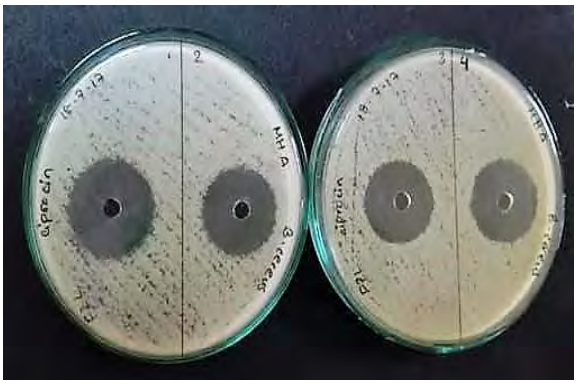
In Table 3.2.2 Ciprofloxacin B degradation of eye drops are observed from set-1 (control) to set-4 (glass tube direct exposure) higher efficiency was observed against *Salmonella typhi* with highest zone of inhibition amongst other gram negative and gram positive bacteria. The least efficiency and smaller zone of inhibition was observed against *Escherichia coli* (STEC).

Table 3.2.2: Antibacterial activity of ciprofloxacin B eye drops against gram positive and gram negative bacteria

Bacterial isolates	**Concentration µl**	**Diameter of zone of inhibition (mm)**			
		Set-1 (Control)	Set-2 (container + carton)	Set-3 (primary packaging)	Set-4 (glass tube direct exposure)
Staphylococcus aureus	2	29.67	29.33	29	28
Bacillus cereus	2	32	29.33	29	28.67
Salmonella typhi	2	36.3	35.7	35	34.3
Escherichia coli (STEC)	2	23.66	21.33	20	19.67
Proteus vulgaris	2	24.33	23.66	21.33	20.67

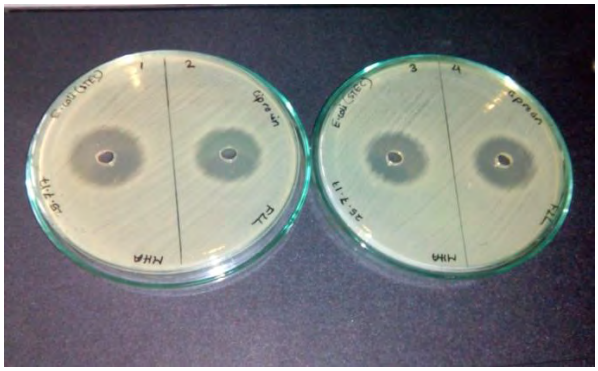


Staphylococcus aureus

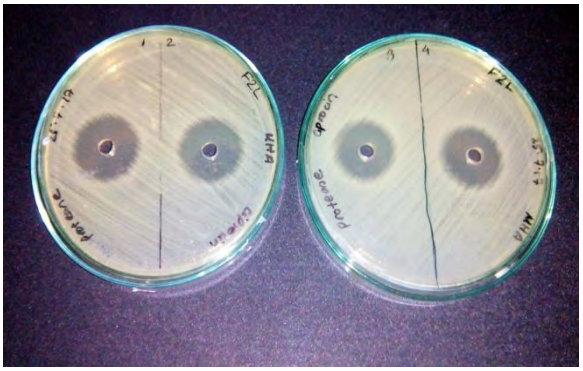


Bacillus cereus

Salmonella typhi



Escherichia coli (STEC)



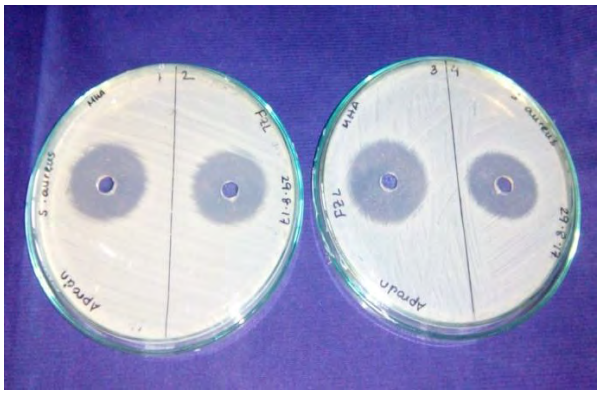
Proteus vulgaris

Figure 3.2.2: Antibacterial activity test ciprofloxacin B eye drops after sunlight treatment

In Table 3.2.3 Ciprofloxacin C degradation of eye drops are observed from set-1 (control) to set-4 (glass tube direct exposure) highest efficiency was observed against *Salmonella typhi* with highest zone of inhibition amongst other gram negative and larger zone of inhibition was also observed against *Bacillus cereus* compared to *Staphylococcus aureus*. And least efficiency and smallest zone of inhibition was observed against *Escherichia coli* (STEC).

Table 3.2.3: Antibacterial activity of ciprofloxacin C eye drops against gram positive and gram negative bacteria

Bacterial isolates	**Concentration µl**	**Diameter of zone of inhibition (mm)**			
		Set-1 (Control)	Set-2 (container + carton)	Set-3 (primary packaging)	Set-4 (glass tube direct exposure)
Staphylococcus aureus	2	28	27	26.33	25
Bacillus cereus	2	32	29.33	29	28.67
Salmonella typhi	2	35	34.6	33	32.3
Escherichia coli (STEC)	2	24.4	23.87	22.4	21
Proteus vulgaris	2	24.6	24	22.33	21.33



Staphylococcus aureus

Bacillus cereus

Salmonella typhi

Escherichia coli (STEC)

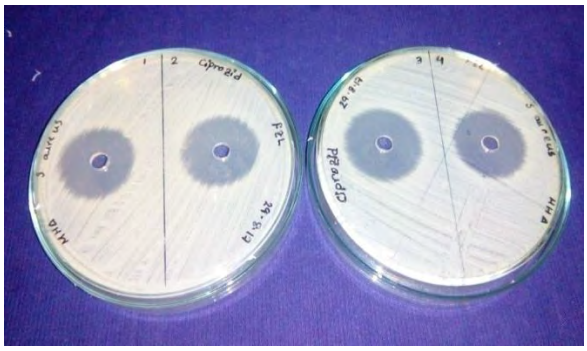
Proteus vulgaris

Figure 3.2.3: Antibacterial activity test ciprofloxacin C eye drops after sunlight treatment

In Table 3.2.4 Ciprofloxacin D a chronological degradation of eye drops are observed from set-1 (control) to set-4 (glass tube direct exposure) highest efficiency was observed against *Salmonella typhi* with highest zone of inhibition amongst other gram negative and larger zone of inhibition was also observed against *Bacillus cereus* compared to *Staphylococcus aureus*. And least efficiency and smallest zone of inhibition was observed against *Escherichia coli* (STEC).

