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Research Article

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Antioxidant and antibacterial activity of human tears

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Abstract

Tears play an important role in keeping us healthy. Tears are made of mucus, water, and oil and each plays an important role in the eye. Tears are the first barrier protecting the cornea against oxidative damage from radiation, atmospheric oxygen, and toxic chemicals The present study is focused with following main objectives to access the antioxidant and antibacterial activity of human tears. Tears had been collected into a capillary tube by using eppendorf tube; the donors were briefly exposed to the vapors of freshly minced onions. These tear specimens were stored at −20°C and tested. The human tear contains both enzymatic and non enzymatic antioxidants. So it prevents oxidative damage occurs in the cornea. Human tears against Gram-positive and Gram-negative strains tested based on agar well diffusion method shows the antibacterial activity of tears. Presence of many proteins like, lysozyme, lactoferin and IgA are reason for its antibacterial activity. Crying is the natural innate immunity present in the eyes to protect from foreign particles. We all take little care about our eyes but the tears make the eyes healthier. So we have to take care about the tears and its components.

Keywords: Tears, Basic biochemical components, Chromatography techniques, Antioxidant activity, Antimicrobial activity.

Introduction

Tears are very important essential secrets produced by the animals to guarantee healthy eyes and vision. Tears can also serve other purposes such as reacting to external stimuli or in response to a certain emotional feeling. Tears are produced in the lachrymal glands (tear ducts) that are in the outer corners of human eyelids. These glands produce tears from blood plasma, selecting some components but not others. Tears serve one primary purpose and that is to clean and lubricate the eyes. This protein-rich liquid goes from the outer edge of the eyeball toward the cornea and lubricates the entire eye surface every time we blink. It keep the surface of our eyeballs dirt free, wet and help to protect our eyes from spoil. Tears are made of mucus, water, and oil and each plays

an important role in the eye. Tears are the first barrier protecting the cornea against oxidative damage from radiation, atmospheric oxygen, and toxic chemicals (Richer *et al., 1998*). Oxidative injury from ROS occurs in the tears and conjunctiva of Sjogren's syndrome patients, and high levels of ROS and oxidative stress have been identified in the tear film of dry-eye patients (Kaviani N *et al., 1995*)**.** The ability of antioxidants to scavenge reactive oxygen species (ROS) is important to protect tissues from light-induced oxidative damage (Halliwell *et al., 1999*). This is particularly true of the ocular tissues because of their exposure to light, which causes production of ROS in situ (Leske *et al.,* 1998*).*

Ascorbic acid (vitamin C), an essential diet-derived antioxidant, is found in a high concentration in the aqueous humor, but there is no agreement regarding its concentration in human tears (Paterson *et al 1987).* In addition, little is known about the antioxidant profile or the "total antioxidant activity" of tears. Vitamin c is known to guard the reducing powers of other antioxidants such as *α*-tocopherol (vitamin E) by rescuing *α*-tocopheryl radicals in membranes. As a reducing agent ascorbic acid may also act as a pro-oxidant by reducing metal ions, which leads to the generation of free radicals through the Fenton reaction. Aside from its reducing properties, ascorbic acid is also essential for collagen synthesis and has anti-inflammatory properties, preventing extensive tissue damage in the eye (Bhattacherjee *et al.,* 1984). Uric acid may make a significant contribution to the total antioxidant activity of biological fluids, including plasma and tears, increased levels are not desirable because these indicate disease rather than health renal failure (Niskanen L *et al.,*1998). GSH is a dominant antioxidant with several functions in the eye. It protects protein thiol groups by affording guard against ROS, is essential for the performance of several glutathione-dependent antioxidant enzymes that counteract ROS, and is answerable for drug detoxification (Brubaker RF *et al.,* 2000). Low levels of cysteine and tyrosine have been detected in tear fluid, and these may contribute to the antioxidant activity of tears.

