IMPACT OF SUNLIGHT ON CHLORAMPHENICOL MEDIATED SYNTHETIC AND HERBAL EYE DROPS IN TERMS OF ANTIMICROBIAL ACTIVITY

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 sisters

Declaration

I hereby declare that this thesis entitled "**Impact of sunlight on chloramphenicol mediated synthetic and herbal eye drops in terms of antimicrobial activity**" is submitted by me, Permita Mazumder, to the Department of Mathematics and Natural Sciences under the supervision and guidance of Kashmery Khan, Lecturer, Department of Mathematics and Natural Sciences, BRAC University. I also declare that the thesis work presented here is original, and has not been submitted elsewhere for any degree or diploma.

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ABSTRACT

Ocular bacterial infections can cause several eye diseases that have potential impact on human health which is a growing concern worldwide. The use of chloramphenicol synthetic eye drops could provide immense coverage against bacterial infections. In addition, herbal eye drops have been emerged as a promising drug in the treatment of ocular diseases as chloramphenicol resistance occurs in many pathogenic species. However, the medicinal quality of drug product can be decreased with the influences of many environmental factors such as sunlight, UV radiation, heat and many other chemical reactions. The purpose of this study was to evaluate the antimicrobial activity of chloramphenicol synthetic and herbal eye drops in the presence of primary and secondary packaging after exposure to sunlight. Photochemical degradation was observed in chloramphenicol synthetic and herbal eye drops due to the sunlight exposure at different time interval. Antimicrobial susceptibility *of Bacillus cereus, Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Salmonella typhi* was carried out using sunlight exposed chloramphenicol synthetic and herbal eye drops by using agar well diffusion method. Chloramphenicol and herbal eye drops effectively degraded by sunlight that also decreasing the antimicrobial activity. In some cases, chemical components degradation was observed in the primary packaging of the chloramphenicol eye drops. It was observed that chloramphenicol synthetic eye drops exhibited more antimicrobial activity than herbal eye drops. Thus, the findings of this study may provide the information of maintaining eye drops in a suitable manner for the molecular stability of the drug compounds. Improvising the knowledge of the photochemical behavior of drugs can provide a proper guidance for handling, packaging, and labeling of drug products in the manufactured company. Furthermore, it may assign the possibility of developing the public awareness to use the drugs by following the appropriate instructions for sustaining a healthy diseased free eye.

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Abbreviations	Elaborations
WHO	**World Health Organisation**
CHLORAM	**Chloramphenicol**
No ZOI	**No zone of inhibition**
NA	**Nutrient Agar**
MHA	**Mueller-Hinton Agar**
ºC	**Degree Celsius**
et al.,	**And others**
ml	**Milliliter**
mm	**Millimeter**

Chapter 1: Introduction

1.1 Background:

Eye infections are stated to be one of the most frequently occurred health hazard around the whole world. Bacteria is the most common agent that causes eye infection in human (Modarres *et al.*, 1998). Ocular bacterial infections are responsible for a series of symptoms and signs, such as the formation of pus, conjunctival hyperemia, lid edema, and even visual impairment (Wang *et al.*, 2015).It is reported that the estimated number of people visually impaired in the world is 285 million; 39 million blinds and 246 million having low vision; 65 % of people visually impaired and 82% of all blind are 50 years and older (WHO, 2012). Bacterial infection is a common cause of conjunctivitis and accounts for up to 50% of all cases of conjunctivitis in adults and 70% to 80% of all cases in children (Bremond-Gignac *et al.*, 2011).Several microorganisms are liable for many serious eye diseases. Bacterial, viral and fungal infections are exhibited by the eye parts that causes many inflammatory disorders. *Staphylococcus aureus* (due to injury), *Pseudomonas aeruginosa* (may be due to contact lens), *Streptococcus, herpes simplex* type I, *varicella zoster, Cytomegalovirus (CMV), adenovirus, Candida species*, and *Actinomyces israelii* are the major bacterial, viral, and fungal agents that infect the different parts of the eyeball causing decreased vision, pain, and red eyes and may even lead to blindness (Malhotra., 2014).There are many antibiotics and steroid preparations available for conducting the Ophthalmic disorders (Namboothiri D.G *et al.*, 2015). Antibiotic eye drops have been used effectively for the treatment of various eye infections and are either prescribed by an ophthalmologist or sold over the counter (Olorode O. A *et al.*, 2017)

Contrariwise, people are turning to natural sources in search of compounds with potent antimicrobial activities these days. The rate at which microbes are developing resistance to synthetic antibiotics is alarming. Inferable from the side effects and resistance that pathogenic microorganisms elevate against common, economically accessible antibiotics, more attention is being paid to extracts and bioactive components that can be isolated from plants used in herbal medicine (Essawi & Srour, 2000). Antibiotic and multi-drug resistance are now a world- wide problem in hospitals, long-stay residential centers and also in the community (Livermore, 2000). To address all these shortcomings, a significant number of therapeutics are being derived from natural sources such as plants by utilizing, systemic and topical novel drugs in assistance with existing products (Woodford, 2005). Many plant materials used as traditional medicine have been

proven to be more effective, and relatively cheaper than their modern counterparts (Mann *et al.,* 2008).

1.2 Eye:

Eye is considered as the window of our spirit. An eye is the most valuable and easily accessible organ of the body. It is a special organ of our body anatomically and in addition physiologically (Akhtar, 2013) .The human eye is basically an organ of vision. This vital organ of vision plays a very important role not only in life but also the Human body (Thogarapalli, 2006). It acquaints with easy access to local drug delivery and is a way to non-invasive clinical treatment of ocular diseases. To treat ocular diseases, topical and localized approaches in the form of solution, suspensions and ointments are more preferred in the treatment of ocular diseases (Sahoo *et al.,* 2008).

1.3 Eye infections:

The eye is well protected from infection that will caused by the conjunctiva and the corneal epithelium. In addition, the tear film contains antimicrobials while the tear flow itself tends to wash away pathogens. The eye also asylums a host of non-pathogenic bacteria that competitively prohibit new bacteria growth. However, these eye-defences can be breached by trauma, improper tearing, or contact lens wear and lead to an infection. An eye infection not only threatens vision, but the orbit can act as an entry portal to the rest of the body and infections can progress to systemic involvement, meningitis, and even death **(**Root, 2007) .Eye infections can be caused by bacterial, viral or other microbiological agents and can affect the eyelids, the cornea and even the optic nerve. The most prominent clinical features of bacterial eye infections include: Conjunctivitis, Cystitis, Keratitis, Blepharitis, canaliculitis, Dacryo-cystitis, Cellulitis and Endophthalmitis.(Modarres *et al.,* 1998). The most common causative bacterial agents in ocular infections include- *Staphylococcus spp. Streptococcus spp, Hemophilus influenza, Pseudomonas aeruginosa,* entric Gram-negative bacilli and some anaerobic bacteria (Modarres, 1998).

1.4 Importance of eye drops to mitigate eye infections:

Eye drops are sterile liquids for instillation into the conjunctiva sac worldwide, they are formulated and packaged in order to maintain their sterility throughout the period of use. The use of eye drops is widespread in all regions of the world especially during the Harmattan or dry season where dust particles, and also other irritants, are easily blown into the eye (Olorode O. A *et al.,* 2017). Most instances of routine bacterial conjunctivitis respond to the commercially available combination of antibiotics, artificial tears, lid scrubs, oral analgesics, and, often, a topical antihistamine to relieve itching and discomfort (Yeung, 2017).Ophthalmic anti-infectives contain in a product formulated especially to be instilled or applied in the eye or eyes. Ophthalmic anti-infectives include eye drops, gels or ointments. Anti-infectives are drugs that can either kill an infectious agent or inhibit it from spreading. Anti-infectives include antibiotics and antibacterials, antifungals, antivirals and antiprotozoals. The range of available eye drop preparations has recently changed in many countries. In the past, silver nitrate was routinely used to prevent infection, but this agent can cause burns of the newborn's eyes. Erythromycin is now more commonly used, but is not available in all countries. The respective advantages of eye drop and ointments include preserved visual acuity and prolonged contact, and a soothing effect (Bremond-Gignac *et al.,* 2011). There are several ophthalmic eye drops such as ciprofloxacin, chloramphencol, moxifloxacin, gatifloxacin, besifloxacin, trifluridine, polymyxin gentamicin, and bacitracin for the treatment of the eye diseases. Chloramphenicol, Hypromellose, Artificial tears, ciprofloxacin and betaxolol eye drops had significant antimicrobial activity with their anti-infective having the highest rapidity in bactericidal activity (Olorode, 2017)

1.5 Chloramphenicol mediated eye drops

Chloramphenicol is a broad-spectrum antibiotic effective against many Gram-positive and Gram-negative bacteria, including several anaerobic organisms (Balbi, 2004). Chloramphenicol is a bacteriostatic antimicrobial compound originally derived from the bacterium *Streptomyces venezuelae,* isolated by David Gottlieb, and introduced into clinical practice in the year of 1949. It was the first antibiotic to be manufactured synthetically on a large scale. Chloramphenicol is effective against a wide variety of microorganisms. Chloramphenicol is used in eye drops or ointment for bacterial conjunctivitis for the treatment of bacterial conjunctivitis (Rimawi & Kharoaf, 2011). Chloramphenicol acts by binding to the 50S ribosomal subunit of bacteria and inhibiting peptidyl transferase, the enzyme catalyzing the formation of peptide bonds between

adjacent amino acids in the growing polypeptide chain (Schwarz *et al.,* 2004).The structure of Chloramphenicol,D-(-)-threo-2,2-dichloro-N-[β-hydroxy-α-(hydroxymethyl)-p nitrophenethlyl] acetamide (Acharya and Gowda, 1979).The molecular formula of Chloramphenicol is $C_{11}H_{12}Cl_2N_2O_5$.

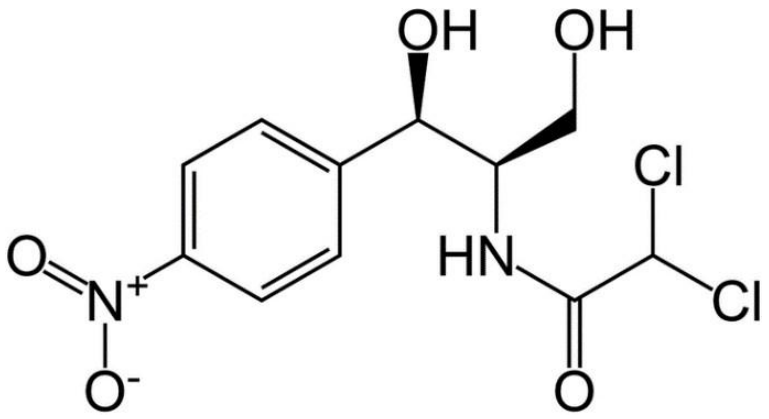


Figure 1.5: chloramphenicol Antibiotics (Image retrieved from : https://www.google.com.bd/search?q=the+structure+of+chloramphenicol+pdf&source=lnms&tbm=isch&sa=X&ved=0ahUKEwjAit_9iMvXAhWHuo8KHZX0AY8Q_AUICigB&biw=1366&bih=647#imgdii=YpE-DPIozIdjMM:&imgrc=4-0XjgT2Y4mSHM:)

The molecule of chloramphenicol is composed of three parts: (I) a p-nitrobenzene moiety, (II) a dichloracetyl moiety, and (III) a 2-amino-propanediol moiety. In more general terms, part I represents an aromatic ring system and part II an aliphatic haloacetyl side-chain. The propanediol moiety possesses two asymmetric carbon atoms. Chloramphenicol itself is the D-threo isomer, which is the only isomer with strong Bacteriostatic properties (Maxwell and Nickel, 1954).Chloramphenicol is once in a while used topically in the treatment of eye infections because of its wide antibacterial spectrum and its penetration of ocular tissues and the aqueous humor (Abachi *et al.,* 2010). It is widely used as eye drops for the treatment of bacterial conjunctivitis caused by *Escherichia coli, Haemophilus influenzae, Staphylococcus aureus, Streptococcus haemolyticus*, *Morax Axenfield* and others. Chloramphenicol is also indicated in typhoid fever,

various salmonella infections, anaerobic infections with *Bacteroides fragilis*, rickettsial infections, and bacterial meningitis. It has been the drug of choice for the treatment of *H. injluenzae* meningitis, especially if the organism is a B-Lactarnase producer (Turk, 1977, Martindale, 1989).

Figure 1.5.1: Chloramphenicol eye drops (Image retrieved from: https://www.google.com.bd/search?q=chloramphenicol+eye+drops&source=lnms&tbm=isch&sa=X&ved=0ahUKEwjPxpuU7M_XAhWJP48KHVsuAdYQ_AUICigB&biw=1366&bih=647#imgrc=p6riB6vs7_sAuM:

It is one of the rare natural compounds which carry a nitro group. Chloramphenicol is a low molecular weight, predominantly lipophilic antibiotic. It is usually well tolerated and is well penetrated in non-inflamed eye better than any other antibiotic regardless of the mode of administration resistance is slow to develop, moderate in nature and not necessarily permanent. The drug readily penetrates into bacterial cells and interferes with protein synthesis. Nevertheless, the molecule has a structure which is one of the simplest of the known antibiotics (Rebstock *et al.*, 1949; Dunitz, 1952).Chloramphenicol is a safe antimicrobial agent effective in treating external

ocular infection. Chloramphenicol performed in advancing microbiological, clinical, and general change in patients with bacterial eye diseases. Chloramphenicol eradicated or controlled bacteria in 76% of culture-positive patients (Bron *et al.*, 1991).

1.5.1 Composition of Chloramphenicol

There are many ingredients that are needed for the composition of chloramphenicol solution.

Table 1.5.1: Amount of Ingredients found in the prepared Ophthalmic Chloramphenicol (0.5%) solution (Abachi *et al.*, 2010)

Chloramphenicol	0.05g
Propylene Glycol	1.0g
Polyethylene Glycol 1500	1.0g
EDTA	0.01g
Cetrimide	0.01g
Sodium Chloride	0.08g
Boric Acid	0.02g
Borax	0.005g
Distilled water	Until 10 ml

1.5.2 Commercial chloramphenicol synthetic eye drops:

Chloramphenicol eye drops manufactured by five different companies were collected from local pharmaceuticals of Bangladesh

Table 1.5.2: Commercial chloramphenicol synthetic eye drops

Chloramphenicol eye drops	Composition	Preservatives
Supraphen	Chloramphenicol BP 0.5% enriched with Hypromellose	Phenylmercuric Acetate 0.02 mg/ml
Opsophenicol	Chloramphenicol 0.5%	Phenylmercuric Acetate 0.002%
Chlorphen	Chloramphenicol BP 0.05%	-
Optical	Chloramphenicol USP 0.5%	-
SQ-Mycetin	Chloramphenicol BP 0.5%	Phenylmercuric Nitrate BP 0.002%

1.5.3 Therapeutic uses of chloramphenicol synthetic eye drops:

More than 50% of the world uses chloramphenicol as a first-line agent in the treatment of conjunctivitis (Melton & Thomas, 2017). Most cases of acute bacterial conjunctivitis resolve spontaneously within 7–10 days, but a broad-spectrum chloramphenicol antibiotic eye drops can decrease disease severity, transmission and also minimize the complication and reinfection rates. The most developed topical antimicrobial, chloramphenicol seems suited to modern life and can

be used as first-line therapy with a reduced dosage regimen and its broad spectrum of action. (Bremond-Gignac *et al.,* 2011).