Table 3.2.4: Antibacterial activity of ciprofloxacin D eye drops against gram positive and gram negative bacteria

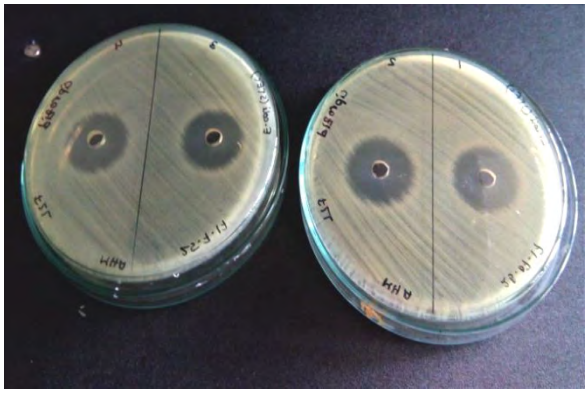
Bacterial isolates	**Concentration µl**	**Diameter of zone of inhibition (mm)**			
		Set-1 (Control)	Set-2 (container + carton)	Set-3 (primary packaging)	Set-4 (glass tube direct exposure)
Staphylococcus aureus	2	29	28.67	28	27.33
Bacillus cereus	2	32	29.33	29	28.67
Salmonella typhi	2	37	36	35.3	29.67
Escherichia coli (STEC)	2	24	23	22.5	21.67
Proteus vulgaris	2	24.67	23.77	22.67	21



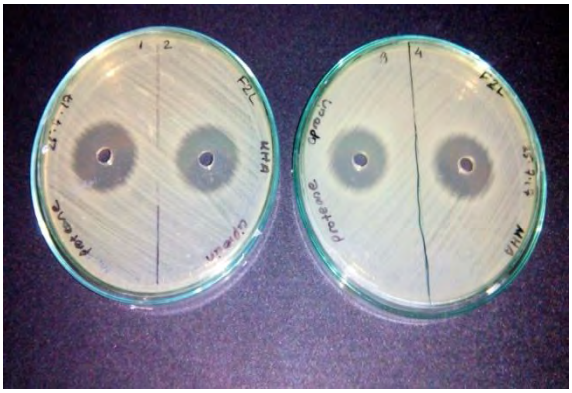
Staphylococcus aureus

Bacillus cereus

Salmonella typhi



Escherichia coli (STEC)



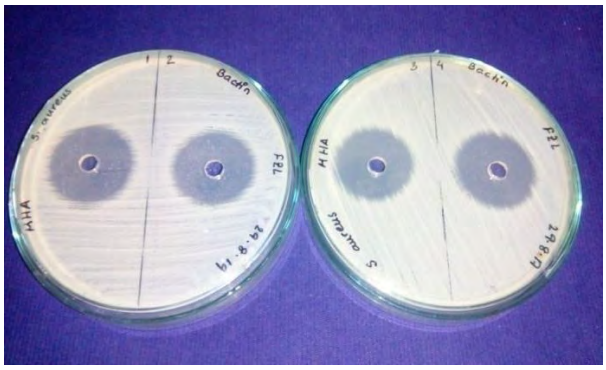
Proteus vulgaris

Figure 3.2.4: Antibacterial activity test ciprofloxacin D eye drops after sunlight treatment

In Table 3.2.3 Ciprofloxacin E degradation of eye drops are observed from set-1 (control) to set-4 (glass tube direct exposure) highest efficiency was observed against *Salmonella typhi* with highest zone of inhibition amongst other gram negative and larger zone of inhibition was also observed against *Bacillus cereus* compared to *Staphylococcus aureus*. And least efficiency and smallest zone of inhibition was observed against *Escherichia coli* (STEC).

Table 3.2.5: Antibacterial activity of ciprofloxacin E eye drops against gram positive and gram negative bacteria

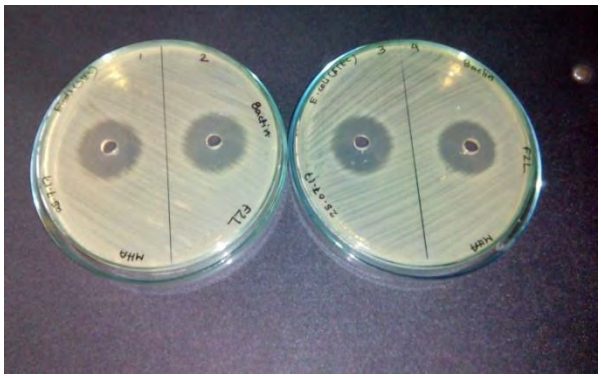
Bacterial isolates	**Concentration µl**	**Diameter of zone of inhibition (mm)**			
		Set-1 (Control)	Set-2 (container + carton)	Set-3 (primary packaging)	Set-4 (glass tube direct exposure)
Staphylococcus aureus	2	29.67	28.67	28	28.33
Bacillus cereus	2	30.33	29.67	27.66	29
Salmonella typhi	2	36	35.3	30	30.6
Escherichia coli (STEC)	2	24.66	24	22.6	23
Proteus vulgaris	2	24.66	24.33	23	23.67



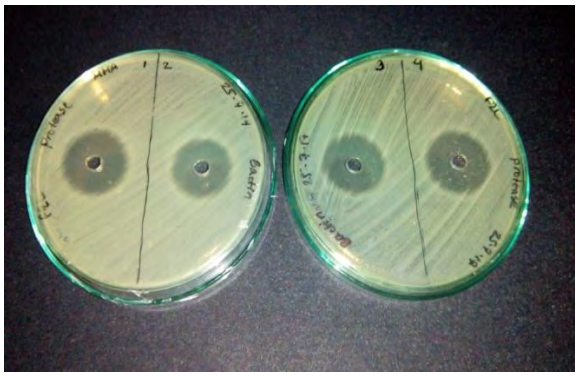
Staphylococcus aureus

Bacillus cereus

Salmonella typhi



Escherichia coli (STEC)



Proteus vulgaris

Figure 3.2.5: Antibacterial activity test ciprofloxacin E eye drops after sunlight treatment

3.3 A comparative study of sunlight- induced ciprofloxacin synthetic eye drops against gram positive and gram negative bacteria

In figure 3.3.1 and 3.3.2 a comparison is antibacterial activity of synthetic eye drops gram positive and gram negative bacteria *Bacillus cereus*, *Staphylococcus aureus* and *Salmonella typhi, Escherichia coli* (STEC) and *Proteus vulgaris* which shows the most potent drug against gram positive bacteria was ciprofloxacin B which shows higher zone inhibition against *Bacillus cereus* and *Staphylococcus aureus* compared other sample of ciprofloxacin synthetic eye drops.