In the anterior chamber of the eye, superoxide dismutase (SOD), catalase, and glutathione-related enzymes act in conjunction with each other to play a critical role in regulating the production of ROS and precluding any tissue damage (Marklund SL *et al* 1998). Antioxidants may play a role in modulating wound healing and inflammatory responses in the cornea (McGahan *et al.,* 1985). When corneal wound is occurs in the eyes, plasmin and plasminogen activators are activated in tear fluid. Activity of these enzymes is useful for corneal wound healing. (Van setten GB *et al.,* 1989). In vitro and in vivo studies have confirmed the antimicrobial activity of tears at the ocular surface and multiple mechanisms are involved. Tear components long recognized to have antimicrobial function include lysozyme, lactoferrin, lipocalin, secretory immunoglobulin A (IgA) and complement (Fleiszig et al., 2003). *Aspergillus, Candida* and *Fusarium*species are common causes of fungal infection in the eye (Farooqet al., 2012). Tears able to cleave chitodextrins in fungal cell walls (Lee-Huang et al., 1999). in addition, a very basic sequence at the N-terminus (referred to as lactoferricin) allows lactoferrin to perform as a cationic detergent and interrupt the cell membrane of some bacteria, fungi and viruses (Farnaud and Evans, 2003). Lysozyme was exposed to be present in tears and to kill Gram-positive bacteria by Alexander Fleming (Fleming, 1922). This enzyme, which is veiled by the main and accessory lachrymal glands, accounts for up to 20–30% of total protein in basal and impulse tears (Albert DM *et al.,* 2008). Tears able to cleave chitodextrins and reported to have anti-HIV activity (Lee-Huang et al., 1999).

Materials and Methods

The different methods of tear collection and stimulations used. In our study and that of (Kuizenga et al., 1987) glass capillary tubes were used for collection, whereas Schirmer strips were used in other studies. Schirmer strips are invasive

and the volume collected is very small and difficult to measure accurately. Evaporation of water from the small tear sample captured may significantly increase the apparent solute concentration (Stephens R *et al., 1990*).

Collection of Tears Sample

Tears had been collected over 5-10 minutes into a capillary tube by using eppendorf tube; the donors were briefly exposed to the vapors of freshly minced onions. These tear specimens were stored at −20°C until tested.

Qualitative Analysis of Basic Component of Tears

Test for Urea: To 2 ml of tears and 5 % of oxalic acid was added. The white precipitate indicates the presence of urea.

Test for Carbohydrates

Molisch's Test: 1ml of sample was added to 2ml of molisch's reagent and the resulting mixture shaken properly. 2ml of concentrated sulphuric acid was then poured carefully down the sides of the test tube. A violet ring at the interphase indicates the presence of carbohydrates.

Test for Protein

Biuret Test: Sample was diluted with distilled water and treated with Biuret reagent. The appearance of pink colour indicates the presence of protein.

Test for Amino Acids

Ninhydrin Test: To a little tears sample, few drops of ninhydrin reagent were added. It was then shaken well and warmed. Purple colour for all amino acid except proline and hydroxy proline, which gives yellow colour.

Test for Arginine

Sakaguchi Test: To 1ml of tears, 1ml of 40% sodium hydroxide and 2 drops of 1% α-napthol were added and mixed well. Then 2-3 drops of bromine was added. The appearance of red colour indicates the presence of arginine.

Test for Tyrosine

Morner's Test: To 1 ml of tears, 3 ml of morner's reagent was added gently and heated in a boiling water bath. The appearance of brick red colour indicates the presence of tyrosine.

Quantitative Analysis of Basic Components of Tears: Urea was estimated by DAM-TSC method, glucose was estimated by OrthoToluidine method and protein was estimated by Lowry's method. Estimation of Ascorbic acid (Vitamin C) & reduced glutathione. uric acid was estimated by Caraway's method and assay of superoxide dismutase (SOD) by method of Kakkar et al., (1984).

Separation and Identification of Monosugars and Amino Acids by Paper Chromatography

The chromatographic paper was taken and a line was drawn above 4cm from the bottom. The standard sugars and amino acids were applied at an interval of 2cm using a capillary tube. The spot were to dry. Newly drops of each solution were made; diluted tear sample was also applied similarly. The paper was tied and then dipped in tray containing running solvent. The run was allowed till the solvent reaches ¾ of the paper. The paper was then removed from the chamber and allowed to dry. The chromatographic conditions had been previously optimized to achieve the best resolution and peak shape. After development, paper was dried. The identity of the bands of monosugars and amino acids in the diluted tear sample was confirmed by locating reagents and their Rf value was calculated.

Separation and Identification of Free Fatty Acids by Thin Layer Chromatography

Apply 10-20 µl fraction of the lipid sample and tears sample in the form of a spot at distance of 2cm starting from the left bottom edge of the activated TLC plate. Develop the plate in the

appropriate developing mixture in an air tight chromatographic glass tank till the solvent front moves upto 4cm below the top edge of the glass plate. Take out the plates, dry them for 5 minutes in air spray the plates with the required detection reagents. Spray the developed plates with ferric chloride and heat them at 100^{o}c for 2-3 minutes in an oven. Locate the position of the lipid spot on the glass plates and measure the distance travelled by the individual lipid component and calculate the Rf values and compare them with those of standards.