Chloramphenicol penetrates the cornea thoroughly, and therapeutically effective concentrations are detectable in the aqueous humor only 15 to 30 minutes after local administration. Eye drops and eye ointment is used in chloramphenicol susceptible organisms in infected eyelid, cornea (corneal ulcer), and lachrymal canal system and anterior of the uvea. It may also be used in Trachoma, Zoster, blepharitis, dacryocystitis and Parinauds conjunctivitis, also used as an adjuvant therapy with other drugs in the treatment of herpes simplex, therapeutic irrigation of the tear ducts and for the prophylaxis of bacterial infection before and after surgical operation and after fire and chemical burns. Chloramphenicol is beneficial in rising the lachrymal canal as a preventive and curative of infections. Some of the cases Sepsis caused by some species of bactericides may respond to chloramphenicol. In addition, safety for use in pregnancy and lactation has not been established. Therefore, it should be used only when considered essential by the physicians. It is also should not be given to new born babies and to the nursing mothers (Drugs, n.d)

Systemic adverse reaction has not been reported within short term topical use of chloramphenicol Eye drops but rarely optic atrophy in children, stinging and burning of the eye, vesicular or maculopapular dermatitis and angioedema in patient sensitive to chloramphenicol. However, after application a slight burning sensation and a bitter taste in the mouth may occur. Allergic reactions in the form of palpebral eczema have been reported in rare cases. Partially irreversible hematodyscrasia (aplastic anaemia, leukopenia, agranulocytosis) has been observed in isolated cases following topical use of chloramphenicol. The prolonged use of antibiotic may result in over growth of non-susceptible organisms and fungi. This medicine should not be used regularly in prolonged period of times. If new infections appear during medication, the drug should be discontinued and appropriate measures should be taken. Chloramphenicol eye drops should never be given for minor infections or for prophylaxis (Drugs, n.d)

1.5.4 Physical factors of chloramphenicol synthetic eye drops

Some pharmaceutical preparations reveal chemical and physical instabilities leading to decomposition. Degradation and deterioration of the formulation of chemical compound. Hence loss of therapeutic benefit of the drug such as instabilities are either produced or catalysed by

environmental factors – such as UV radiation, humidity, heat and light, through chemical process of hydrolysis, oxidation and photolysis (Okeniyi *et al.,* 2006).

Photolysis: Exposure of drug molecule to light may produce photolytic degradation products. The rate of photo degradation depends upon the intensity of incident light and quantity of light absorbed by drug molecule. Photolytic degradation is carried out by exposing the drug substance to or drug product to a combination of visible and UV light. Most of the compounds will degrade as solutions when exposed to high-energy UV exposure. Example- the formulation of chloramphenicol eye drops 0.5% on exposure to UV-light induces photolysis (Shinde *et al.,* 2013). Photolysis of the antibiotics compounds was recorded as a result of the energy gained by absorption of sun light. The light absorbing ability of the compound also determined the process of photolysis (Jasim *et al.,* 2010). With the increase in the UV and sun light treatment duration, the rate of degradation increases, resulting in the gradual inactivation of the antibiotic (Singh and Gupta, 2014).The half-life of chloramphenicol is about 50 times stable in red light than in sunlight or ultraviolet light (Okeniyi *et al.,* 2006)

Temperature: Chloramphenicol Synthetic eye drops are liable to heat or topical temperature. Degradation caused by exposure to temperatures include bond breakage ex- pyrolysis. Any degradation mechanism that is enhanced at elevated temperatures are thermolytic pathways include hydrolysis, dehydration, isomerization, decorboxylation, rearrangement, polymerization reactions, pyrolysis. Thermal degradation study is carried out at 40^{o}C. The mostly accepted temperature is 700C at low and high humidity for 1-2 months.

Hydrolysis: Hydrolysis is the most common degradation chemical reaction over wide range of pH. Water either as solvent or as moisture in the air comes in contact with pharmaceutical dosage form is responsible for degradation of most drugs. Hydrolysis of most of drugs is depending upon the relative concentration of hydronium and hydroxyl ions (Shinde *et al.,* 2013) Changes are occurred in PH degrees for hydrolysis of chloramphenicol at different temperatures. This indicates that the degradation of chloramphenicol is linearly dependent on buffer concentration (borax), the primary pathway for the degradation of chloramphenicol is the hydrolysis of the amide linkage, forming the. Corresponding amine and dichloroacetic acid (Abachi *et al.,* 2010). AMPD (2-amino-1-(4-nitrophenyl) propane-1, 3-diol) is the main degradation product of chloramphenicol is produced during hydrolysis (Alaani *et al.,* 2014)

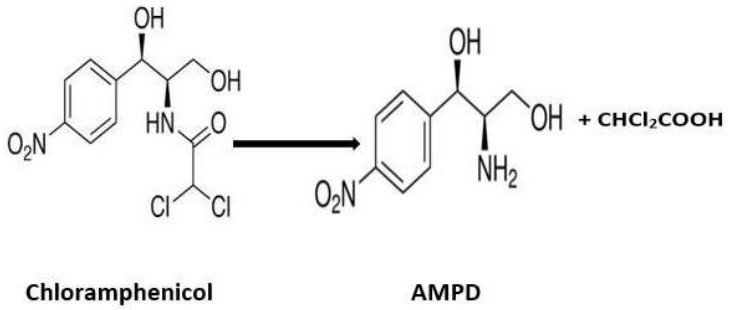


Figure 1.5.2: The hydrolysis of Chloramphenicol

Oxidation: The mechanism of oxidative degradation of drug substance involves an electron transfer mechanism to form reactive anions and cations. The mechanism of oxidative degradation of drug substance involves an electron transfer mechanism to form reactive anions and cations. Drugs In pharmaceuticals most common form of oxidative decomposition is oxidation through a free radical chain process.

1.5.5 Antimicrobial properties of chloramphenicol synthetic eye drops

Broad-spectrum chloramphenicol antibiotic eye drops are commonly used to effectively treat ocular infection (Bremond-Gignac *et al.,* 2011).The ophthalmic chloramphenicol Fluoroquinolone antibiotics act via direct inhibition of bacterial DNA synthesis, preventing the action of DNA gyrase and topoisomerase IV, which blocks DNA replication and eventually leads to damage to bacterial DNA and cell death (Okeri *et al.,*2008). This compound has a broad range of activity against the Gram-positive organisms (Hubicka *et al.,* 2013; Hayder *et al.,* 2012) and the Gram-negative bacteria (Hubicka *et al.,* 2013).

By conducting by agar diffusion test of chloramphenicol retained its antimicrobial efficacy against *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Reddy C. *et al.,* 2011)

In a Study , chloramphenicol are antimicrobial antibiotics with having broad spectrum activities against a wide range of bacteria including *S. aureus, Pseudomonas sp, Proteus* and many coliform bacilli (Rosenthal *et al.,* 2006) . In another study, it was found that chloramphenicol inhibits the growth of the most common causative pathogens seen with bacterial conjunctivitis include

Staphylococcus aureus, *Streptococcus pneumonia*, *Haemophilus* i*nfluenza*, and *Moraxella catarrhalis* (Bremond-Gignac *et al.,* 2011).

On the other side, (Jasim *et al.,* 2010) studied the effects of the sun light degradation of chloramphenicol and it was observed that the zone of inhibition of degraded chloramphenicol was smaller than the controls which was not exposed to sunlight. In another study, it was observed that the antibacterial activity of ciprofloxacin was suppressed by UV and Sun light. With the increase in the UV and sun light treatment duration, the rate of degradation increases, resulting in the gradual inactivation of the antibiotic. (Singh and Gupta, 2014).

However, in recent years, the spread of antibiotic resistance and its potential impact on human health have caused a growing concern worldwide (Zuorro *et al.,* 2013). Unselective and extensive use of antibiotics and selective pressure on bacterial strains is highly considered to be the reason behind such evolution (Goudrazi *et al.,* 2015; Habeeb *et al.,* 2007). Growing resistance has been observed among ocular bacteria as with other systemic pathogens. The factors contributing to the development of drug resistance include overuse of antibiotics for systemic infection as well as overuse of topical antibiotics in the eye (Bremond-Gignac *et al.,* 2011).

1.6 Herbal eye drops

Many antibiotic and steroid preparations are available for the treatment of ophthalmic disorders. However prolonged use of this synthetic antibiotic may have potential side effects (Namboothiri D.G *et al.,* 2015). The ayurvedic system of medicine has described a number of medicinal plants useful in the treatment of ocular diseases and disorders (Mitra *et al.,* 2000). The herbal eye drops formulation is equipped for beneficial effects in inflammatory and allergic conditions of the eyes. They are cheap, reliable and have little side effects than the costly synthetic drugs, many of which have adverse effects and are beyond the reach of poor patients. The efficacy of many traditional herbal medicines in curing ocular diseases are now being gradually recognized in modern science as well. Herbal eye drop is a polyherbal formulation indicated for anti-inflammatory and antihistaminic and antimicrobial effect (Gupta *et al.,* 2012). The shortcomings of the Health sector and as well as unwanted effects of present treatment modalities could be combated by Holistic approach of Ayurveda and Traditional Medicinal systems prevailing in the country by treating successfully many infectious and many eye pathologies (Silva *et al.,* 2015).

1.6.1 Itone herbal eye drops

Itone is a polyherbal eye drop mixture of aqueous distillates of nineteen traditionally used ingredients that sum up to impart potency to the formulation and make it a useful adjunct in various ocular pathologies. Itone is an ayurvedic proprietary polyherbal eye drop which is developed on the basis of ayurvedic principles which is available for the last 25 years in Indian Market and has been recognized to have medicinal value for various ocular ailments (Velpandian *et al.*, 2013). It is a potent formulation that is useful in pink eyes, conjunctivitis, swollen eyelid bump, sty in eye, eye strain, red eyes, tired eyes, dry eyes, watery eyes, trachoma, myopia, corneal ulcers, hypermetropia, lenticular opacity, itching and burning sensation in the eyes (OTC search, n.d.). The effects of Itone eye drops against both gram-positive and gram-negative bacteria make it a promising drug in the treatment of ocular diseases due to infections (Mitra *et al.*, 2000). Clinical evaluation of Itone eye drops is necessary to estimate the clinical potency (Biswas *et al.*, 2001).

Figure 1.6.1: Itone herbal eye drops (Image retrieved from: https://www.google.com.bd/search?q=itone+ayurvedic+eye+drops&source=lnms&tbm=isch&sa=X&ved=0ahUKEwj48cu7z9bXAhVJK48KHQRvCC8Q_AUICigB&biw=1366&bih=598#imgrc=948-SrELfrYHeM :)

1.6.2 Composition of Itone Herbal eye drops

The polyherbal formulation (Itone™) consists of a fixed combination of the aqueous distillates of nineteen traditionally used ingredients

Table 1.6.1: Ingredients of Itone herbal eye drops with activity

Ingredients	Quantity (in mg)	Ingredients	Quantity (in mg)
*Azadirachta indica (*nimba)	562.50	*Ocimum sanctum* (tulsi Patra)	562.50
*Moringa pterygosperma (*sobhanjana)	562.50	*Rosa centifolia* (satapatri)	150.00
Eclipta alba (bhringaraj)	562.50	*Carum copticum* (yamani)	69.50
*Boerhaavia diffusa (*punarnava)	562.50	*Terminalia chebula* (haritaki)	46.25
Vitex negundo (nirgundi)	562.50	*Terminalia belerica* (vibhitaka)	46.25
Emblica officinalis (dhatriphala)	46.25	*Amomum subulatum* (ela)	27.75
Curcuma longa (haridra)	92.50	*Santalum album* (sweet Chandan)	69.50
Cinnamomum Camphora(Karpuram)	6.95	Mukta (pearl)	0.93
Mentha piperata (menthol)	1.85	saindhava laban (rock salt)	13.88
Madhu (honey)	92.50		

1.6.3 Therapeutic uses of Itone herbal eye drops

Itone eye drops have been successfully used to treat many infectious and degenerative eye pathologies (Silva *et al.,* 2015). The constituents of Itone eye drops are known to possess antimicrobial and anti-inflammatory properties and are used in traditional medicine for the treatment of a variety of ocular disorders and eye drops offered the anti-inflammatory effect was almost comparable to that of betamethasone may be due to the inhibition of inflammatory mediators by the ingredients present in the formulation. The antioxidant effect also exhibited by Itone eye drops might prove beneficial in the treatment of ocular disorders such as acute and chronic conjunctivitis, eye strain, dacryocystitis and pterygium (Mitra, 2000). It has been also reported to be a useful adjunct in various ocular diseases like trachoma, blepharitis, keratitis, corneal ulcers etc and also maintaining the visual acuity and helpful in computer vision syndrome (Velpandian *et al.,* 2013)

Itone ayurvedic eye drops formulation are rich source of tannin and tannin like compounds which are responsible for antimicrobial and antioxidant properties of ayurvedic eye drops (Soni *et al.,* 2015). The Itone eye drops showed significant antiangiogenic and anti-inflammatory activities and noticeable anti-cataract activity (Velpandian *et al.,* 2013). It is an ayurvedic eye drops was developed for dry eye syndrome (DES) systematically following contemporary methods and parameters right from quality assurance of ingredients (Srikanth *et al.,* 2015).

Volatile and fixed oils of *Ocimum sanctum* (tulsi Patra) are known to possess anti-inflammatory activity (Singh and Agrawal, 1991).Essential oil of *Ocimum sanctum* (tulsi Patra) possesses bactericidal activity against gram-positive and gram-negative bacteria (Prasad and Rao, 1987).

The extract of *Cinnamomum camphora* (karpuram) showed antibacterial activity against gram-positive and gram-negative organisms (Naqvi *et al*., 1985).

Rosa centifolia (Satapatri) is well-known for its cooling property, when applied to the eyes. It provides relief from eyestrain and computer vision syndrome and exhibits antibacterial activity. Honey is generally recommended for sore eyes. It is also reported to prevent infection and promote healing, as it has ingredients similar to antibiotics (Mitra, 1985).

1.6.4 Antimicrobial activity of Itone herbal eye drops

Acute Bacterial conjunctivitis is a common and usually a self-limiting condition caused by direct eye contact with infected secretions. The most common isolates are *Stapylococcus pneumonia*, *Stapylococcus aureus*, *Haemophilus influenzae*, and *Moraxella catarrhalis* their growth are inhibiting by administering Herbal eye drops (Silva *et al.*, 2015).

In a study, Itone eye drops possessed antibacterial activity against *Salmonella typhosa, Micrococcus pyogenes* and *Escherichia coli* (Krishnan and Badhwar, 1953). In another study, itone eye drops were potent against *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella,Proteus, Candida albicans, Shigella flexneri* (Chand *et aI.*, 1994).

In another experiment, the Itone eye drops were found to be most potent against *Bacillus thuringienesis* and *Escherichia coli* amongst the other gram positive bacteria and gram negative bacteria under studies, respectively. Moderate action was seen against *Bacillus subtilis, Salmonella typhi, Proteus mirabilis, Bacillus polymyxa* and *Staphylococcus aureus*. A comparatively weak antibacterial action was shown against *Pseudomonas aeruginosa and Klebsiella pneumonia* (Dhamankar *et al.*, 2014).