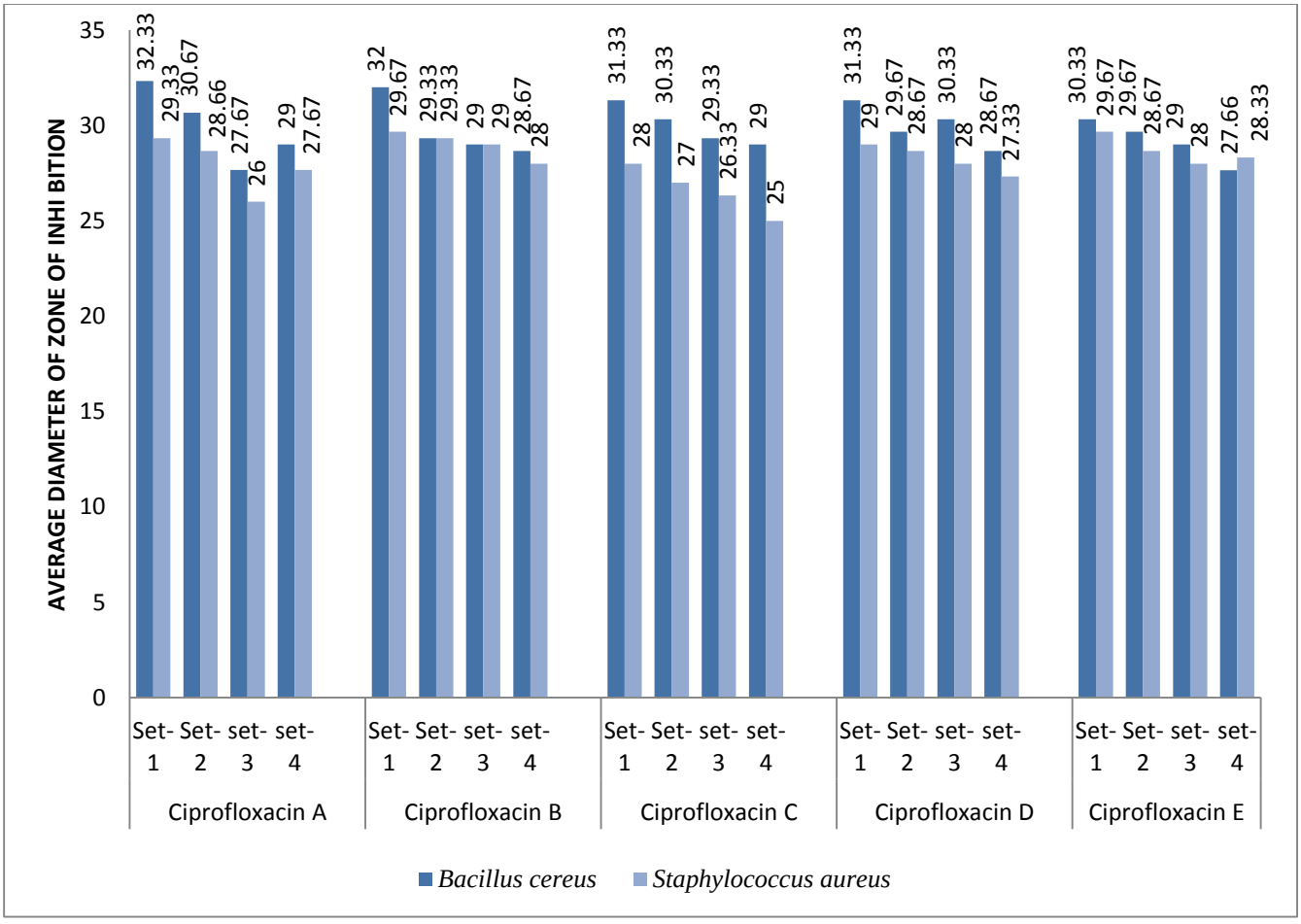


Figure 3.3.1: Comparison of antibacterial activity of synthetic eye drops against gram positive bacteria (*Bacillus cereus* and *Staphylococcus aureus)*

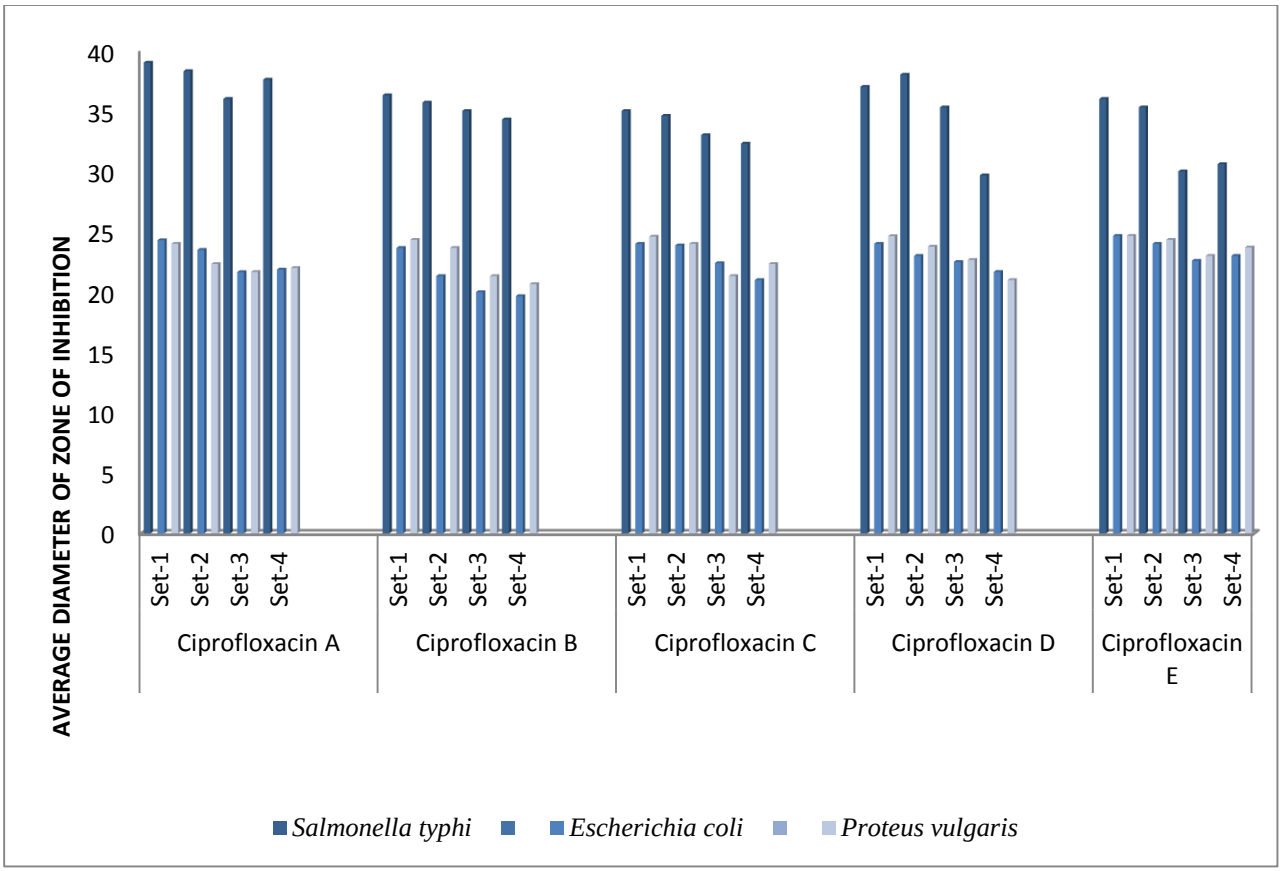
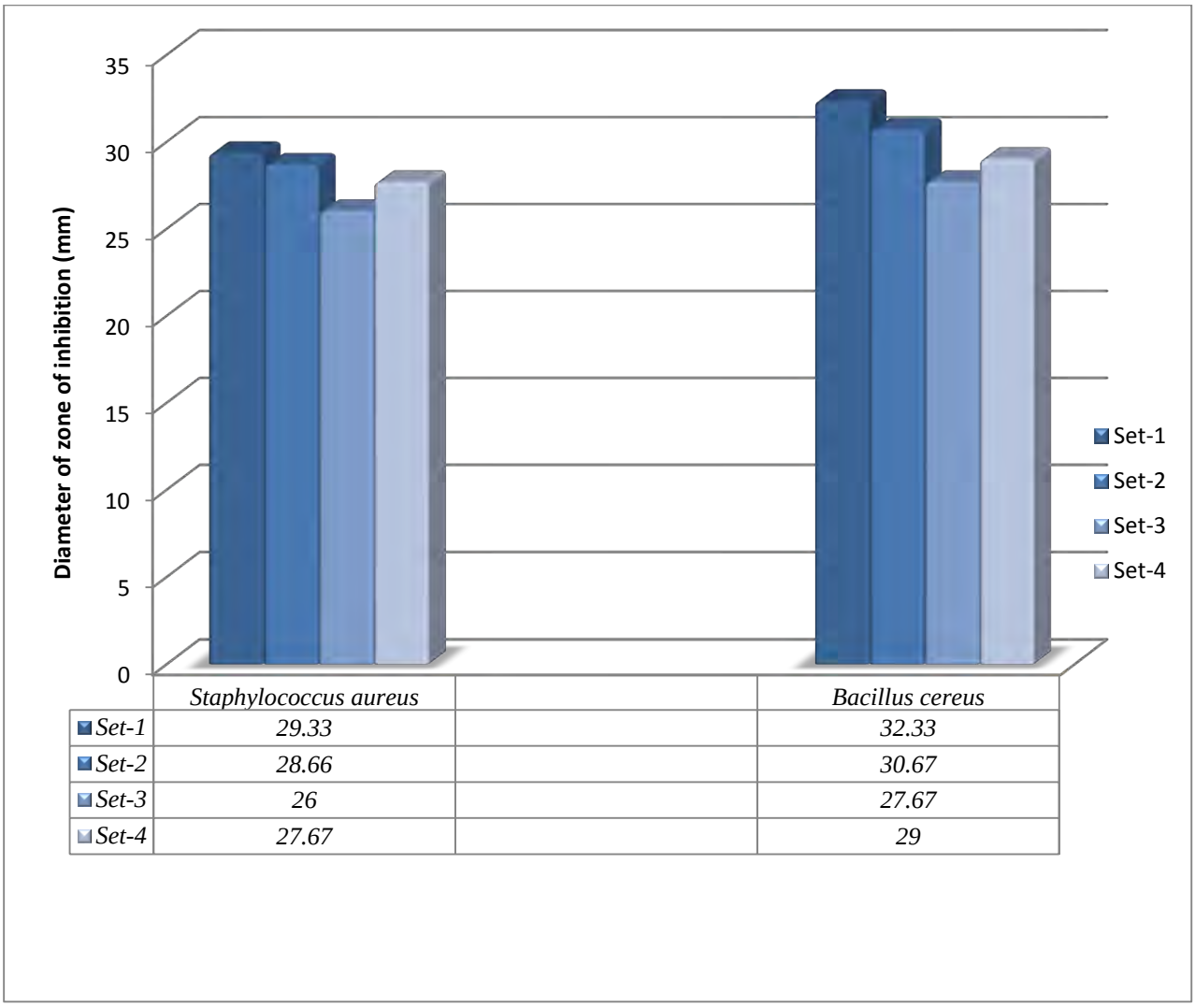