Anti-Bacterial Activity

Antibacterial activity of tears on gram negative and gram positive bacteria. The bacterial strains used for the experiment were collected as pure cultures. Both Gram positive and Gram-negative organisms were taken for the test and they are listed in the below.

Gram Positive Bacteria
Staphylococcus aureus

Gram Negative Bacterias
Pseudomonas aeruginosa
Vibrio cholerae
Escherichia coli

Culture Medium and Their Composition: The Muller Hinton Agar Medium was used normally to demonstrate the antimicrobial activity and to make subculture of the test organisms.

Preparation of the Medium

To prepare required volume of this medium, calculated amount of Muller Hinton agar medium was taken in a bottle with a cap and distilled water was added to it to make the required volume. The contents were then autoclaved to make a sterile solution.

Preparation of Subculture

In an aseptic condition under laminar air cabinet, the test organisms were transferred from the pure cultures to the agar slants with the help of a transfer loop to have fresh pure cultures. The inoculated strains were then incubated for 24 hours at 37˚C for their optimum growth. These fresh cultures were used for the sensitivity test.

Preparation of the Test Plate

The test organisms were transferred from the subculture to petridish containing about 10 ml of melted and sterilized Muller Hinton agar medium. The bacterial suspension was taken by a loop a mixed with normal saline with the help of vortex machine. Then a sterilized cotton bud was taken and dipped into the bacterial suspension. Then the bacterial sample is applied to the petridish with the help of this cotton bud.

Procedure

The agar diffusion method was employed for the determination of antibacterial activities of tears. The bacterial strains were cultured in a nutrient broth for 24 hours. Then, previously prepared 1ml of suspension bacteria was spread on petridish containing MH Agar medium. In Agar medium were punctured to form holes. Add, 10µl, 20µl, 30µl of tear samples are diluted with DMSO to make the final volume to 30µl. Methanol was used as negative control and Chloramphenicol (30mcg/disk) as positive reference standard. All the plates were incubated at 37°C for 24 hours. Antibacterial activity was evaluated by measuring the zone of inhibition in millimetres. Antibacterial activity was evaluated by measuring the zone of inhibition in millimetres.

Results and Discussion

Qualitative Analysis of Basic Components of Tears

Table 1 shows the qualitative analysis screening for basic components of the tears was carried out using standard qualitative tests. The sample was screened for the presence of basic components of tears such as glucose, proteins, urea and some amino acids like arginine and tyrosine.

Table 1 Basic Qualitative Analysis of Tears

Tests	Tears Sample
Carbohydrates	+
Reducing sugar	+
Protein	+
Urea	+
Amino acids	+
Arginine	+
Tyrosine	+

Quantitative Analysis of Urea, Glucose and Protein in Tears

Table 2 represents the quantitative analysis of carbohydrate, proteins and urea. It plays an important role in nutrients and protection of eyes. Also helps to know the proper function of tear ducts. The presence of urea is representing the proper excretory function of tears glands. Urea is the end product of protein metabolism. Result shows the normal function of protein metabolism occur in the tears.Glucose is the only carbohydrate source present in the tears for healthy corneal functions of eyes. Because it does not have any blood supply in it. So all the nutrients are only supplied through the tears. The presence of glucose also shows that the tear ducts are in proper function.

Table 2 Quantitative Analysis of Urea, Glucose and Protein in Tears

S. No	Particulars	Values in mg/100ml of tears sample
1.	Urea	0.5 ± 0.06
2.	Glucose	6.0 ± 0.72
3.	Protein	440 ± 35.2

Antioxidant Status of Tears

Antioxidant is the broad spectrum of compounds which provides primary defends to the cornea of eyes. These are protective agents which donate its electron to reactive oxygen species and prevent them from attacking the lipid bilayer of the cell membrane present in the cornea of eyes. Production of free radicals quickly increases while poisonous chemical accumulates in the body. Under these conditions supplements of antioxidants through diet promotes the scavenging activity of antioxidants. (Hajieva *et al., 2006*).