1.7 Bacterial strains sensitive to chloramphenicol synthetic and herbal eye drops

Bactericidal activity of chloramphenicol synthetic eye drops and Itone herbal eye drops were observed against the following bacterial species isolated from clinical samples:

1.7.1 Bacillus cereus

Bacillus cereus is a ubiquitous, gram-positive, endospore-forming rod. Infective Endophthalmitis involves eyes typically have poor vision outcomes and a large proportion of patients (75%–91%) experience a loss of light perception, phthisis bulbi, evisceration, or enucleation is caused by *Bacillus cereus* after Cataract Extraction Surgery. conjunctival chemosis, subconjunctival hemorrhage, and corneal edema also caused by *Bacillus cereus* (Chan *et al.*,2003)

1.7.2 Staphylococcus aureus

Staphylococcus aureus is a facultative anaerobic, gram-positive coccus, which appears as grape-like clusters when viewed through a microscope, and has round, usually golden-yellow colonies. Conjunctivitis, which is also known as pink eye, is common, affecting about 6 million people annually is caused by *Staphylococcus aureus.*

1.7.3 Escherichia coli

Escherichia coli (commonly abbreviated *E. coli*) is a gram-negative, rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms (endotherms) which is responsible for bacterial conjunctivitis, eye lid infection and also keratitis (Abid and Ewadh,2012).

1.7.4 Salmonella typhi

Salmonella typhi a gram-negative, motile, rod-shaped bacteria. *Salmonella typhi* is responsible for Endogenous endophthalmitis is a rare but serious condition that occurs when bacteria cross the blood-ocular barrier and multiply within the eye (Sinha *et al.*, 2012)

1.7.5 Proteus vulgaris

Proteus vulgaris is a gram-negative, rod-shaped bacterium. Dacryocystitis is a common inflammation of the lacrimal sac, usually from an underlying bacterial infection. Most cases can be attributed to common ocular flora as *Proteus vulgaris.*

1.8 Objectives

The specific objectives of this study included the following:

- To analyse photolytic degradation of chloramphenicol mediated synthetic and herbal eye drops by exposing them to sunlight.
- To reveal antimicrobial activity of degraded of chloramphenicol mediated synthetic and herbal eye drops by exposing them to sunlight with comparison among them.

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 (Biosafety 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 chloramphenicol synthetic 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.

2.2Materials:

2.2.1 Equipment's:

- 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.

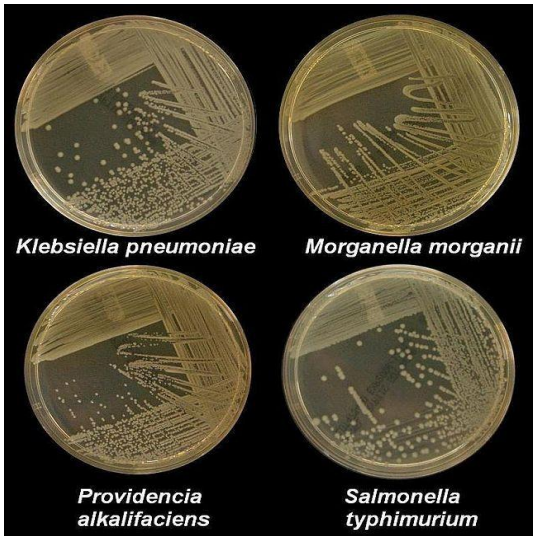


Figure 2.3: Nutrient Agar

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 are 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, sulphur 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.

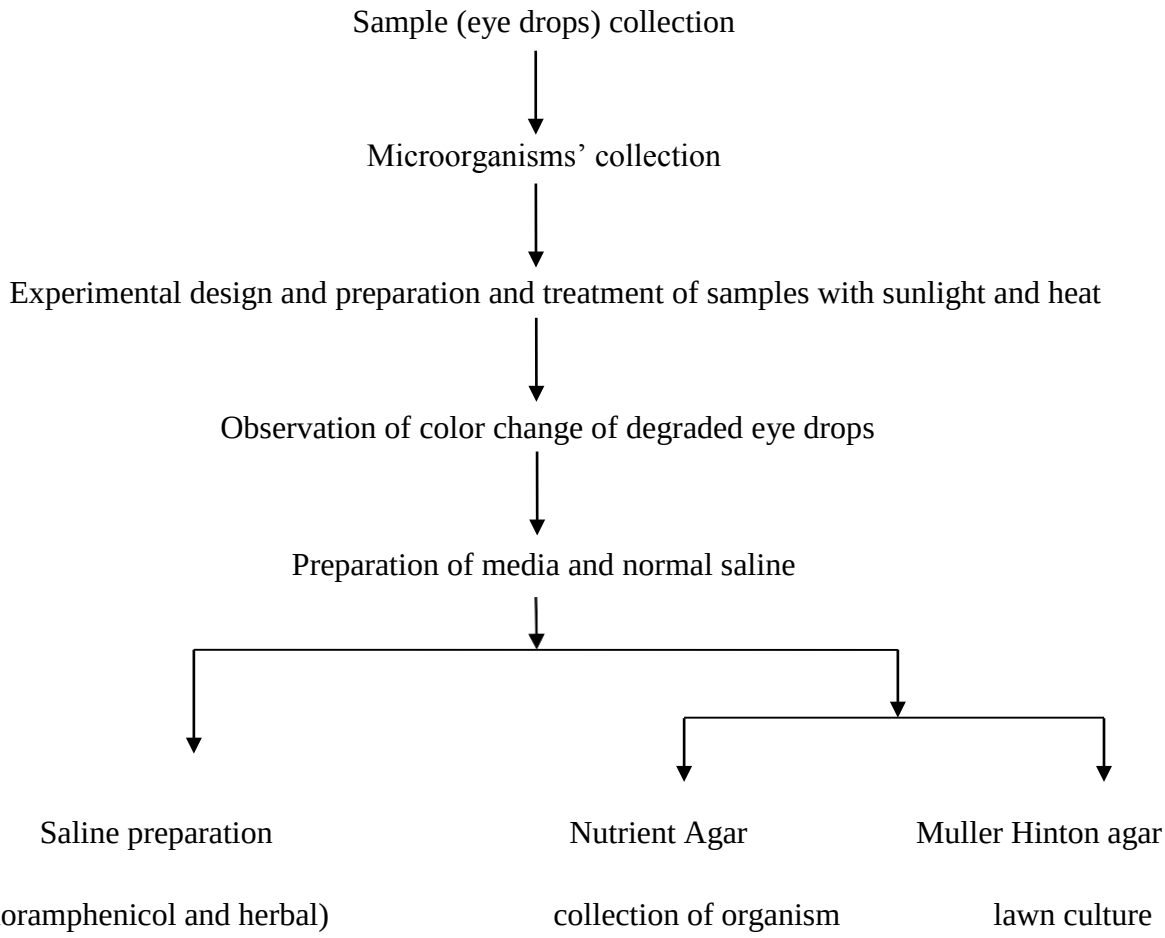
The use of a suitable medium for testing the susceptibility of microorganisms to sulfonamides and trimethoprim is essential. Antagonism to sulfonamide activity is demonstrated by para-aminobenzoic acid (PABA**)** and its analogs. Reduced activity of trimethoprim, resulting in smaller inhibition zones 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 antimicrobics.

1.4.1 Muller Hinton Agar (MHA)composition:

Table 2.4.1: 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 chloramphenicol synthetic eye drops:

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

Table 2.6.1: Chloramphenicol synthetic eye drops

Eye drop samples	Collection Area
Chloramphenicol A	Mohakhali
Chloramphenicol B	Mohakhali
Chloramphenicol C	Mohakhali
Chloramphenicol D	Niketon
Chloramphenicol E	Niketon

2.6.2 Sample collection of herbal eye drops:

Herbal Eye drops were collected from India.

Table 2.6.2: Herbal eye drops

Eye drop samples	Collection Area
Herbal eye drops	India

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

An 8 ml aliquot of the five different commercially prepared samples of USP 0.5% chloramphenicol synthetic and herbal eye drops of set 1, 2, 3 and 4 were used in this study.

- **Set-1**: Chloramphenicol 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

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

- **Set-4**: Chloramphenicol 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 negative (*Escherichia coli* (STEC), *Salmonella typhi*, *Proteus vulgaris)* and gram positive bacteria (*Staphylococcus aureus*, *Bacillus cereus*). 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 commercially 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

Table 2.6.3: Isolated microorganisms

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

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 2.6.6: Isolated 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. These were used later, when required.

2.6.8 Preparation of Muller Hinton Agar plates:

To make Muller Hinton Agar 38 gm of Muller Hinton Agar Powder was dissolved in 1000ml 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 inoculum 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 9ml 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}.

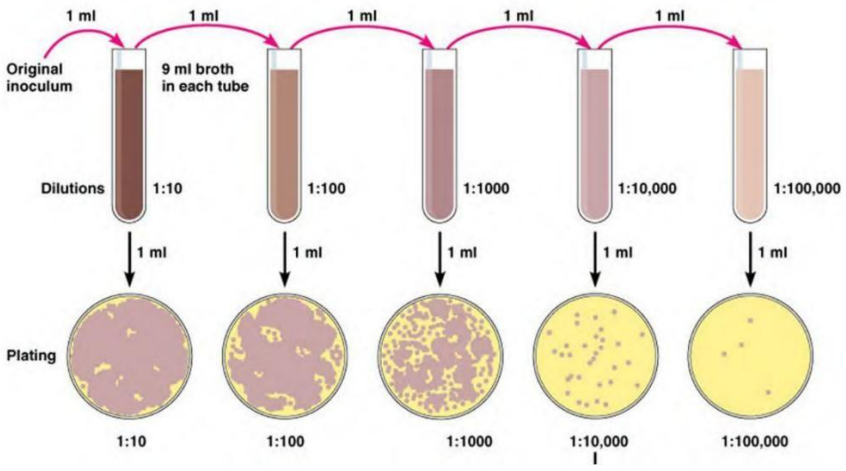


Figure 2.6.9: Serial dilution

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 MH plate and lawn culture:

Using sterile cotton swab into the inoculum 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 a bacterial inoculum is applied from 9ml 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. (Jorgensen JH, 2009).

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.

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 chloramphenicol 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.

Chapter 3: Results

3.1 Observing the change in color of sunlight treated chloramphenicol synthetic eye drops

The change in the appearance after sunlight-induced degradation of five chloramphenicol 0.5% eye drops of five different companies A, B, C, D and E were noted before and after of exposure to sunlight. Before exposure to sun the appearances of all the samples that remained in secondary packaging, primary packaging and transparent vial tubes were clear and colorless. After 10 hours exposure of sunlight all samples showed significant changed in color.

The results obtained are presented below-

Table 3.1.1: Monitoring of change in color of sunlight treated five chloramphenicol eye drops samples

Product name	Sets	Initial condition	2 hours sunlight exposure	10 hours sunlight exposure
A	Set -1 (control)	-	-	-
	Set – 2 (container + carton)	Clear, colorless	Colorless, opaque	Colorless, opaque
	Set – 3 (primary packaging)	Clear , colorless	Colorless, opaque	Slight yellowish
	Set – 4 (glass tube direct exposure)	Clear, colorless	Slight yellowish	Deep straw
B	Set -1 (control)	-	-	-
	Set – 2 (container + carton)	Clear, colorless	Colorless, opaque	Colorless, opaque
	Set – 3 (primary packaging)	Clear , colorless	Colorless, opaque	Yellowish

	Set – 4 (glass tube direct exposure)	Clear, colorless	Slight yellowish	Deep straw
Product name	**Sets**	**Initial condition**	**2 hours sunlight exposure**	**10 hours sunlight exposure**
C	Set -1 (control)	-	-	-
	Set – 2(container + carton)	Clear, colorless	Colorless, opaque	Colorless, opaque
	Set – 3 (primary packaging)	Clear , colorless	Colorless, opaque	Slight yellowish
	Set – 4 (glass tube direct exposure)	Clear, colorless	Colorless, opaque	yellowish
D	Set -1 (control)	-	-	-
	Set – 2 (container + carton)	Clear, colorless	Colorless, opaque	Colorless, opaque
	Set – 3 (primary packaging)	Clear , colorless	Colorless, opaque	Colorless, opaque
	Set – 4 (glass tube direct exposure)	Clear, colorless	Colorless, opaque	Slight yellowish
E	Set -1 (control)	-	-	-
	Set – 2 (container + carton)	Clear, colorless	Colorless, opaque	Colorless, opaque
	Set – 3 (primary packaging)	Clear , colorless	Colorless, opaque	Colorless, opaque
	Set – 4 (glass tube direct exposure)	Clear, colorless	Colorless, opaque	yellowish

Set-1(control)

Set-2

Initial condition

sunlight exposure (2 hours)

sunlight exposure (10 hours)

Set-3

Initial condition

sunlight exposure (2 hours)

sunlight exposure (10 hours)

Set-4

Initial condition

sunlight exposure (2 hours)

sunlight exposure (10 hours)

Figure 3.1: Sunlight exposure of set 2, set 3 and set 4 of brand A eye drops

Set-1(control)

Set-2

Initial condition

sunlight exposure (2 hours)

sunlight exposure (10 hours)

Set-3

Initial condition

sunlight exposure (2 hours)

sunlight exposure (10 hours)

Set-4

Initial condition

sunlight exposure (2 hours)

sunlight exposure (10 hours)

Figure 3.2: Sunlight exposure of set 2, set 3 and set 4 of brand B eye drops

Set-1(control)

Set-2

Initial condition

sunlight exposure (2 hours)

sunlight exposure (10 hours)

(Transformed to vial)

Set-3

Initial condition

sunlight exposure (2 hours)

sunlight exposure (10 hours)

(Transformed to vial)

Set-4

Initial condition

sunlight exposure (2 hours)

sunlight exposure (10 hours)

Figure 3.3: Sunlight exposure of set 2, set 3 and set 4 of brand C eye drops

Set-1(control)

Set-2

Initial condition

sunlight exposure (2 hours)

sunlight exposure (10 hours)

(Transformed to vial)

Set-3

Initial condition

sunlight exposure (2 hours)

sunlight exposure (10 hours)

(Transformed to vial)

Set-4

Initial condition

sunlight exposure (2 hours)

sunlight exposure (10 hours)

Figure 3.4: Sunlight exposure of set 2, set 3 and set 4 of brand D eye drops

Set-1(control)

Set-2

Initial condition

sunlight exposure (2 hours)

sunlight exposure (10 hours)

(Transformed to vial)

Set-3

Initial condition

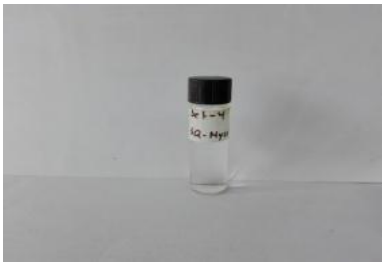
sunlight exposure (2 hours)

sunlight exposure (10 hours)

(Transformed to vial)

Set-4

Initial condition



sunlight exposure (2 hours)

sunlight exposure (10 hours)

Figure 3.5: Sunlight exposure of set 2, set 3 and set 4 of brand E eye drops

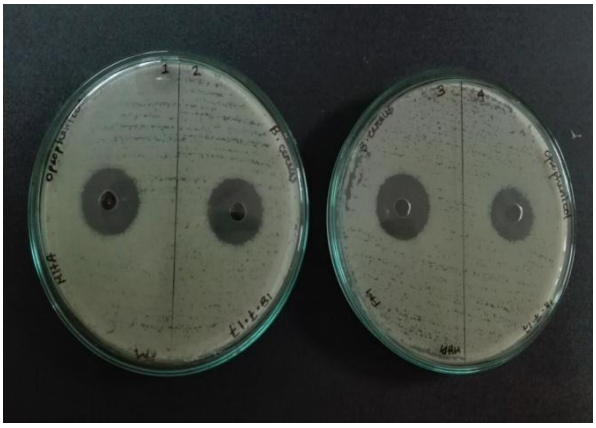
3.2 Antibacterial activity of synthetic eye drops after treatment with sunlight

Antibacterial activity of five different companies of chloramphenicol eye drops such as A,B,C,D and E were tested against five microorganisms such *Escherichia coli (ETEC), Proteus vulgaris, Staphylococcus aureus, Salmonella typhi and Bacillus cereus.* The results obtained are presented below.