Figure 3.3.2: Comparison of antibacterial activity of synthetic eye drops against gram negative bacteria (*Salmonella typhi, Escherichia coli* (STEC) and *Proteus vulgaris)*

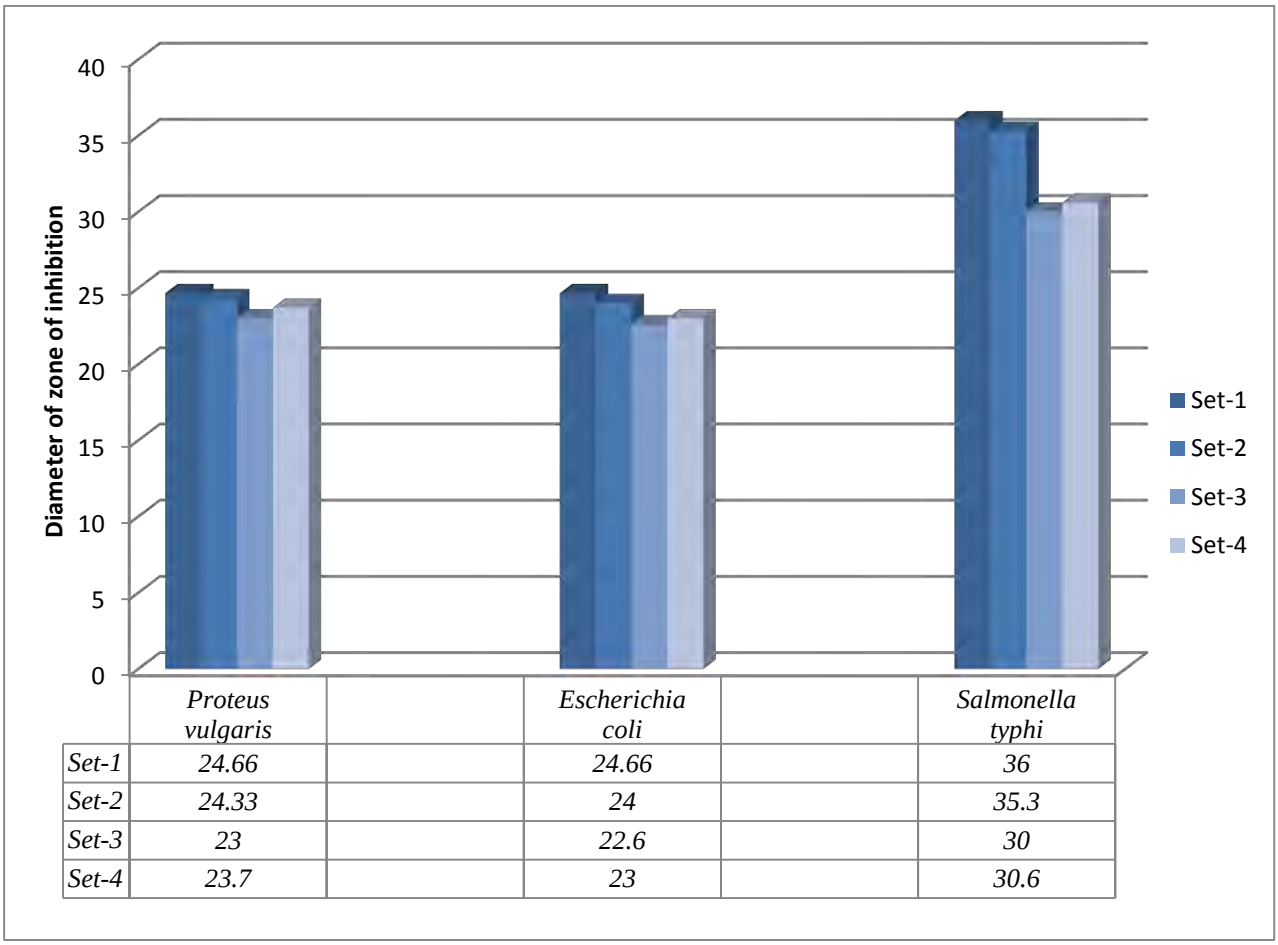
3.4 Observation of antibacterial activity of sunlight induced ciprofloxacin ophthalmic preparation against gram positive and gram negative bacteria

In this figure 3.4.1 and 3.4.2 in set-1(control) is compared with other set-2, 3 and 4 for this particular ophthalmic preparation, in set-3 which was exposed to sunlight with primary packaging showed the smallest zone of inhibition against gram positive bacteria. So, due to poor packaging the ophthalmic component in set-3 showed greater chemical degradation than set-4 this was exposing to sunlight in transparent glass tubes