Non Enzymatic Antioxidants

Nutrient Antioxidant

Anti-oxidant from our diet plays an important role in helping endogenous antioxidants for the neutralization of oxidative stress. The nutrient antioxidant deficiency is one of the causes of numerous chronic degenerative pathologies. Each nutrient is unique in terms of its structure and antioxidant function. The non-enzymatic antioxidants are also called as nutrient antioxidants. These are compounds which cannot be produced in the body and must be provided through foods or supplements, such as vitamin E, vitamin C, Vitamin A etc.

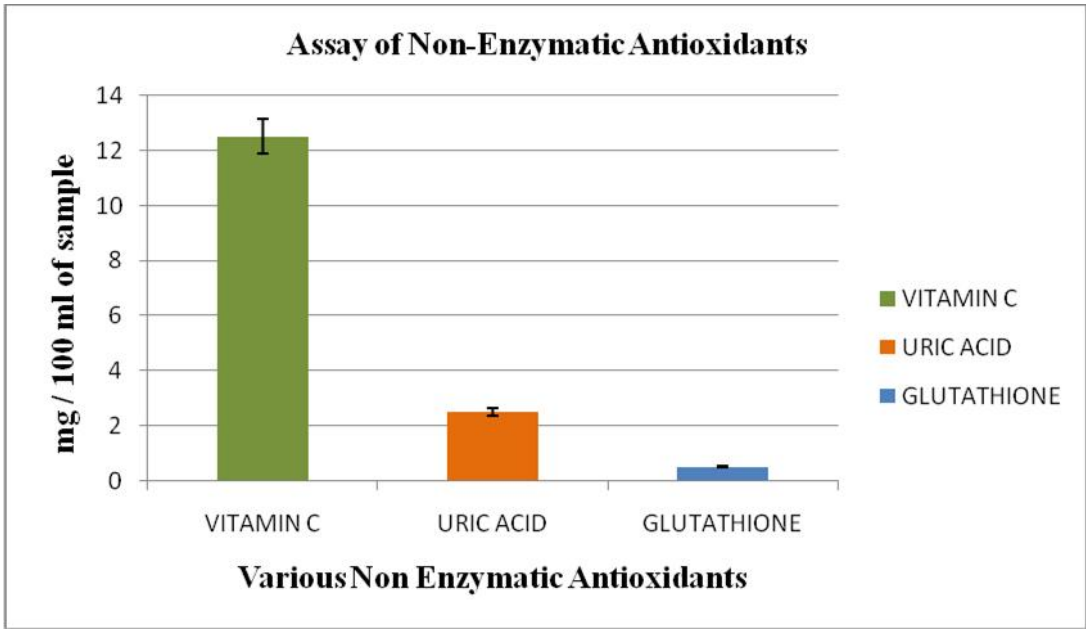


Fig. 1 Activity of Non-Enzymatic Antioxidants in tears

Vitamin C

Fig. 1 shows the non enzymatic antioxidants activity of human tears. Here, Vitamin C is significantly higher (12.5mg/100ml of tears) than other non enzymatic antioxidants such as uric acid and glutathione. In tears, uric acid and ascorbic acid account for around half the total antioxidant activity. The high concentrations of ascorbic acid in the aqueous humour, together with its ability to absorb UV light, have led to its referral as a physiological "sunscreen" (Ringvold A *et al* 1996), preventing the penetration of UV light and protecting tissues from photo-induced oxidative damage .As a scavenging species, ascorbic acid is oxidized by ROS in a two-step process which detoxifies or stabilizes hydroxyl and superoxide anion radicals (Richer SP *et al.,* 1998).

Uric acid

Uric acid or, more correctly, at physiological pH values, its anion urate, is a degradation product of purines. It is now well established that uric acid acts as an antioxidant and contributes to radical scavenging systems, thereby protecting from damage by oxidative stress. Uric acid is present not only in serum or plasma, but also in sweat, nasal fluid and in eye fluids (Lam *et al.*, 1995). A correlation between the uric acid concentrations of these body fluids with age revealed a significant relationship between uric acid concentration in aqueous humour and age. Uric acid is only responsible for 10% of the total antioxidative capacity in aqueous humour as ascorbic acid represents the major antioxidant in this eye fluid in humans (Richer *et al* 1998). Superoxide radicals occur during its biosynthesis and therefore uric acid may participate in these diseases. But, uric acid acts as an antioxidant and could contain a defensive effect.

Glutathione

When overall GSH concentrations in the cornea are lower relative to ascorbic acid concentrations, GSH plays a major role in corneal defence. It is involved in maintaining the barrier function of the corneal endothelium, controlling normal hydration levels, protecting cell membrane integrity, and degrading xenobiotics agents. In extra tissues, GSH levels are maintained by a mixture of GSH uptake, *de novo* synthesis from its pioneer amino acids, GSH renewal from GSSG by glutathione reductase, and GSH efflux. Surprisingly, there is limited information regarding the molecular pathways involved in maintaining GSH homeostasis in the cornea.