Table 3.2.1: Antimicrobial effects (Inhibition zones) produced by chloramphenicol synthetic eye drops (2 µl) Against *Bacillus Cereus*

Product Name	sets	Average diameter of zone of inhibition(mm)
A	1	26.33
	2	24
	3	23
	4	21.33
B	1	22.66
	2	21
	3	20.67
	4	19.33
C	1	22.33
	2	21.33
	3	21
	4	19
D	1	25.33
	2	23.66
	3	23.33
	4	22.33
E	1	27.66
	2	25
	3	23.33
	4	24.66

A



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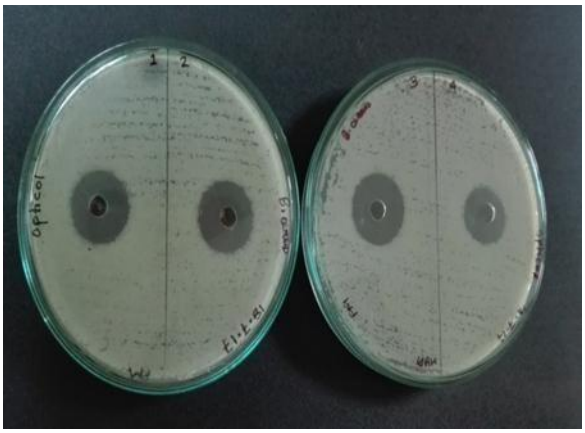

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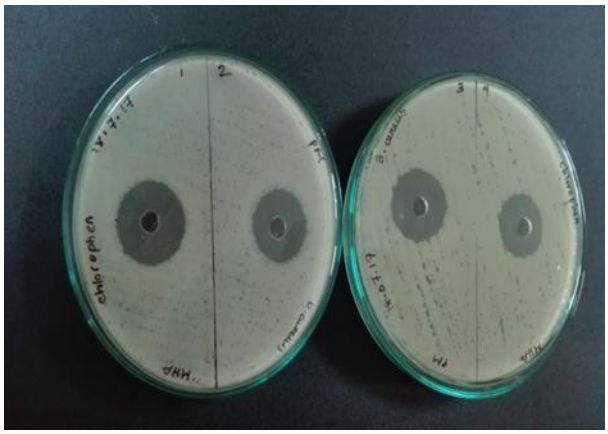
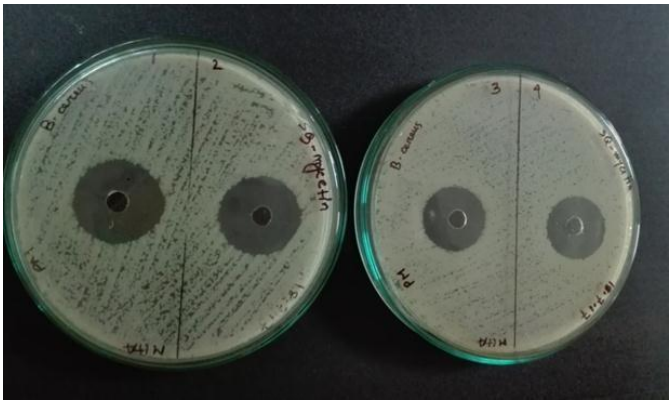


D

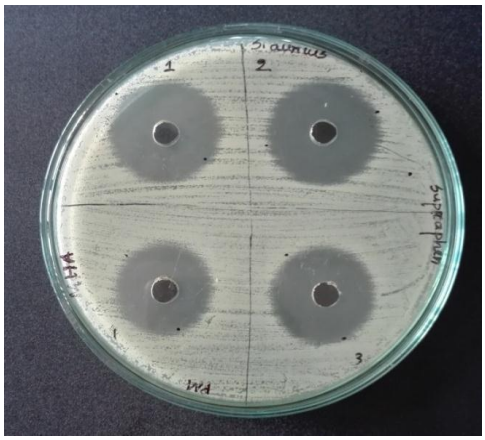


E

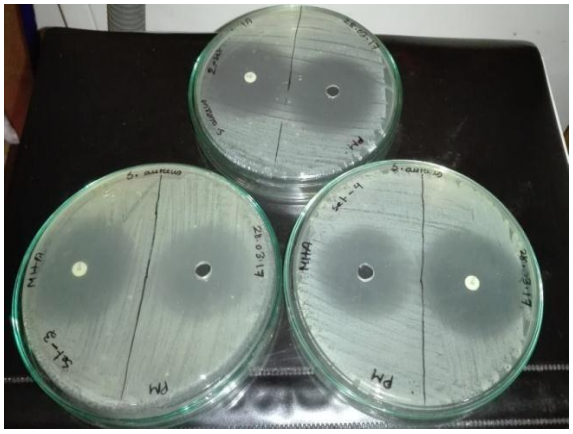
Figure 3.6: Antimicrobial effects of brand A and B eye drops against *Bacillus cereus*

Table3.2.2: Antimicrobial effects (Inhibition zones) produced by chloramphenicol synthetic eye drops (2µl) Against *Staphylococcus aureus*

Product Name	sets	Average diameter of zone of inhibition(mm)
A	1	31.6
	2	28.6
	3	26
	4	23
B	1	35
	2	34.6
	3	28.6
	4	22
C	1	27
	2	25.3
	3	24.6
	4	20.3
D	1	25.6
	2	24.3
	3	21
	4	23.33
E	1	28
	2	27.3
	3	25
	4	22.3



A



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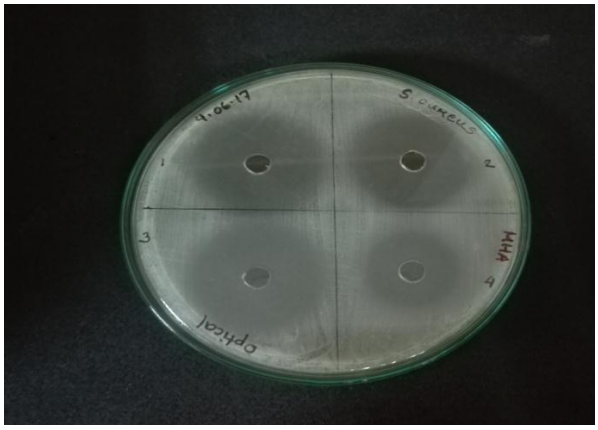
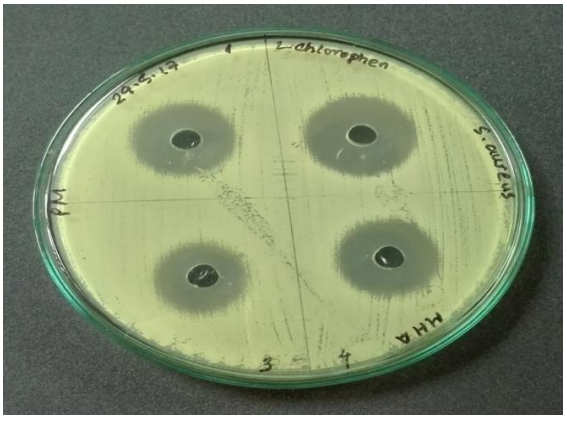
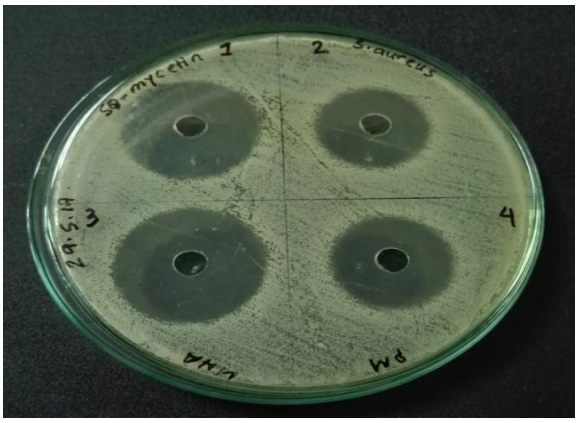


C



D

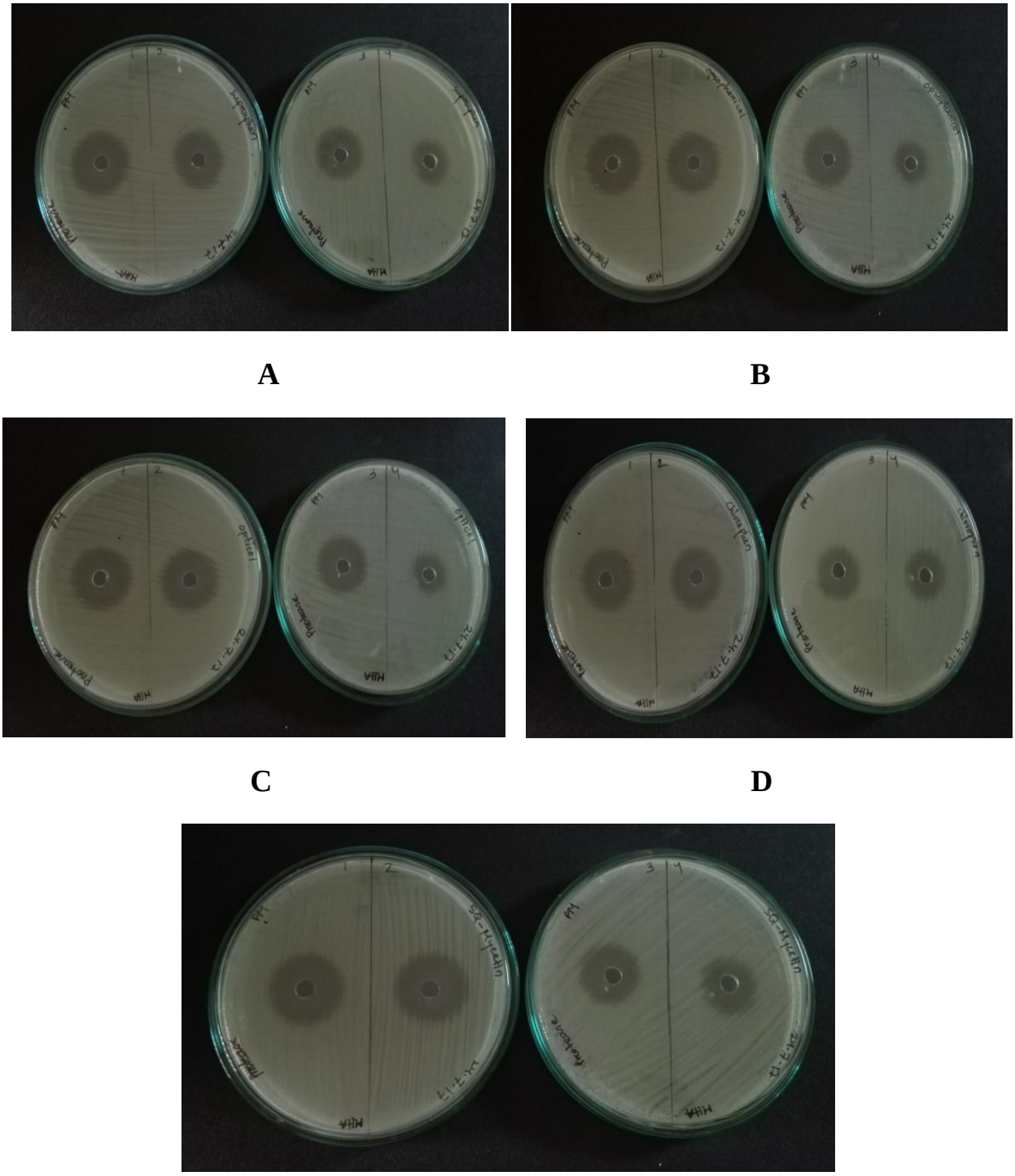


E

Figure 3.8: Antimicrobial effects of brand A,B,C,D and E against *Staphylococcus aureus*

Table 3.2.3: Antimicrobial effects (Inhibition zones) produced by chloramphenicol synthetic eye drops (2µl) Against *Proteus vulgaris*

Product Name	sets	Average diameter of zone of inhibition(mm)
A	1	22.67
	2	19
	3	18.33
	4	15
B	1	21.33
	2	20.67
	3	20.33
	4	19.33
C	1	21.33
	2	21
	3	20.67
	4	16
D	1	20.33
	2	19.33
	3	18.67
	4	16
E	1	21.33
	2	20.33
	3	19.33
	4	16.67



A **B**

C **D**

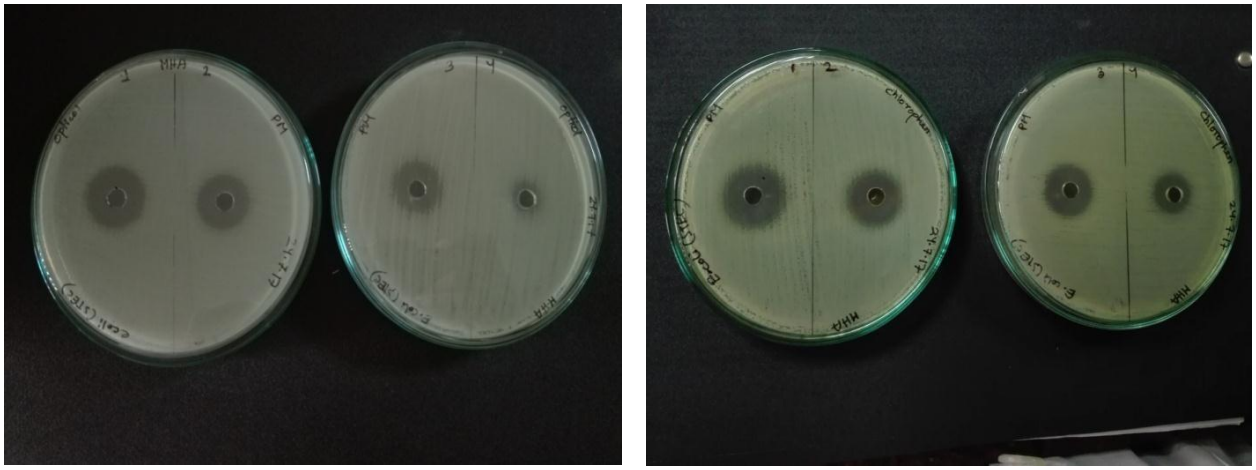
E

Figure 3.9: Antimicrobial effects of brand A,B,C,D and E eye drops against *Proteus vulgaris*

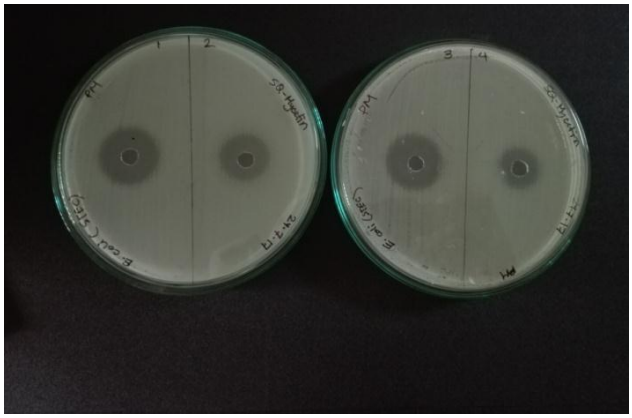
Table 3.2.4: Antimicrobial effects (Inhibition zones) produced by chloramphenicol synthetic eye drops (2µl) Against *Escherichia coli*