	Staphylococcus aureus		*Bacillus cereus*
Set-1	*29.33*		*32.33*
Set-2	*28.66*		*30.67*
Set-3	*26*		*27.67*
Set-4	*27.67*		*29*

Figure 3.4.1: Observation of antibacterial activity of sunlight exposed synthetic eye drops ciprofloxacin A after antibacterial activity test against gram positive bacteria *Staphylococcus aureus* and *Bacillus cereus*



	Proteus vulgaris		*Escherichia coli*		*Salmonella typhi*
Set-1	*24.66*		*24.66*		*36*
Set-2	*24.33*		*24*		*35.3*
Set-3	*23*		*22.6*		*30*
Set-4	*23.7*		*23*		*30.6*

Figure 3.4.2: Observation of chemical degradation of sunlight exposed synthetic eye drops ciprofloxacin A after antibacterial activity test against gram negative bacteria *Salmonella typhi, Escherichia coli and Proteus vulgaris*

3.5 Monitoring the changes in color of sunlight-treated herbal eye drops samples:

The change in the appearance after sunlight-induced degradation of herbal eye drops preparation was noted before and after of exposure to sunlight. Before exposure to sun the appearances of all the samples of set-2, set-3, and set-4 were clear and colorless. After 10 hours exposure of sunlight all samples showed significant changed in color. The results obtained are presented below:

3.5.1 Table: Change of appearance after exposure to sunlight of herbal eye drops

Time interval	Set - 1(control)	Set -2 (container + carton)	Set -3(primary packaging)	Set -4 (glass tube direct exposure)
Initial condition	-	Clear , colorless	Clear , colorless	Clear , colorless
2 hours sunlight exposure	-	No color change	No color change	No color change
				
10 hours sunlight exposure	-	No color change	No color change	No color change
				

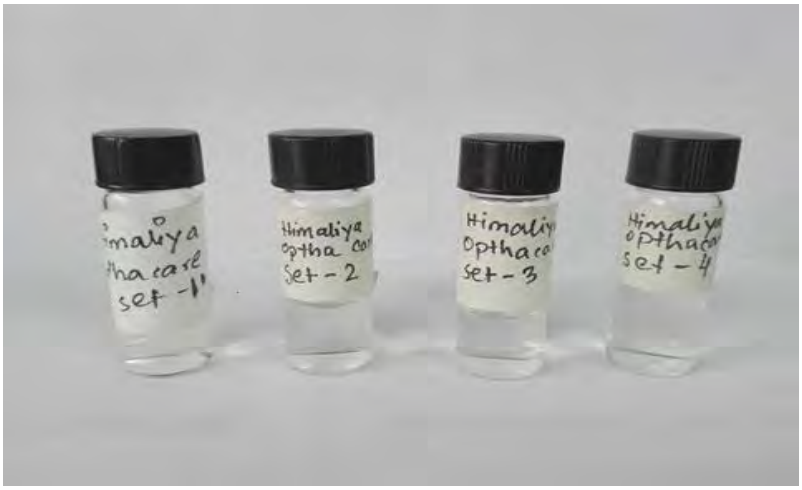


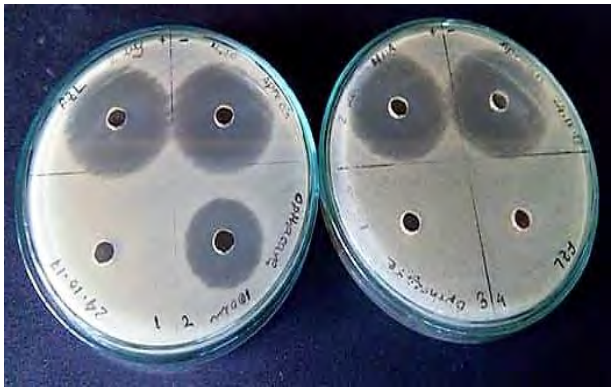
Figure 3.5.1: Comparison of the color changes in herbal eye drops after sunlight treatment

3.6 Comparison of antibacterial activity test of herbal eye drops after sunlight treatment against gram positive and gram negative bacteria

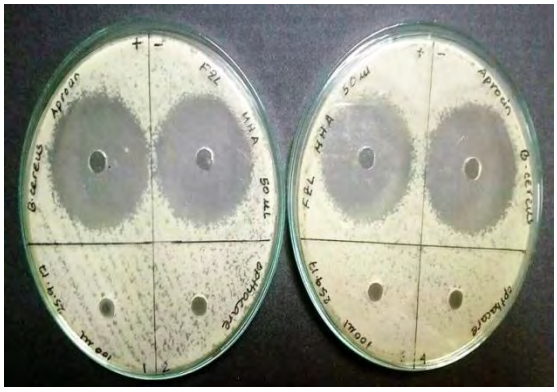
Antibacterial activity of herbal eye drop was tested against five microorganisms such *Staphylococcus aureus, Salmonella typhi, Bacillus cereus, Escherichia coli* and *Proteus vulgaris.* In table 3.5.2 herbal eye drops did not show any antibacterial activity against both gram positive and gram negative bacteria and a small zone of inhibition was observed against *Staphylococcus aureus* in set-2 (container+ carton) was seen.

Table 3.6.1: Antibacterial activity of herbal eye drops eye drops

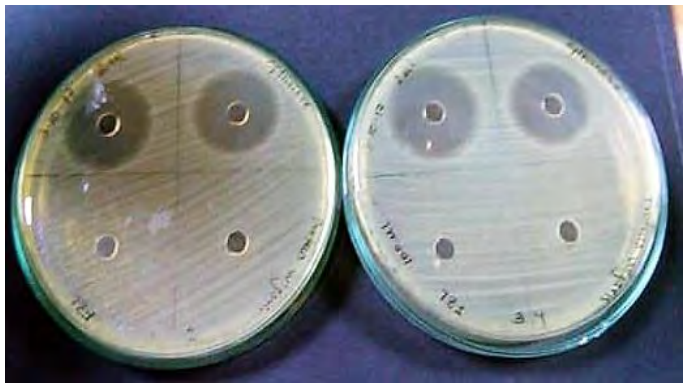
Bacterial isolates	**Concentration µl**	**Diameter of zone of inhibition (mm)**			
		Set-1 (Control)	Set-2 (container + carton)	Set-3 (primary packaging)	Set-4 (glass tube direct exposure)
Staphylococcus aureus	2	**No ZOI**	**28.33**	**No ZOI**	**No ZOI**
Bacillus cereus	2	**No ZOI**	**No ZOI**	**No ZOI**	**No ZOI**
Salmonella typhi	2	**No ZOI**	**No ZOI**	**No ZOI**	**No ZOI**
Escherichia coli (STEC)	2	**No ZOI**	**No ZOI**	**No ZOI**	**No ZOI**
Proteus vulgaris	2	**No ZOI**	**No ZOI**	**No ZOI**	**No ZOI**



Staphylococcus aureus



Bacillus cereus



Salmonella typhi

Escherichia coli (STEC)

Proteus vulgaris

Figure 3.5.2: Antibacterial activity test herbal eye drops after sunlight treatment

3.7 A comparative study of potency and efficacy of herbal drops and synthetic eye drops of against gram positive bacteria

In figure 3.6 a comparison of herbal eye drop and synthetic eye drop are shown. Here in case the herbal eye drops did not show any zone of inhibition against *Bacillus cereus* and synthetic eye drops showed zone of inhibition. In case of *Staphylococcus aureus the* herbal eye drops of set 1 (control), 3 (primary packaging) and 4 (transparent glass tube packaging) were resistant but set – 2 (container + carton) showed zone of inhibition of 28.25mm. In synthetic sunlight treated eye drops and sunlight non-treated synthetic eye drops were compared with herbal eye drops, synthetic eye drops against *Staphylococcus aureus* sunlight treated sample had zone of inhibition of 31.33 mm and sunlight non- treated sample had zone of inhibition 29 mm, against *Bacillus cereus sunlight* treated sample had zone of inhibition of 28 mm and sunlight non- treated sample had zone of inhibition 25 mm.