Previous work has investigated GSH uptake by measuring S-GSH in the different layers of the cornea and found that the highest concentrations of S-GSH were detected in the stroma (Huang LC *et al.*, 2006).

Enzymatic Antioxidant assay

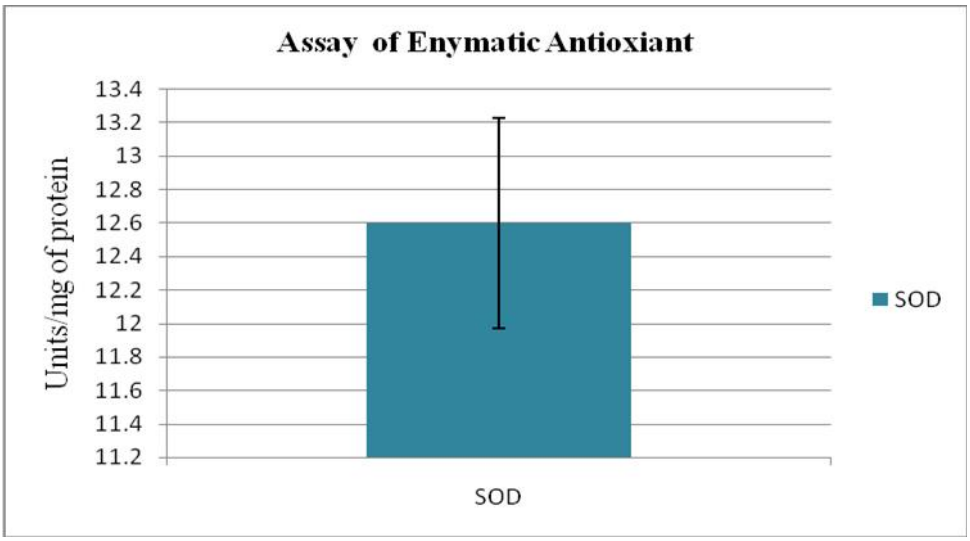


Fig. 2 Antioxidant Activity of SOD in Tears

The assays of enzymatic antioxidants in the tears show a notable amount of enzymatic antioxidant in it. Among them the level of SOD is significant shown in Fig. 2. 100ml of tear sample contains 12.5 units/mg of protein. One of the major enzymatic antioxidant directly involved in the neutralization of ROS is: superoxide dismutase (SOD). SOD, the first line of defence against free radicals, catalyses the dismutation of superoxide anion radical (O2) into hydrogen peroxide (H2O2) by reduction. One SOD molecule takes two superoxide molecules namely A and B, removes the extra electron of superoxide A, and places it on the superoxide B. As a result, superoxide A loses the electron and returns to being a normal oxygen molecule. Superoxide B ends up with two additional electrons, attracts two hydrogen ions and becomes hydrogen peroxide, a less hurtful ROS. It naturally present in the tissues usually control ROS levels, but surplus ROS react with nearby proteins, lipids or other cellular components, leading to unpredictable, cumulative and often deleterious effects on normal cell function. Oxidative injury from ROS occurs in the tears and conjunctiva of Sjogren's syndrome patients, and high levels of ROS and oxidative stress have been identified in the tear film of dry-eye patients **(**Kaviani N *et al., 1995***).**

Separation and Detection of Monosugars and Amino Acids by Paper Chromatography

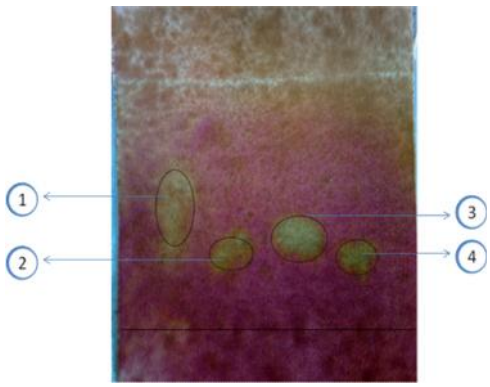


Fig. 3 Paper Chromatography for Monosugars

Table 3 Rf Value of Monosugars

S.No	Lane	Rf values
1.	Std 1 - Galactose	0.56
2.	Std 2 - Glucose	0.32
3.	Std 3 - Fructose	0.37
4.	Unknown - Diluted Tears Sample	0.30 (Glucose)

Fig. 3 shows the presence of monosugar (glucose) in the tears sample and glucose is the only carbohydrate energy source present in the tears. The major energy requirement for the corneal cells is adenosine tri phosphate, and it's produced from the glucose molecule by many steps of oxidative processes.