Product Name	sets	Average diameter of zone of inhibition(mm)
A	1	20.67
	2	16.67
	3	16
	4	12.67
B	1	18
	2	17.33
	3	11.33
	4	10.33
C	1	20.67
	2	17.67
	3	16
	4	10.67
D	1	21
	2	20
	3	19.33
	4	15.67
E	1	20
	2	16.67
	3	16.33
	4	12

A **B**



C **D**

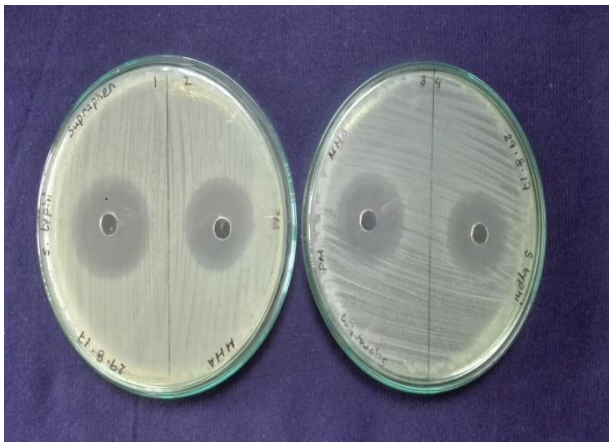


E

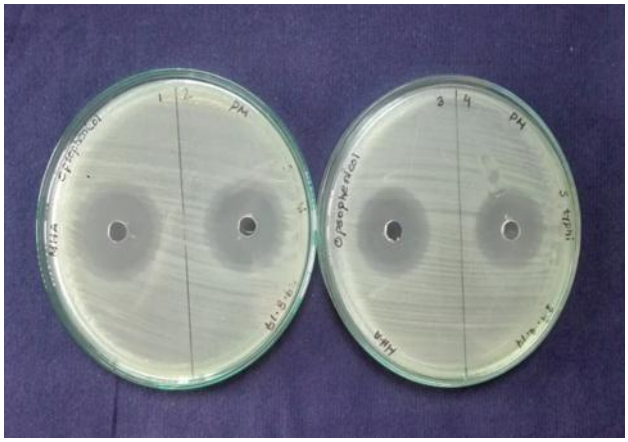
Figure 3.10: Antimicrobial effects of brand A,B,C,D and E eye drops against *Escherichia coli*

Table 3.2.5: Antimicrobial effects (Inhibition zones) produced by chloramphenicol synthetic eye drops (2µl) Against *Salmonella typhi*

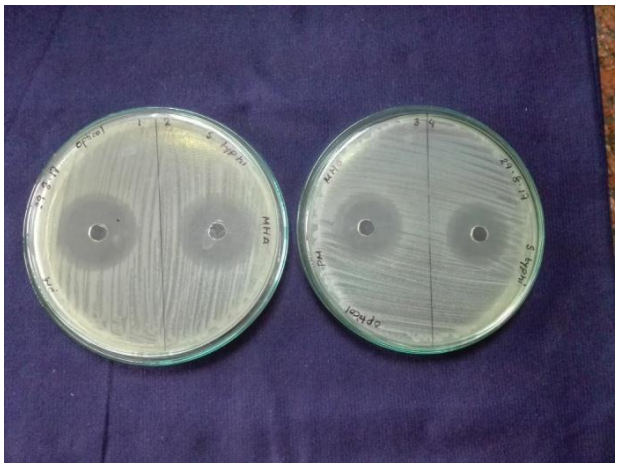
Product Name	sets	Average diameter of zone of inhibition(mm)
A	1	25.33
	2	23.67
	3	22.33
	4	18
B	1	26
	2	25.33
	3	22
	4	18.67
C	1	24.33
	2	23.33
	3	19.33
	4	17
D	1	23
	2	22.67
	3	20.33
	4	21.66
E	1	25.33
	2	24.66
	3	24
	4	20.33



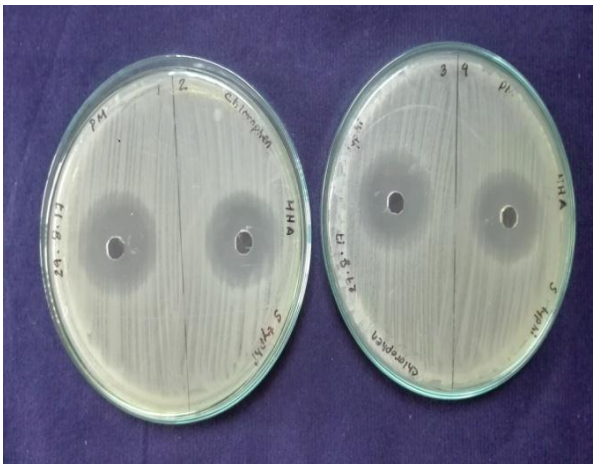
A



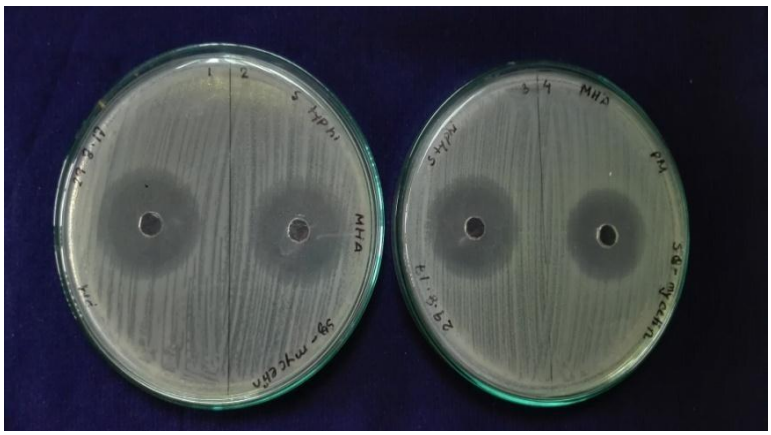
B



C



D



E

Figure 3.11: Antimicrobial effects of brand A,B,C,D and E eye drops against *Salmonella typhi*

3.3 Changes of antibacterial potency of chloramphenicol synthetic eye drops after sunlight exposure against gram positive and gram negative bacteria

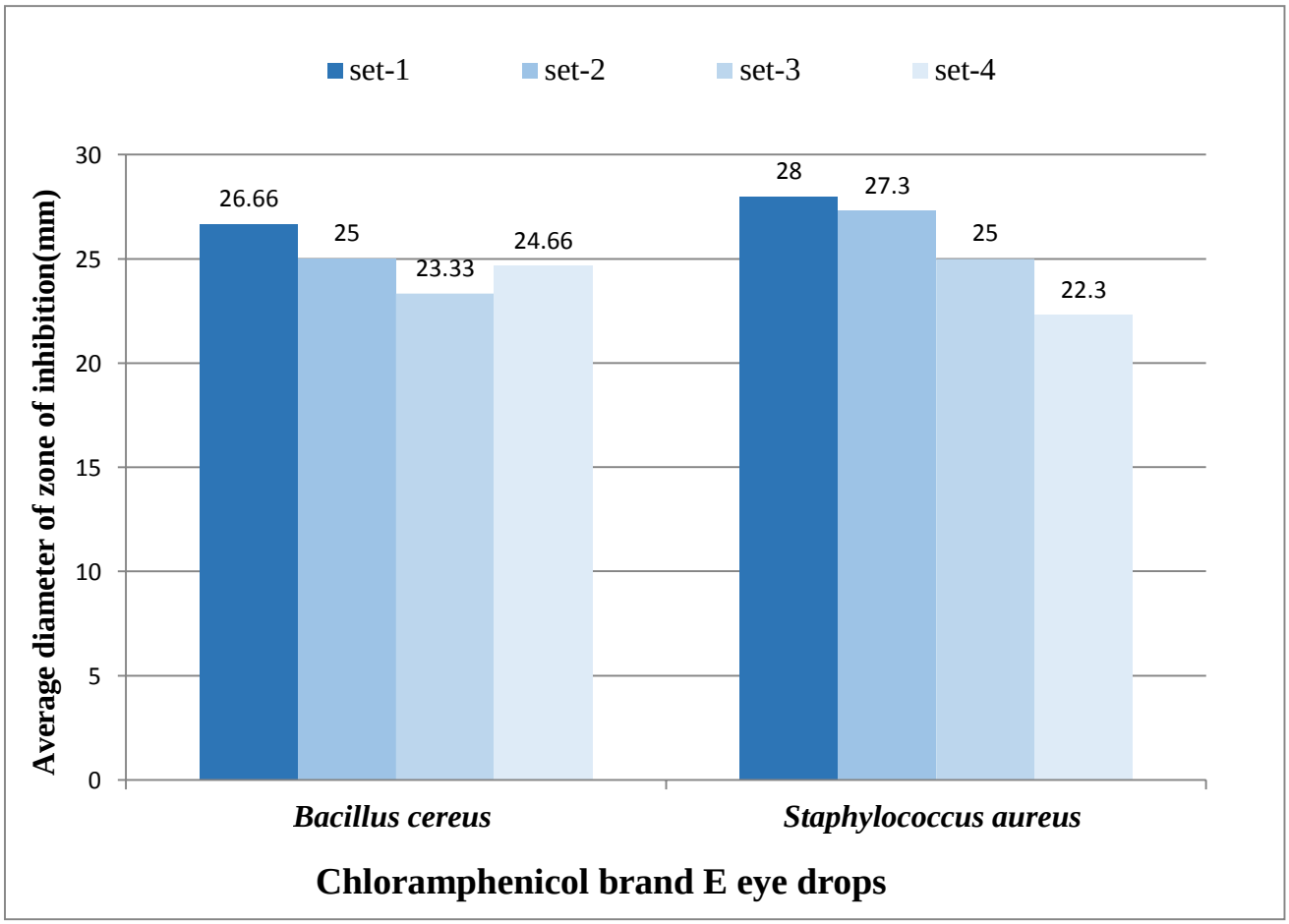


Figure 3.12: Changes of antibacterial potency of chloramphenicol synthetic eye drops after sunlight exposure

The graph above illustrate that chemical components degradation occurred in brand E eye drops against *Bacillus cereus*. After exposure to sunlight, brand E eye drops showed degradation by observing their zone of inhibition which was gradually decreased but samples remained in transparent vial tube having more antibacterial activity which was 24.66 mm diameter of zone of inhibition than the samples of primary packaging and antibacterial activity was 23.33 mm of zone of inhibition against *Bacillus cereus*

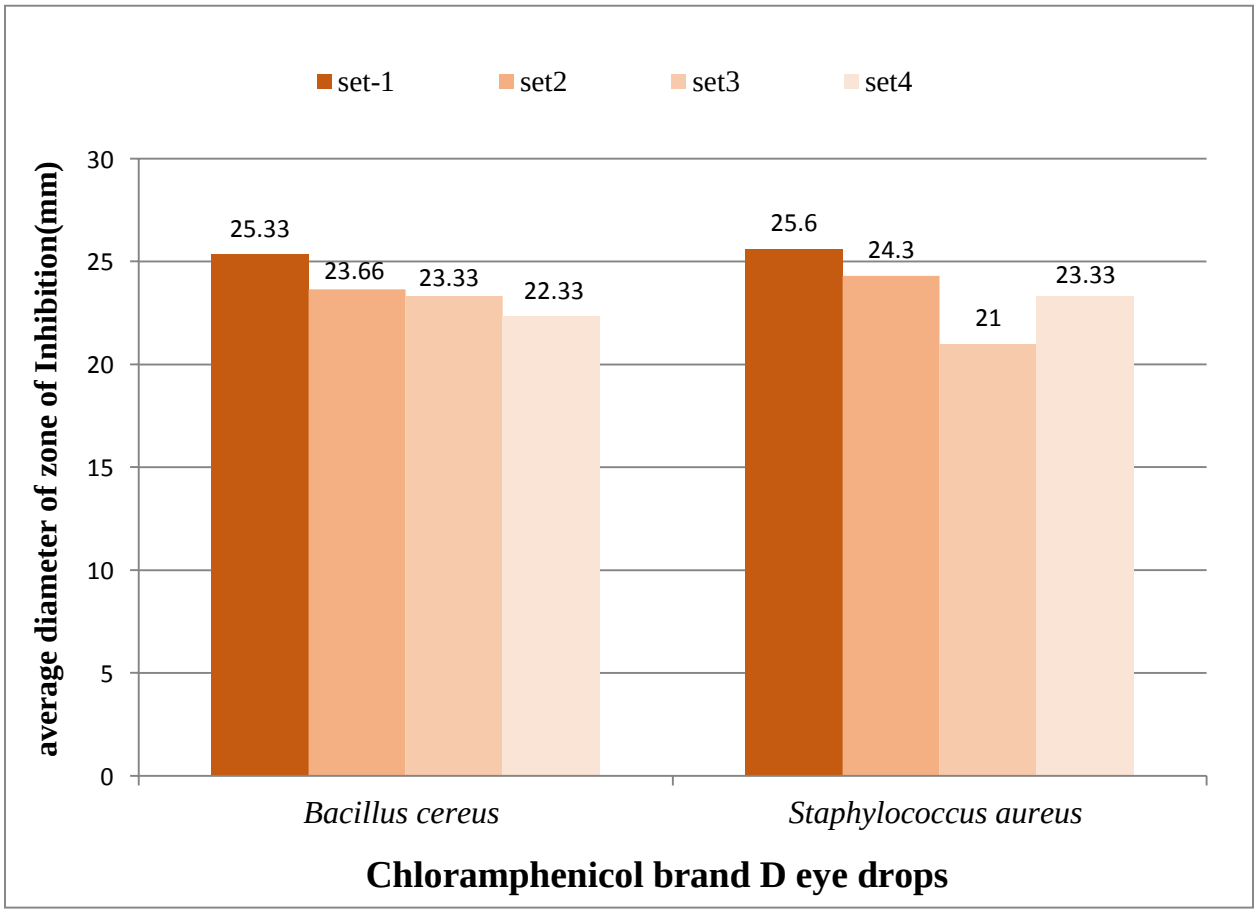


Figure 3.13: Changes of antibacterial potency of chloramphenicol synthetic eye drops after sunlight exposure

The graph above illustrate that chemical components degradation occurred in brand D eye drops against *Staphylococcus aureus.* After exposure to sunlight, brand D eye drops showed degradation by observing their zone of inhibition which was gradually decreased but samples remained in transparent vial tube having more antibacterial activity which was 23.33 mm diameter of zone of inhibition than the samples of primary packaging and antibacterial activity was 21mm against *Staphylococcus aureus.*