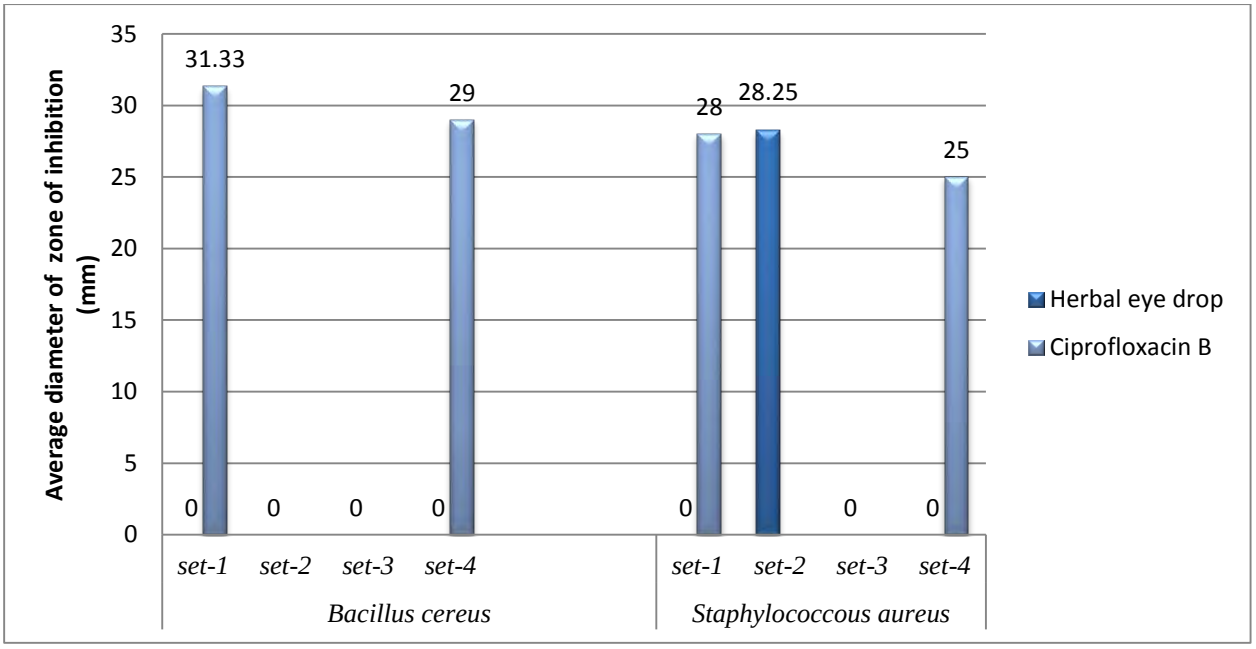


Figure 3.7: Comparison of potency and efficacy of herbal and synthetic eye drop against gram positive bacteria *Bacillus cereus* and *Staphylococcus cereus*

3.8 A comparative study of potency and efficacy of herbal drops and synthetic eye drops of against gram negative bacteria

In this figure 3.7 in herbal eye drops did not show any zone of inhibition against gram negative bacteria *Escherichia coli, Salmonella typhi* and *Proteus vulgaris.* On the other hand, a sunlight treated eye drops and sunlight non- treated synthetic eye drops were compared with herbal eye drops, synthetic eye drops showed higher potency against *Escherichia coli* sunlight treated sample had zone of inhibition of 24 mm and sunlight non- treated sample had zone of inhibition 21 mm, against *Salmonella typhi* sunlight treated sample had zone of inhibition of 35 mm and sunlight non- treated sample had zone of inhibition 32.3 mm and *Proteus vulgaris* sunlight treated sample had zone of inhibition of 24.6 mm and sunlight non- treated sample had zone of inhibition 22.33 mm

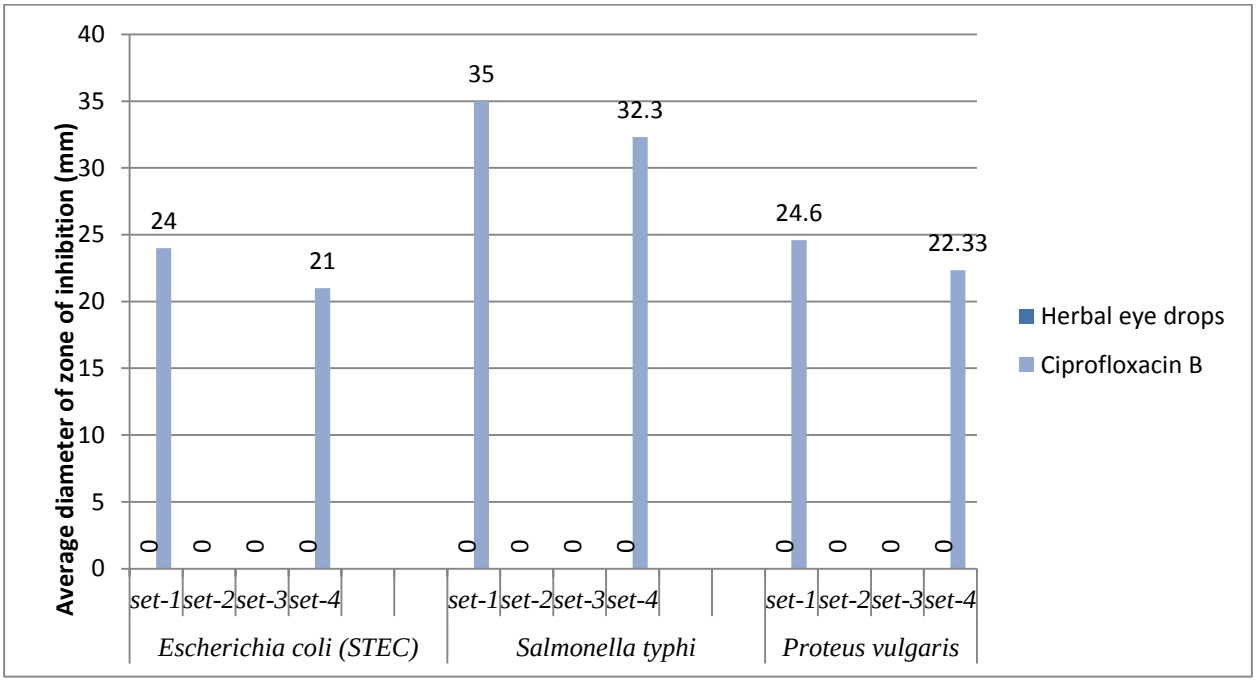


Figure 3.8 Comparison of potency and efficacy of herbal and synthetic eye drop against gram negative bacteria *Escherichia coli, Salmonella typhi* and *Proteus vulgaris*