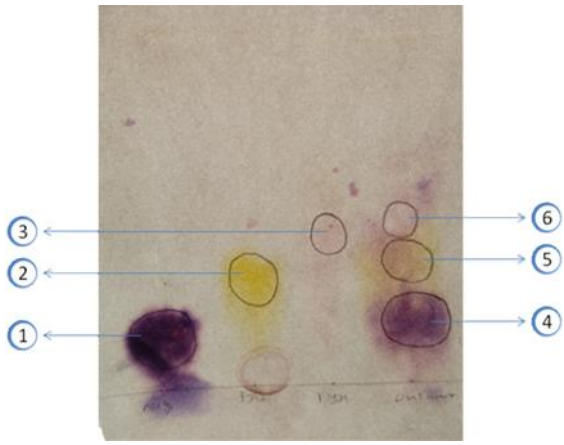


Fig. 4 Paper Chromatography for Amino Acids

Table 4 Rf Value of Various Amino acids

S.No	Lane	Rf values
1.	Std 1 - Arginine	0.21
2.	Std 2 – Proline	0.35
3.	Std 2 – Tyrosine	0.45
3, 4 & 5	Unknown - Diluted Tears Sample	0.24 (Arg), 0.38 (Pro) & 0.47 (Tyr)

Fig. 4 shows the presence of amino acids like, Arginine, Proline, and Tyrosine in tears sample. These amino acids are helps in the formation of protein and involved in the wound healing mechanism of the cornea. Amino acids are most important components in the formation of non enzymatic antioxidants like glutathione and help to reduce the risk of oxidative stress.

Separation and Detection of Lipids by TLC

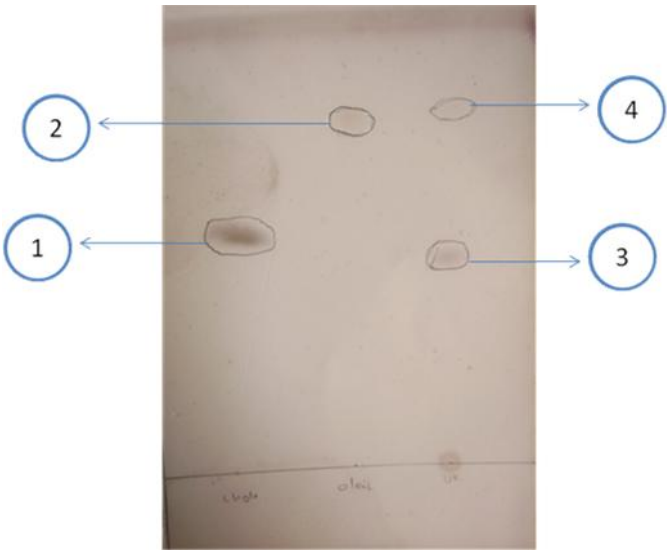


Fig. 5 TLC for Lipids

Table 5 Rf Value of Cholesterol and Oleic acid

S.No	Lane	Rf values
1.	Std 1 - Cholesterol	0.46
2.	Std 2 - Oleic acid	0.69
3 & 4	Unknown - Diluted Tears Sample	0.43 (Cholesterol) & 0.72 (Oleic acid)

The presence of lipids in the sample was confirmed by the corresponding standards Rf values. Fig. 5 showed that the formation of brown spot confirmed the presence of cholesterol and oleic acid when compared with standard cholesterol and oleic acid. Lipids are the most essential compound to prevent the evaporation of tears. If lipids are not present the concentration of tear components will be higher than normal level. Because of the evaporation of water content present in the tears.