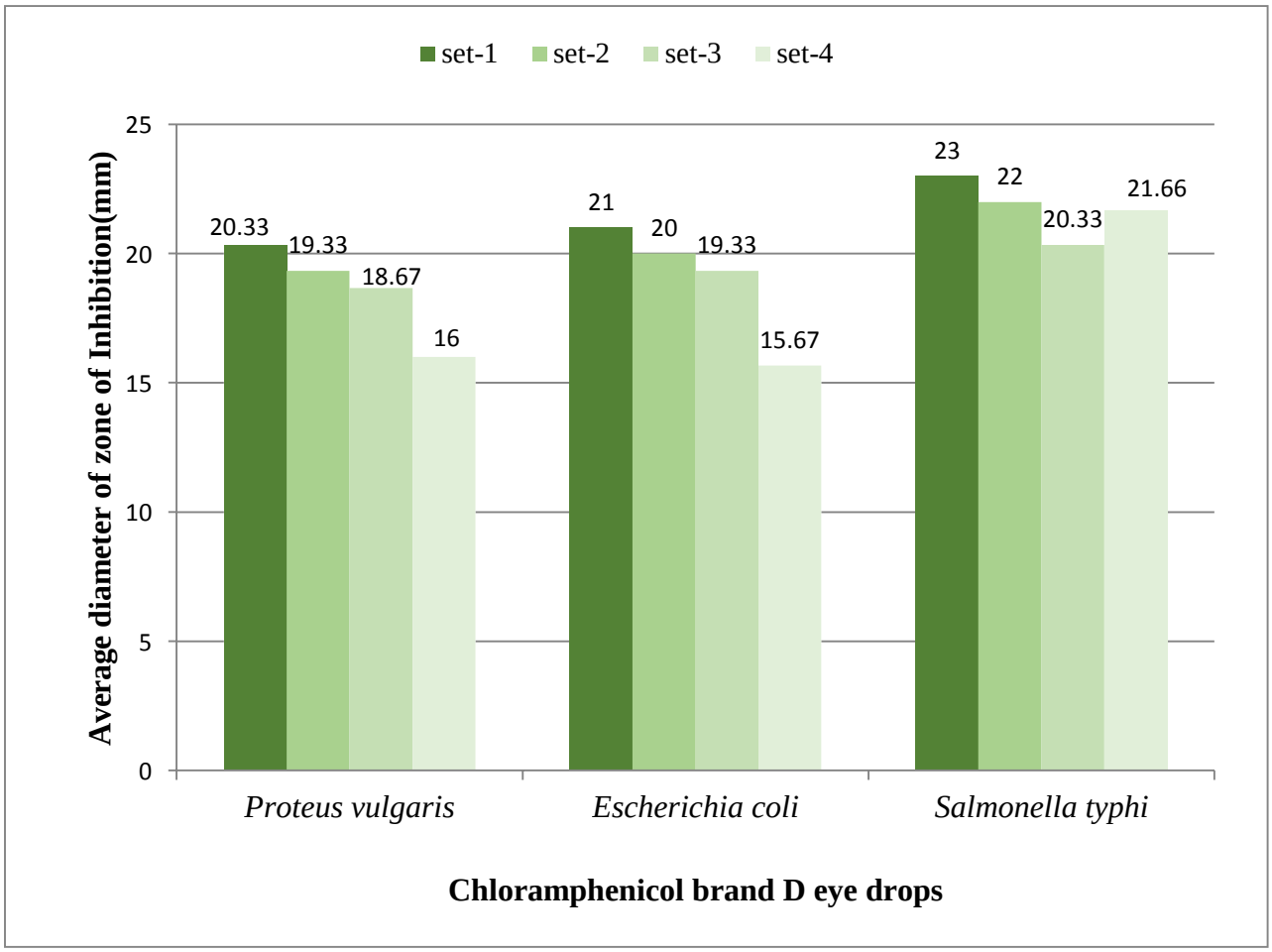


Figure 3.14: Changes of antibacterial potency of chloramphenicol synthetic eye drops after sunlight exposure

The graph illustrate that chemical components degradation occurred in brand D eye drops against *Salmonella typhi.* After exposure to sunlight, brand D eye drops showed degradation by observing their zone of inhibition which was gradually decreased but samples remained in transparent vial tube having more antibacterial activity which was 21.66 mm diameter of zone of inhibition than the samples of primary packaging and antibacterial activity was 20.33mm against *Salmonella typhi.*

3.4 Analyzing antimicrobial activity against gram positive and gram negative bacteria

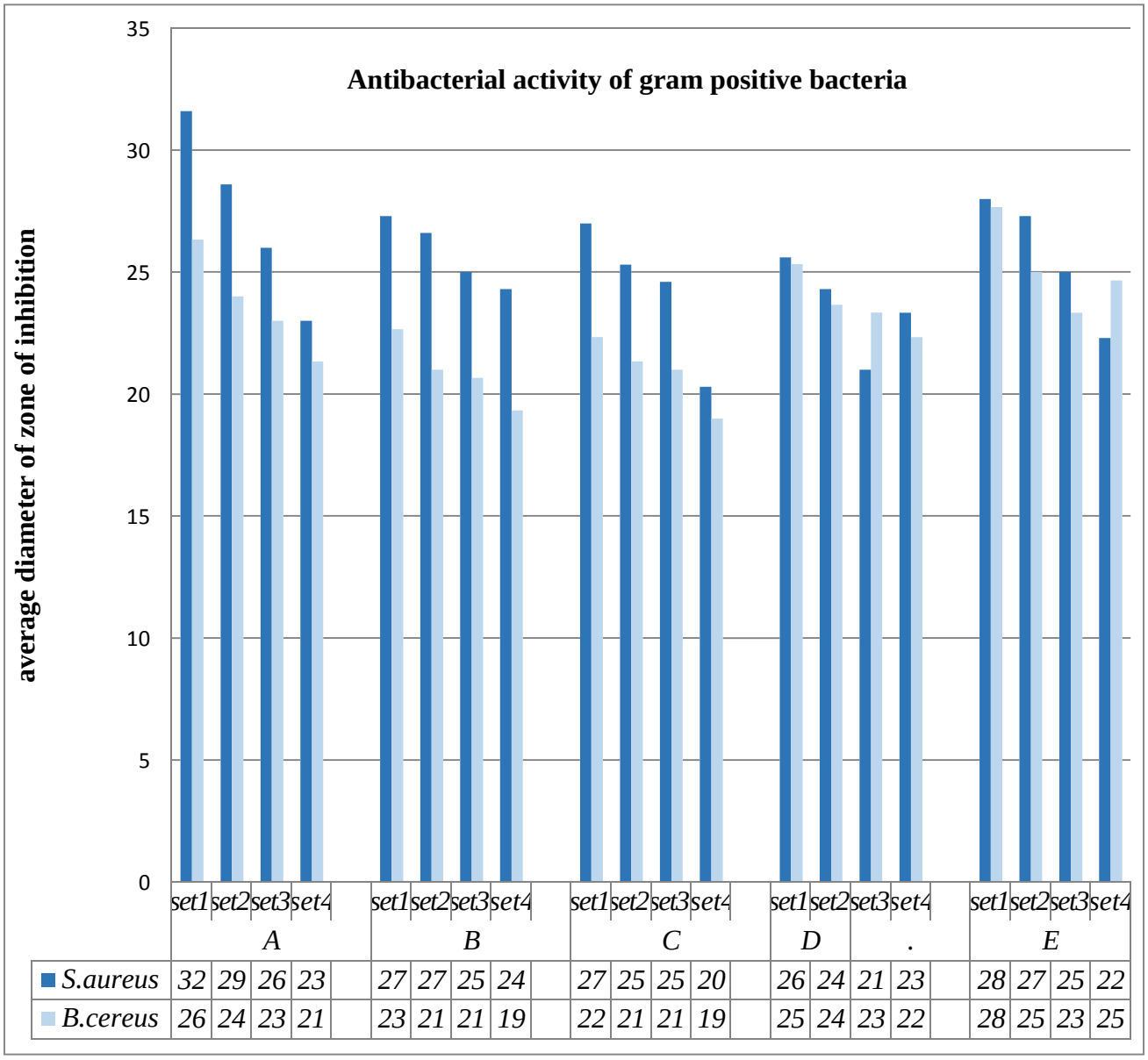


Figure 3.15: Antibacterial activity of Brand A, B, C, D and E eye drops against *Bacillus cereus* and *Staphylococcus aureus*

The graph above illustrates the antibacterial activity of gram positive bacteria where *Staphylococcus aureus* showed the highest zone of inhibition than the *Bacillus cereus*.

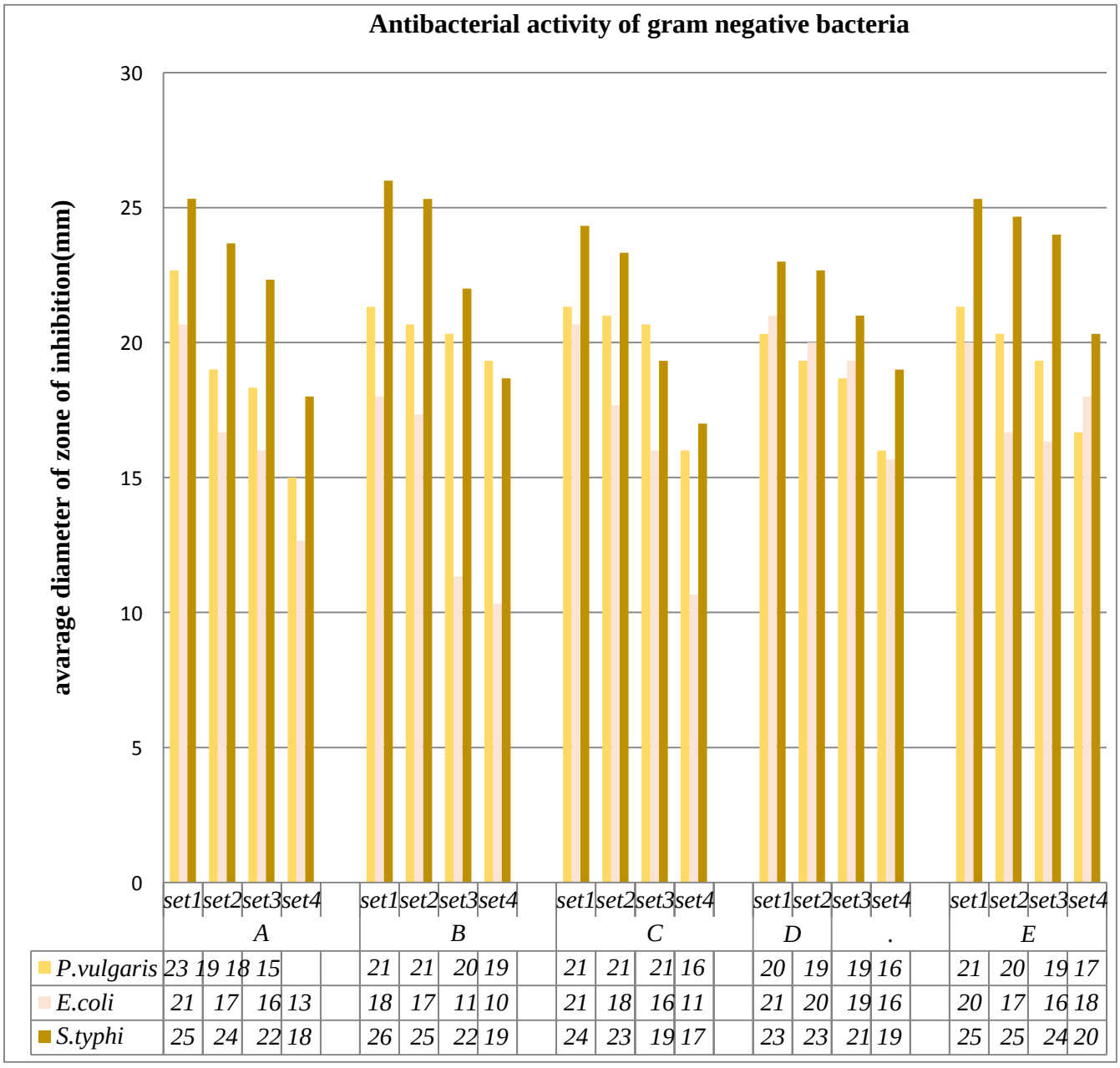


Figure 3.16: Antibacterial activity of brand A, B, C, D and E eye drops against *Proteus vulgaris, Escherichia coli and Salmonella typhi*

The graph above illustrate the antibacterial activity of gram negative bacteria where *Salmonella typhi* showed the highest zone of inhibition than *Proteus vulgaris* and *Escherichia coli*

3.5 Comparison study of chloramphenicol synthetic eye drops against gram positive and gram negative bacteria

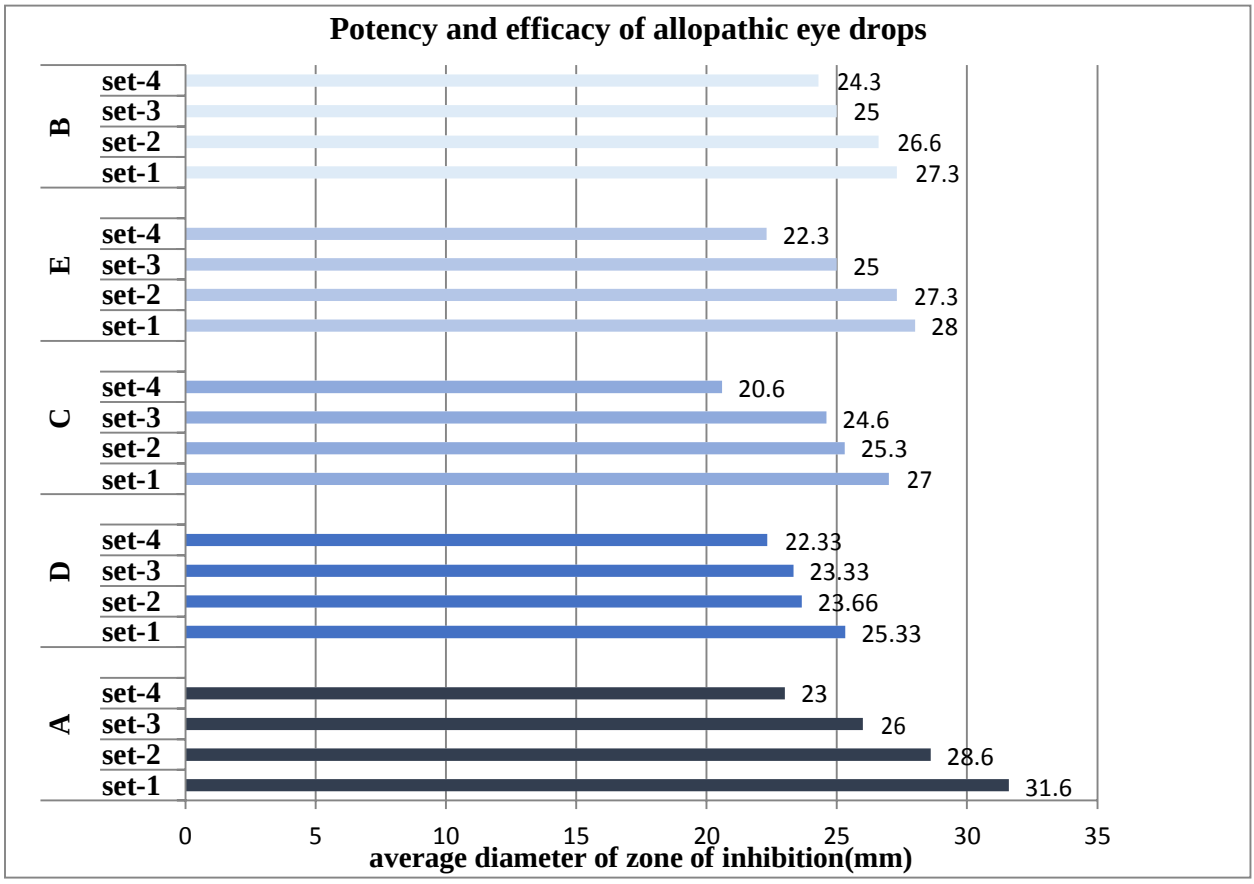


Figure 3.17: Potency and efficacy of chloramphenicol synthetic eye drops against gram positive and gram negative bacteria

The graph above illustrate that chloramphenicol brand A eye drops showed the highest potency against all gram positive and gram negative bacteria

3.6 Observing the Change in color of sunlight treated herbal eye drops eye drops after exposure to sunlight

The change in the appearance after sunlight-induced degradation of one herbal aqueous solution of the Itone eye drops preparation was noted before and after of exposure to sunlight. Before exposure to sun the appearances of all the samples that remained in secondary packaging, primary packaging and transparent vial tubes were clear and colorless. After 10 hours exposure of sunlight all samples remain same in color. Significant color change was not observed.