Chapter 4 - Discussion

The photo-degradation of synthetic and herbal eye drops is observed in this study. Changes after sunlight-induced degradation of ciprofloxacin and herbal eye drops was noted before and after of exposure. Before exposure to sunlight all the samples were colorless and opaque. After 2 hours of sunlight exposure all samples ciprofloxacin A, ciprofloxacin B, ciprofloxacin C, ciprofloxacin D, ciprofloxacin E and herbal eye drop of set-2 (container + carton), set-3 (primary packaging) and set-4 (transparent glass test tubes direct exposure) retained its original color. After 5 hours of sunlight exposure an observation of all samples were taken in set-2 (container + carton) of all samples were opaque and colorless. In Set-3 (primary packaging) ciprofloxacin A, ciprofloxacin B, ciprofloxacin C, ciprofloxacin D and herbal eye drops) did not change their color but in ciprofloxacin E a slight yellowish color change was observed. In set-4 (transparent glass test tubes direct exposure) ciprofloxacin A, ciprofloxacin B and ciprofloxacin E showed a slight yellowish color which indicates that due to sunlight exposure the component in glass tubes started to degrade, in ciprofloxacin C, ciprofloxacin D and herbal eye drops did not change any color.

After 10 hours exposure of sunlight the color of all samples of set-2 (container + carton) ciprofloxacin A, ciprofloxacin B, ciprofloxacin C, ciprofloxacin D, ciprofloxacin E and herbal eye drops retained its original color. The color of the set -2 (container + carton) samples did not change significantly due to the presence of secondary packaging (carton). In case of set-3 (primary packaging), the color of ciprofloxacin A, ciprofloxacin B and ciprofloxacin E solutions turned into light yellow and no color change was observed in ciprofloxacin C, ciprofloxacin D and herbal eye drops. The results suggest that packaging is very important factor for the stability of ciprofloxacin 0.3% eye drops the container alone is not enough to protect the eye drops from sunlight, which is evident by the production of colored degradation products in most of the eye drops with primary packaging (Ahmed, *et al*., 2013). In set -3 (primary packaging) some container contains transparent plastic packaging, by which the sunlight directly penetrates through the container causing change in color. On the other hand, in some ciprofloxacin A, ciprofloxacin C, ciprofloxacin D and herbal eye drops contains white plastic packaging for which no color degradation were observed. The change in appearance of the ciprofloxacin A, ciprofloxacin B, ciprofloxacin C, ciprofloxacin D and ciprofloxacin E in set -4 (transparent glass test tubes direct exposure) was most prominent and all of the preparations turned into a deep yellow color. This result

proves that, among all sets the most color degradation was observed in set-4 (transparent glass test tubes direct exposure). It indicates that direct exposure of ciprofloxacin eye drops (0.3%) to sunlight in transparent glass test tubes degrades the product more than other sets. The direct ray of sunlight penetrates through the transparent packaging directly causing the product to degrade (Kullavanijaya & Lim, 2005). The photo-degradation by natural sunlight of ciprofloxacin in eye drops preparation produces an ethylenediamine derivative of ciprofloxacin by opening of the piperzinyl ring (Das & Subodh, 2005). The structural degradation of ciprofloxacin eye drops exposed to natural sunlight confirmed by electro spray mass spectrometric that indicated 20-30% loss of potency of ciprofloxacin and slightly lesser antimicrobial activity (Jiben *et al*., 2005).

In herbal eye drops no change of color was seen because in herbal eye drops do not contain any synthetic components. All herbal extracts are present in herbal eye drops which eventually do not show any color degradation.

Determination of antibacterial activity of sunlight-treated treated ciprofloxacin eye drops (0.3%) and herbal eye drops was done in order to detect the effectiveness of degraded samples against all the tested human pathogens that were evaluated by well diffusion method against two gram positive bacteria - *Staphylococcus aureus* and *Bacillus cereus*; three gram negative bacteria - *Salmonella typhi, Escherichia coli* (STEC) and *Proteus vulgaris.* The zone of inhibition of set–2 (container + carton), set– 3 (primary packaging) and set–4 (transparent glass test tubes direct exposure) samples were compared to that of Set-1(control) after exposure to sunlight. The zone of inhibition of set-1, set -2, set -3 and set -4 of ciprofloxacin A, ciprofloxacin B, ciprofloxacin C, ciprofloxacin D, ciprofloxacin E and herbal eye drops against gram positive and gram negative bacteria were measured. It is evident that the antibacterial activity decreased from set-1 (control) to set-4 (transparent glass test tubes direct exposure).

In case of *Staphylococcus aureus* isolates the most effective eye drops was ciprofloxacin B were a chronological degradation was observed from set-1 to set- 4. The sample ciprofloxacin B showed the largest zone of inhibition in set-1(control), set-2 (container + carton), set-3 (primary packaging) and Set-4 (transparent glass test tubes direct exposure) that are 29.67, 29.33, 29 and 28 mm among the other eye drops samples. So a comparison of antibacterial test of antibiotic

eye drops samples against *Staphylococcus aureus* after sunlight treatment was shown in figure 3.3.1 were the highest effectiveness was observed in sample ciprofloxacin B and least effectiveness was noted in sample ciprofloxacin C.

On the other hand, herbal eye drops were resistance against *Staphylococcus aureus* except set-2 (container + carton) which showed a zone of inhibition 28.33 mm. Many literature reviews suggest that the herbal eye drops has bactericidal and bacteriostatic properties that inhibit the growth of *Escherichia coli, Hemophilus influenza, Proteus spp, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pyogenes, Salmonella species* and *Vibrio cholera* (Mitra *et al.*, 2000).

In *Bacillus cereus* isolates, the highest effective sample was ciprofloxacin B compared to other samples. The zone of inhibition of sample ciprofloxacin B of set-1 (control), set-2 (container + carton), set-3 (primary packaging) and set-4 (transparent glass test tubes direct exposure) were 32, 29.33, 29 and 28.67 respectively. In the figure 3.3.1 the highest zone of inhibition was observed in ciprofloxacin B and the lowest effectiveness was seen in Ciprofloxacin E.

The herbal eye drops set-1(control), set-2 (container + carton), set-3 (primary packaging) and set-4 (transparent glass test tubes direct exposure) did not show any zone of inhibition against *Bacillus cereus*.

For determination of antibacterial activity of the eye drops against gram negative bacteria three strains were taken for this study - *Salmonella typhi, Escherichia coli* (STEC) and *Proteus vulgaris.* These organisms showed different antibacterial activity against ciprofloxacin (0.3%) commercial eye drops preparations and herbal eye drops preparation. Firstly, against *Salmonella typhi* isolates the most effective formulation was ciprofloxacin B. The largest zone of inhibition was obtained from ciprofloxacin B set-1(control), set-2 (container + carton), set-3 (primary packaging) and set-4 (transparent glass test tubes direct exposure) that are 36, 35.7, 35 and 34.5 mm among the other eye drops preparation. A comparison chart was shown in figure 3.3.2 were the untreated sample set-1(control) showed the highest zone of inhibition and the sunlight exposed samples set-2 (container + carton) , set-3 (primary packaging) and set-4 (transparent glass tubes) gradually showed smaller zone compared to set-1 (control). The smallest zone of inhibition was observed in set-4 (transparent glass tubes). So, the

lowest efficacy was observed in set-4 in all samples. The best drug that was most efficient against *Salmonella typhi* ciprofloxacin B and the least efficient drug with small of inhibition was ciprofloxacin E.