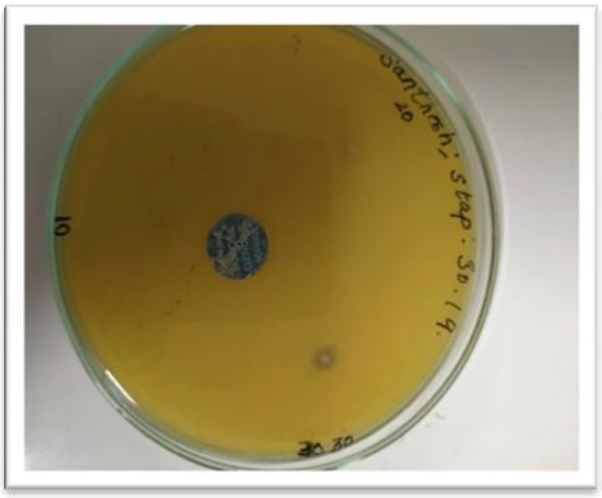
Anti-Microbial Activity

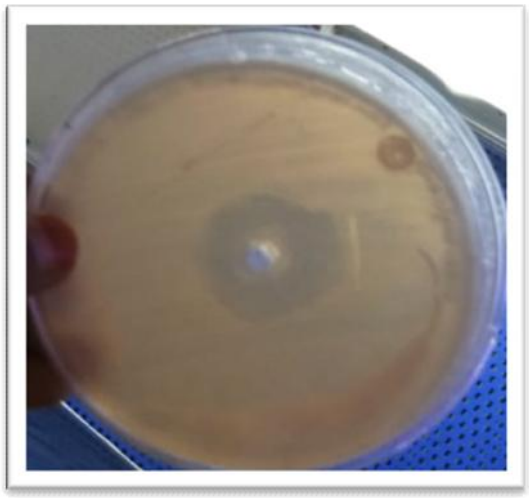
Anti-bacterial activity

The present study represents the most systematic study on antimicrobial properties of tears against common pathogenic bacteria by supporting the view that tears is a potent anti-microbial agent. The antibacterial activity of the human tears were studied in different concentration (10,20 and 30µl) against four bacterial ATCC strains, one Gram-positive *Staphylococcus aureus (*ATCC 25923) as well as Gram negative strains such as *Escherichia coli (*ATCC 25922) , *Pseudomonas aeruginosa (*ATCC 25668) and *Vibrio cholerae (*39050) were used for the evaluation of antibacterial activity.