The results obtained are presented below-

Table 3.6.1: Monitoring of change in color of sunlight treated herbal Eye drops samples

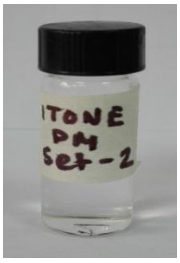
Product name	Sets	Initial condition	2 hours sunlight exposure	10 hours sunlight exposure
Itone	Set -1 (control)	-	-	-
	Set – 2 (container +two layer of carton)	Clear, colorless	Clear, colorless	Clear, colorless
	Set – 3 (primary packaging)	Clear , colorless	Clear , colorless	Clear , colorless
	Set – 4 (glass tube direct exposure)	Clear, colorless	Clear, colorless	Clear, colorless

Set-1(control)

Set-2

Initial condition

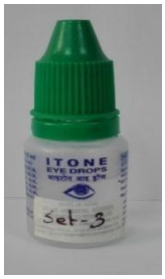
sunlight exposure (2 hours)



sunlight exposure (10 hours)

Set-3

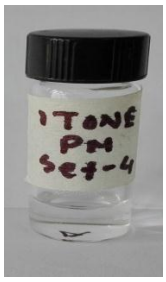
Initial condition



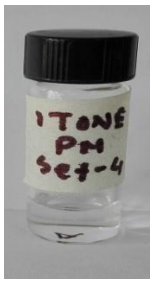
sunlight exposure (2 hours)

sunlight exposure (10 hours)

Set-4



Initial condition



sunlight exposure (2 hours)

sunlight exposure (10 hours)

Figure 3.18: Sunlight exposure of set 2, set 3 and set 4 of herbal eye drops

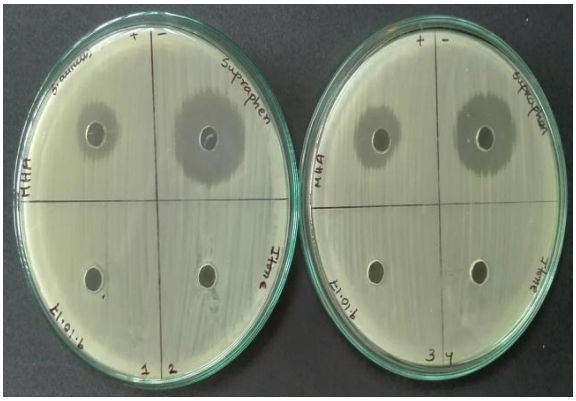
Figure 3.19: Observation of all the samples that are transferred to vial after exposure to sunlight

3.7 Antibacterial activity of herbal eye drops after treatment with sunlight

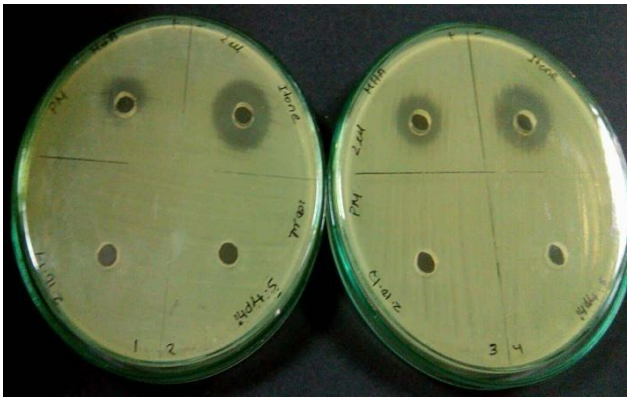
Antibacterial activity of Itone herbal eye drops were tested against five microorganisms such *Escherichia coli (ETEC), Proteus vulgaris, Staphylococcus aureus, Salmonella typhi and Bacillus cereus.* The results obtained are presented on the next page.

Table 3.7.1: Antimicrobial effects (Inhibition zones) produced by herbal eye drops against five microorganisms

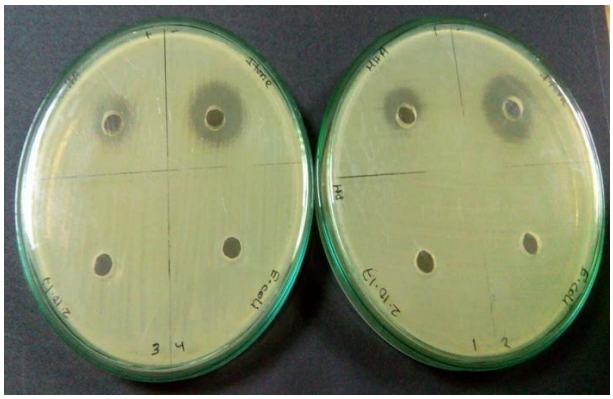
Antibacterial activity of eye drops	Itone herbal eye drops			
	Microorganisms	**sets**	**Concentration(µl)**	**Average diameter of zone of Inhibition(mm)**
	Bacillus cereus	1	100	18
		2	100	16.33
		3	100	15.67
		4	100	10.33
	Staphylococcus aureus	1	100	No ZOI
		2	100	No ZOI
		3	100	No ZOI
		4	100	No ZOI
	Escherichia coli	1	100	No ZOI
		2	100	No ZOI
		3	100	No ZOI
		4	100	No ZOI
	Proteus vulgaris	1	100	No ZOI
		2	100	No ZOI
		3	100	No ZOI
		4	100	No ZOI
	Salmonella typhi	1	100	No ZOI
		2	100	No ZOI
		3	100	No ZOI
		4	100	No ZOI



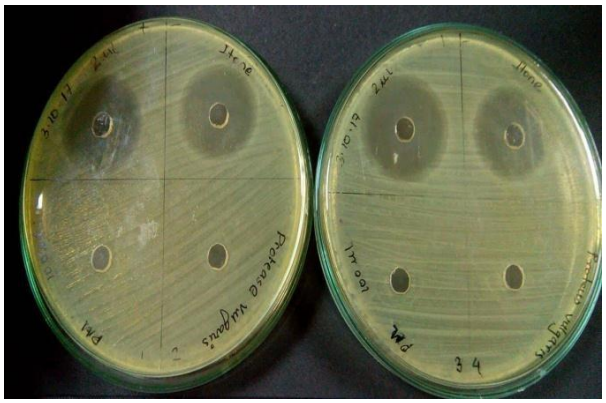
(a)



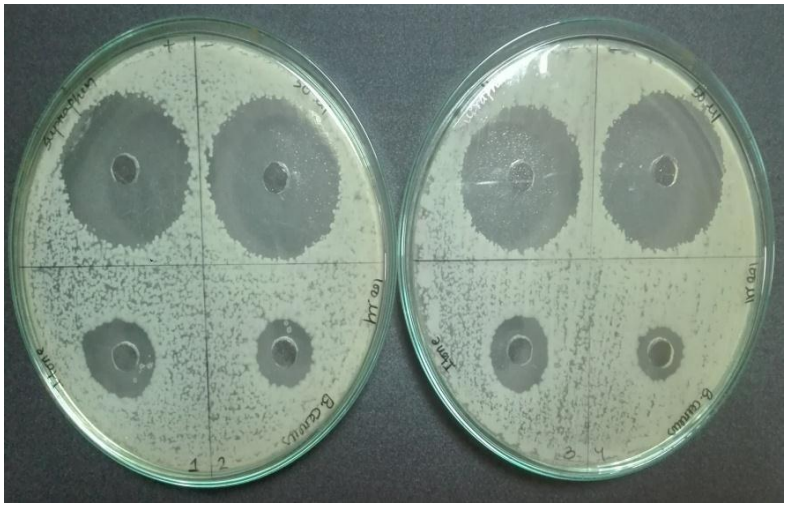
(b)



(c)



(d)



(e)

Figure 3.20: Antibacterial effects of Itone herbal eye drops against *Staphylococcus aureus,Salmonella typhi,Escherichia coli,Proteus vulgaris and Bacillus cereus*

3.8 Antibacterial activity of Itone herbal eye drops

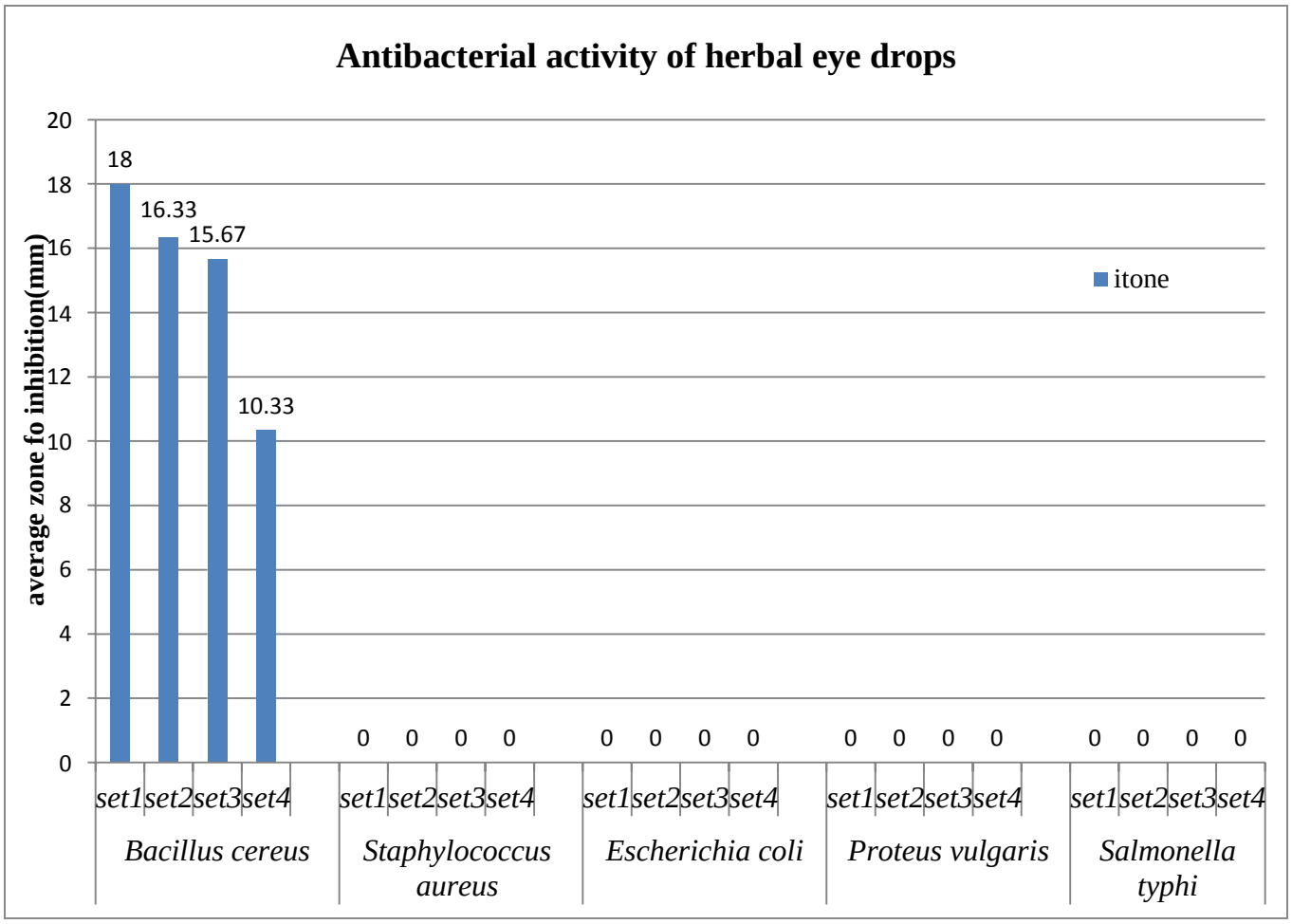


Figure 3.21: Antibacterial activity of Itone herbal eye drops against gram positive and gram negative bacteria

The graph illustrates that only *Bacillus cereus* showed the zone of inhibition

3.9 Antibacterial activity of chloramphenicol synthetic and herbal eye drops against gram positive bacteria

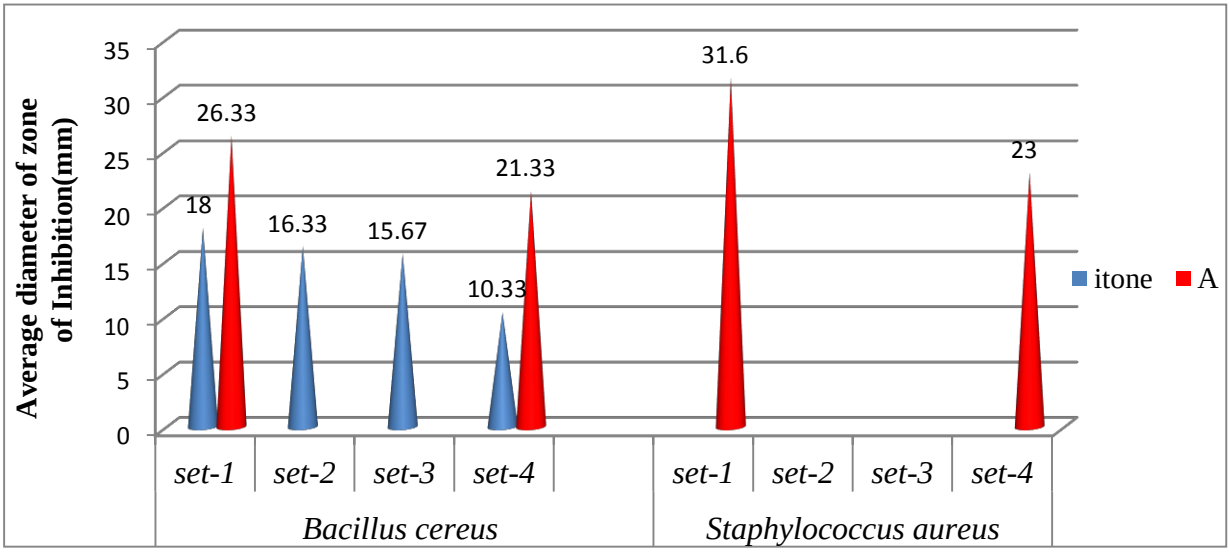


Figure 3.22: Antibacterial activity of chloramphenicol synthetic and herbal eye drops against gram positive bacteria

The graph demonstrates that chloramphenicol brand A eye drops showed better antibacterial Activity than Itone herbal eye drops