Secondly, against *Escherichia coli* (STEC) isolate the most effective eye drops sample was ciprofloxacin C. The largest zone of inhibition was obtained from ciprofloxacin C set-1 (control), set-2 (container + carton), set-3(primary packaging) and set-4(transparent glass test tubes direct exposure) 24.4, 23.87, 22.4 and 21. In comparison figure 3.3.2 with ciprofloxacin eye drops ciprofloxacin had the highest efficiency. The smallest zone of inhibition was obtained from ciprofloxacin D.

Lastly, against *Proteus vulgaris* isolate the most efficient eye drops was ciprofloxacin C. The sample showed the largest zone of inhibition in set-1 (control), set-2 (container + carton), set-3 (primary packaging) and set-4 (transparent glass test tubes direct exposure) that are 24.6, 24, 22.33 and 21.33 mm in comparison to the other eye drops preparation. In figure 3.3.1 the chart shows that highest zone of inhibition were obtained from ciprofloxacin C and the smallest zone of inhibition was obtained from ciprofloxacin A.

On the other hand, the commercially available herbal eye drops preparation did not have any bactericidal effect on human tested gram negative bacterial isolates *Salmonella typhi, Escherichia coli (STEC)* and *Proteus vulgaris*. While many antibacterial agents have similar spectra of activity, they are not equally potent against several organisms and vary in their pharmacokinetic properties (Irsch & Guyton, 2009). The herbal eye drops revealed marked antibacterial and antifungal activity the effects of this eye drops against both gram-positive make it a promising drug in the treatment of ocular diseases due to infections and inactive against gram-negative bacteria (Mitra *et al*., 2000).

In case of both gram positive and gram negative bacteria, a significant difference is observed in case of set-3 (primary packaging). Ciprofloxacin (0.3%) commercially prepared eye drops ciprofloxacin A showed a decrease in zone of inhibition compared to Set – 4 (transparent glass test tubes direct exposure). In study, the antibacterial activity decreased from control to transparent glass test tubes direct exposure. But in case of ciprofloxacin A greater degradation was observed in set-3 which showed the smallest zone of inhibition against gram positive

bacteria and gram negative bacteria. This result suggests that the structure of the degradation product of ciprofloxacin exposed to natural sunlight was also confirmed by the photolytic breakdown of the piperazine ring in ciprofloxacin that produces the ethylenediamine derivative (Das & Subodh, 2005). Due to lack of quality control in packaging the ophthalmic component tend lose their photo-stability by heat, UV rays and sunlight exposure (Turro *et al*., 2010). The photo products of a drug are destructive and can cause photo-toxic, photo-allergic, or photo-sensitization responses upon administration (Kullavanijaya and Lim, 2005).

In a comparative study between the ciprofloxacin (0.3%) eye drops and herbal eye drops, the sunlight degraded ciprofloxacin (0.3%) samples showed changes in color after 10 hours of exposure in few set-3 (primary packaging) and all set-4 (transparent glass test tubes direct exposure). The reason for changes in color of ciprofloxacin (0.3%) eye drops samples, were found to degrade an ethylenediamine derivative of ciprofloxacin when exposed to natural sunlight (Sun *et al*., 2008). And no changes in color were seen in herbal eye drops. This is because herbal components used in manufacturing this herbal eye drops preparation did not possess any sunlight degrading components. So, for these reason the herbal eye drops retained their original color. For further studies, antibacterial test was done against gram positive and gram negative bacteria, ciprofloxacin (0.3%) eye drops both sunlight treated and untreated showed their efficiency with chronological decrease in zone of inhibition from set-1 (control) to set-4 (transparent glass test tubes direct exposure). This result suggests that the eye drops being sunlight degraded still retained some antibacterial property. On the other hand, herbal eye drops had no antibacterial activity against both gram negative and gram positive bacteria. Ayurvedic system of medicine has described a number of medicinal plants useful in the treatment of ocular diseases and disorders (Chandra *et al*., 2017). Herbal eye drops contains both antibacterial and antifungal activity but some herbal eye drops are not equally active against gram negative and gram positive bacteria (Mitra *et al.,* 2000). Except in case of *Staphylococcus aureus* zone of inhibition was obtained from set-2 (container + carton). The probable reasons could be that the eye drops had double protection which did not degrade the component after sunlight exposure and other sets degraded due to sunlight and lost their efficacy. So, the herbal eye drops has very less potential to fight against bacterial pathogens compared to synthetic eye drops.

Conclusion:

In a comparative study the among five commercially available eye drops (ciprofloxacin A, ciprofloxacin B, ciprofloxacin C, ciprofloxacin D and ciprofloxacin E) before and after treatment sunlight treatment were effective against *Staphylococcus aureus, Bacillus cereus, Salmonella typhi, Escherichia coli (STEC)* and *Proteus vulgaris*. The zone of inhibition decreased gradually from set-1 (control) to set-4 (transparent glass test tubes direct exposure) due to sunlight treatment. Among the synthetic eye drops the best effective drug against all above mentioned bacteria was ciprofloxacin B. A comparison was done between ciprofloxacin (0.3%) eye drops and herbal eye drops to identify the effectiveness of herbal eye drops against the above mentioned organisms. As synthetic drugs has many side-effects like photo-toxic, photo-allergic reaction and prolong use of antibiotics can cause antibiotic resistance (Horspool & Armesto, 1992). So in contrast to that herbal eye drops were taken to consideration and antibacterial test was done which showed very surprising results. The herbal eye drops had no antibacterial activity against *Staphylococcus aureus, Bacillus cereus, Salmonella typhi, Escherichia coli (STEC)* and *Proteus vulgaris.* A very low efficiency was observed against *Staphylococcus aureus* in set-2 (container + carton). So, the overall results shows that the ciprofloxacin (0.3%) eye drops before and after sunlight degradation were active against pathogenic bacteria causing ocular infections. But the herbal eye drops were not comparable to ciprofloxacin eye drops (0.3%) as it has low to no efficacy.

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Appendix - I

Media composition

Compositions of the media used in this study are provided below. The media were autoclaved at 121°C for 15 min at 121psi.

1. Nutrient Agar (HiMedia, India)

Ingredients	Amounts (g/L)
Peptic digest of animal tissue	5.0
Beef extract	1.5
Sodium chloride	5.0
Yeast extract	1.5
Agar	15.0

2. Mueller-Hinton Agar (HiMedia, India)

Ingredients	Amounts (g/L)
Beef infusion	300
Casamino acids	17.5
Starch	1.5
Agar	17.0

Appendix – II

Instruments

Autoclave	Wisd Laboratory Instruments Made in Korea
Water Bath WiseBath^{R}	Wisd Laboratory Instruments DAIHAN Scientific Co., Ltd Made in Korea
Shaking Incubator	Model: JSSI-1000C JS RESEARCH INC. Made in Rep. of Korea
Incubator	Model: DSI 3000 Digisystem Laboratory Instruments Inc. Made in Taiwan
Vortex Mixer	Model: VM-2000 Digisystem Laboratory Instruments Inc. Made in Taiwan
Table Top Centrifuge	Model: DSC-200A-2 Digisystem Laboratory Instruments Inc. Made in Taiwan
Electronic Balance	RADWAG Wagi ELEktroniczne Model: WTB 200
Refrigerator (4^{0}C)	Model: 0636 Samsung