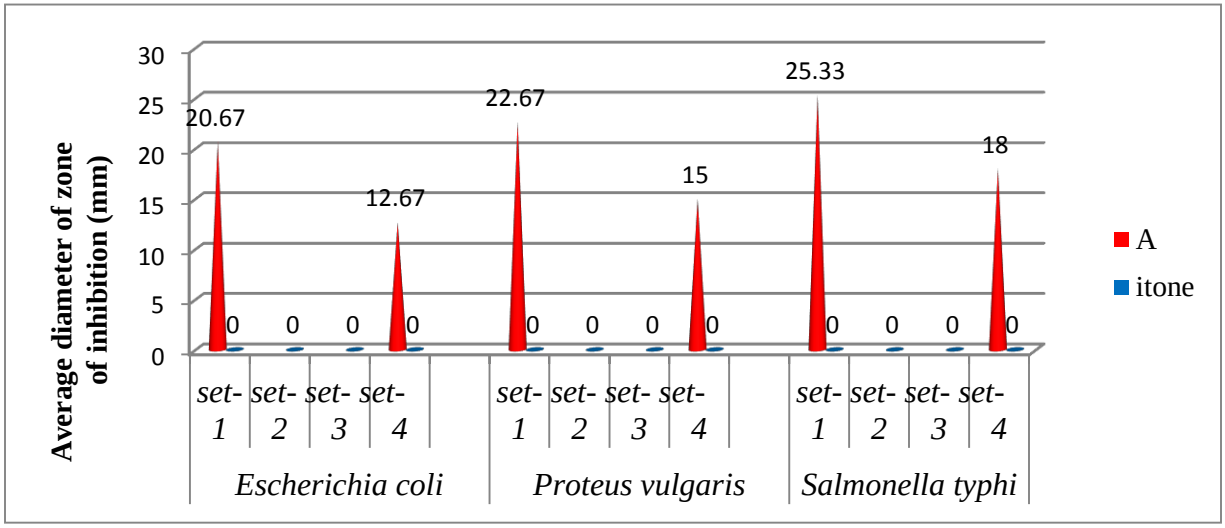


Figure 3.23: Antibacterial activity of chloramphenicol synthetic and herbal eye drops against gram negative bacteria

The graph demonstrates that chloramphenicol brand A eye drops showed best antibacterial activity and the Itone herbal eye drops showed no antibacterial activity

3.10 Bactericidal activity of chloramphenicol synthetic and herbal eye drops against gram positive and gram negative bacteria

Table 3.10.1 Bactericidal activity of chloramphenicol synthetic and herbal eye drops against gram positive and gram negative bacteria

	Microorganisms	**Bactericidal activity of eye drops**				
		A	B	C	D	E
Chloramphenicol eye drops	*Bacillus cereus*	Highly sensitive	sensitive	sensitive	sensitive	Highly sensitive
	Staphylococcus aureus	Highly sensitive	Highly sensitive	sensitive	sensitive	Highly sensitive
	Escherichia coli	sensitive	sensitive	sensitive	sensitive	sensitive
	Proteus vulgaris	sensitive	sensitive	sensitive	sensitive	sensitive
	Salmonella typhi	Highly sensitive	Highly sensitive	sensitive	sensitive	sensitive
Herbal eye drops		**Bactericidal activity of eye drops**				
		Itone	-	-	-	-
	Bacillus cereus	sensitive	-	-	-	-

Chapter 4: Discussion

The photochemical degradation was observed in chloramphenicol synthetic eye drops due to the sunlight exposure. There was a significant change in appearance of the eye drops solutions that were exposed to the sun. Before exposure to sun the appearances of samples those remained in both primary and secondary packaging (container and carton) and some samples that were in only primary packaging (container only), others were in the clear transparent vial tubes were clear and colorless. After that, 2 hours exposure of sunlight, all samples mostly those were in primary packaging (container only) and transparent vial tubes started changing their color from their original color. Afterwards, 10 hours exposure of sunlight the color of samples those remained in both primary and secondary packaging (container and carton) turned into opaque. In the case of primary packaging (container only), where samples remained in the transparent container turned into bright yellow and those were in the white container turned into very slight yellowish in color. In the case of transparent vial tubes, the color of all the sample solutions turned into light yellow to deep yellow. Lastly, those samples stored in the refrigerator considered as Controls (Set-1) stayed in clear and colorless solutions. As compared with controls (Set-1), samples remained in secondary packing (container and carton), primary packaging (container only) and transparent vial tubes showed a remarkable change in color.

The results indicate that packing is very important factor for keeping the stability of chloramphenicol 0.5% eye drops. The color of the samples that remained in container and carton did not change significantly from its original color because it was protected from the sunlight by its secondary packaging. On the other side, the container alone is not enough efficient to protect the sunlight which is noticed by the production of colored degradation products in the most of samples that remained in primary packaging and samples remained in the transparent tube shows the most prominent color change by turning light yellow to deep yellow (Ahmed *et al.*,2013)

Thermal degradation of chloramphenicol occurs at a faster rate if it is stored in unrefrigerated conditions and therefore inadequate refrigeration and prolonged shelf lives of chloramphenicol generics are conducted with high levels of chloramphenicol thermal breakdown product (Aboshiha *et al.,* 2014).The degradation of chloramphenicol is the mixture of chemical pathways and mechanism initiated by light. The rate of decomposition of chloramphenicol may therefore be dependent on the source of light and light intensity. The colorless chloramphenicol aqueous solution changed to deep yellow color solution, with precipitation on exposure to sunlight and UV

light in the presence of air (Okeniyi *et al.,* 2006). Pharmaceutical drugs or products that are prone to degradation on exposure to UV-light. There are many active pharmaceuticals ingredients (drug substances) that are very much sensitive to heat or topical temperature and degradation frequently leads to loss of potency in their product especially in liquid formulations and UV-light is one of the means that degrades the formulation because of having the photolytic reaction. Photolytic degradation occurs when the drug substance or drug product expose to a combination of visible and UV light. During manufacturing process or packing or on storage drugs are liable to photo oxidation. Most of the compounds will degrade as solutions when exposed to high-energy UV exposure. Hydrolysis is another process where it is reacting with water that causes Drug degradation. Water in the air that could be as solvent or as moisture comes in contact with pharmaceutical dosage is the biggest responsible for the degradation of most drugs (Shinde *et al.,* 2013). Mercuric phenyl borate (or acetate) is often used as a preservative (Martindale 28th edn; British Pharm. Codex, etc.) in the formulation of the chloramphenicol eye drops. As this compound may affect the photochemical reaction, its influence was investigated but proved to be small (Vries *et al.,* 1984).

In this study, 2-amino-1-(4-nitrophenyl) propane-1, 3-diol (a hydrolytic degradation product of chloramphenicol) were used as a measure of the degradation of the chloramphenicol content in the eye-drops by changing their color form colorless to deep yellow and phenylmercuric nitrate (0.02mg/ml) and phenylmercuric acetate (0.02 mg/ml) is used in the formulation of almost all of the commercial eye drops of chloramphenicol that may affect the photochemical reactions.

On the other hand, the photochemical degradation was not observed in Itone eye drops after sunlight exposure. There was not significant change in appearance of the eye drops solutions that were exposed to the sun. All of the samples exposed no significance change in color. As the Itone herbal eye drops contains aqueous distillates of nineteen traditionally used ingredients including *Azadirachta indicam* (nimba-5%), *Moringa pterygosperma* (sobhanjana-5%), *Eclipta alba* (bhringaraj-5%), *Boerhaavia diffusa* (punarnava-7.5%), *Carum copticum* (yamani-2%), *Terminalia chebula* (haritaki-5%), *Terminalia belerica* (vibhitaka-5%),*Emblica officinalis* (dhatriphala-5%), *Santalum album* (sweet chandan-5%), mukta (pearl-1%), *Ocimum sanctum* (tulsi patra-5%), *Vitex negundo* (nirgundi-5%), *Curcuma longa* (haridra-5%), *Mentha piperata* (menthol-2%), *Cinnamomum camphora* (camphor-3%), *Amomum subulatum* (ela-5%),*Rosa*

centifolia (satapatri-7.5%), saindhava laban (rock salt-2%) and madhu (honey-10%) and all these ingredients were natural constituents that may not involve in any photochemical reactions and therefore, they did not change in color.

Chloramphenicol, widely used antibiotic can be effectively degraded by the UV radiation with hydrogen peroxide treatment that decreasing the antibacterial activity of chloramphenicol solutions (Zuorro *et al.*, 2013)

In the present investigation, the five degraded chloramphenicol synthetic eye drops from the marketed eye drop preparations which were designated as A, B, C, D and E respectively showed zones of inhibition when the bioassay was carried out by agar spot method for antimicrobial activity against five clinically obtained isolates such as *Escherichia coli (ETEC), Proteus vulgaris, Staphylococcus aureus, Salmonella typhi and Bacillus cereus.* However, when the agar diffusion method was followed to find the antimicrobial activity of the five chloramphenicol eye drops against the pathogens, the zone of inhibition of samples that remained in secondary packaging, primary packaging and transparent vial tubes were compared with samples that stored in refrigerator after exposure to sunlight. The measured zone of inhibition of stored samples, samples in secondary packaging, primary packaging and transparent vial tubes of product A against *Bacillus cereus* was 26.33, 24, 23, 21.33 mm in diameter respectively (Table-2).Thus, the antibacterial activity decreased gradually from the stored samples to samples in transparent vial tubes against *Bacillus cereus*. It was asserted the almost similar trend in other four products B, C, D and E (Table-3, 4, 5, and 6). Moreover, the antimicrobial activity was decreased in the sunlight induced degradation of chloramphenicol synthetic eye drops preparations. As the sun light degraded its medicinal quality it is no longer able to kill extensive amount of microorganisms.

A significant characteristics were noticed in some of the eye drops samples that remained in primary packaging. In this primary packaging, eye solutions remained in the container only that directly exposed to the sun and showed more decreasing level of medicinal quality along with the less antimicrobial activity than the eye solutions remained in transparent vial tubes. In case of product E, for gram positive bacteria., the zone of inhibition of stored samples, samples in secondary packaging, primary packaging and transparent vial tubes against *Bacillus cereus* was 26.66, 25, 23.33 and 24.66 mm in diameter respectively and against *Staphylococcus aureus* was 28,27.3,25and 22.3 mm in diameter respectively (figure-13). According to the previous study in

this experiment, chloramphenicol synthetic drugs showed degradation by observing their zone of inhibition which was gradually decreased like antimicrobial activity against *S.aureus* but in *Bacillus cereus*, where aqueous solution remained in primary packaging (container only) having less antibacterial activity which is 23.33 mm diameter of zone of inhibition than the samples remained in transparent vial tubes having antibacterial activity is 24.66 mm in diameter so therefore primary packaging showed less medicinal quality of the drug.

In case of product D, for gram positive bacteria., the zone of inhibition of stored samples, samples in secondary packaging, primary packaging and transparent vial tubes against *Bacillus cereus* was 25.33, 23.66, 23.33 and 22.33 mm in diameter respectively and against *Staphylococcus aureus* was 25.6,24.3,21and 23.33 mm in diameter respectively (figure-14). According to the previous study in this experiment, chloramphenicol synthetic drugs showed degradation by observing their zone of inhibition which was gradually decreased like antimicrobial activity against *B.cereus* but in *Staphylococcus aureus*, where aqueous solution remained in primary packaging (container only) having less antibacterial activity which is 21 mm diameter of zone of inhibition than the samples remained in transparent vial tubes having antibacterial activity is 23.33 mm in diameter so therefore primary packaging showed less medicinal quality of the drug

In case of product D, for gram negative bacteria., the zone of inhibition of stored samples, samples in secondary packaging, primary packaging and transparent vial tubes against *Proteus vulgaris* was 20.33, 19.33, 18.67 and 16 mm in diameter respectively, against *Escherichia coli* was 21, 20, 19.33 and 25.67 mm in diameter respectively and against *Salmonella typhi* was 23, 22, 20.33 and 21.66 mm in diameter (figure-15). According to the previous study in this experiment, chloramphenicol synthetic drugs showed degradation by observing their zone of inhibition which was gradually decreased like antimicrobial activity against *Proteus vulgaris* and *Escherichia coli* but *Salmonella typhi* in primary packaging (container only) having less antibacterial activity which is 20.33 mm diameter of zone of inhibition than the samples remained in transparent vial tube having antibacterial activity is 21.66 mm in diameter so therefore primary packaging showed less medicinal quality of the drug.

Knowledge of the 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 light (Ahmad *et al.*, 2016).

In this study, primary packaging could not give the protection of the products in some cases and thus degradation occurred due to the sunlight exposure. There is a possibility that the Primary packaging may not be handled in proper way by manufactured company so that it miscarried to keep the stability of the drugs. Therefore, samples those remained in the primary packaging showed less antimicrobial activity.

However, the degraded chloramphenicol synthetic eye drops under this study exhibited excellent antimicrobial activity against all the selected bacteria. For *Staphylococcus aureus,* highest zone of inhibition was produced by chloramphenicol synthetic eye drops which is 31.6 mm in diameter so therefore, the eye drops were found to be most potent against *Staphylococcus aureus* (31.6mm) and *Salmonella typhi* (26mm) amongst the other gram positive bacteria and gram negative bacteria respectively. Moderate action was seen against *Escherichia coli, Proteus vulgaris* and (22.67mm, 20.67mm). Overall, it was observed that, inhibitory action of the eye formulation was highest for gram positive bacteria, followed by gram negative bacteria. It was observed that as the sunlight almost degrade medicinal quality of the eye drops so that they were gradually losing their efficacy but to some extent few chloramphenicol eye drops showed the potency against the microorganisms.

On the other side, the degraded herbal eye drops were appeared with exposing less antimicrobial activity against only one selected bacteria. In the case of herbal eye drops, only Bacillus cereus showed the zone of inhibition. The measured zone of inhibition of stored sample, secondary packaging, primary packaging and transparent vial tubes of Itone herbal eye drops against Bacillus cereus was 18, 16.33, 15.67 and 10.33 respectively and also Itone herbal eye drops became resistance to all other bacteria. Therefore, the antimicrobial activity of chloramphenicol synthetic eye drops are more effective than antimicrobial activity of herbal eye drops.

The most important challenge faced by Herbal formulations arises from lack of complete evaluation of its constituents, due to its complex nature. Evaluation of constituents is necessary to ensure quality, purity and stability of the finished product. Stability study provides evidence on how quality of a drug substance or product varies with time under influence of variety of environmental factors such as, temperature, humidity and light and also to establish a retest period for the drug substance or product and recommended storage conditions. So it can be said that stability study is necessary as an assessment of product quality and so storage condition becomes

a most important aspect which effects on shelf-life of the product (Patgiri *et al.,* 2014). The quality assessments of herbal formulations are of paramount importance in order to justify their acceptability in modern systems of medicine. One of the major problems faced by the herbal drug industry is the unavailability of rigid quality control profiles for herbal materials and their formulations. Now a day's most of the herbal and traditional formulations are lacking in defined quality control parameters (Silva *et al.*, 2015).

Many eye drops product available in the market are likely to vary in quality and therapeutic efficacy due to differences in composition of the product, differences in methods of manufacture, differences in methods of storage, differences in geographical origin and season of plants parts collected, lack of adequate documentation of production (Shenoy and Yoganasimhan, 2008).

Conclusion:

The degraded chloramphenicol synthetic eye drops under this study exhibited better antimicrobial activity against all the selected bacteria than the Itone herbal eye drops. So, the overall results showed that the chloramphenicol eye drops before and after sunlight degradation were active against pathogenic bacteria causing ocular infections. On the other hand, the herbal eye drops were not comparable to chloramphenicol eye drops as it had 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 (4^0C)	Model: 0636 Samsung