A. Gram Positive Bacteria: *Staphylococcus aureus* with tears sample and standard

Tears sample **Control**

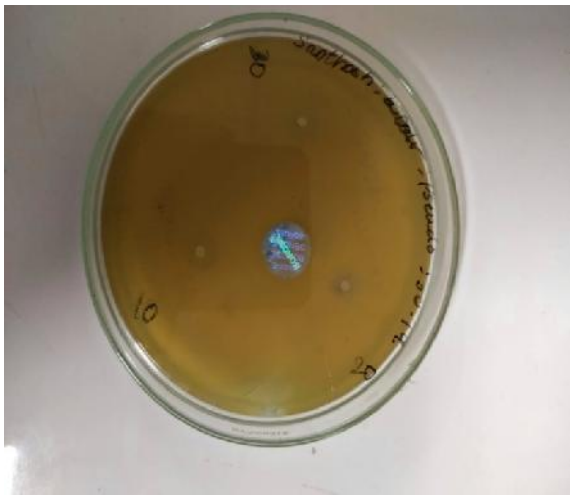


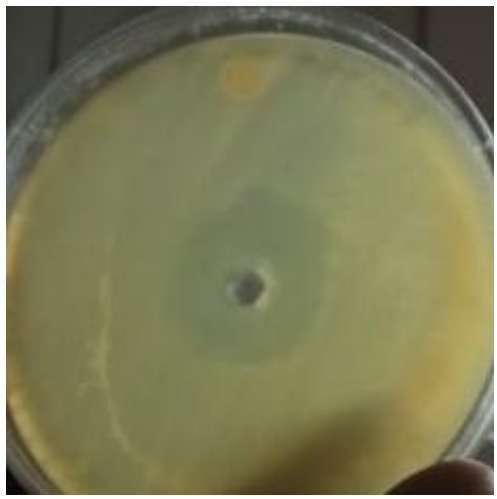


B. Gram Negative Bacteria

a. *Pseudomonas aeruginosa* with tears sample and standard

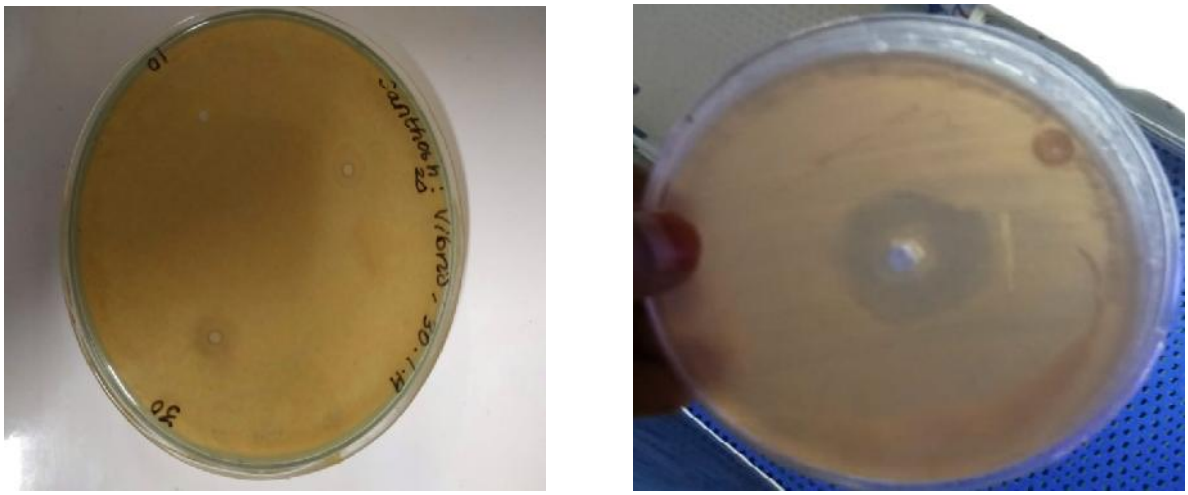
Tears sample **Control**





b. *Vibrio cholerae* with tears sample and standard

Tears Sample **Control**



c. *Escherichia coli* with tears sample and standard

Tears sample **Control**

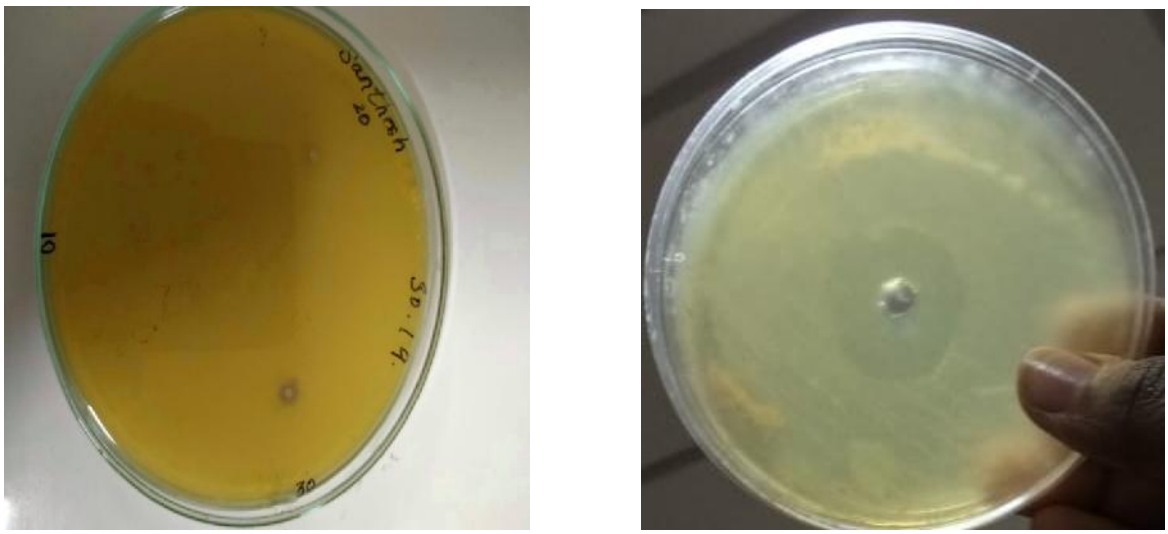


Fig. 6 Antibacterial Activity of Tear

Fig. 6 shows the Anti-bacterial activity of human tears against Gram-positive and Gram-negative strains tested based on agar well diffusion method and their zone of inhibition was measured and given in mm diameter.

Table 6 Zone of Inhibition in mm Diameter

S.No.	Microorganism	Concentration			Antibiotic Chloramphenicol
		10µl	20µl	30µl	
1.	*Staphylococcus aureus*	nil	5mm	9mm	20mm
2.	*Pseudomonas aeruginosa*	3mm	6mm	10mm	19mm
3.	*Vibrio cholerae*	5mm	9mm	14mm	20mm
4.	*Escherichia coli*	nil	5mm	8mm	24mm

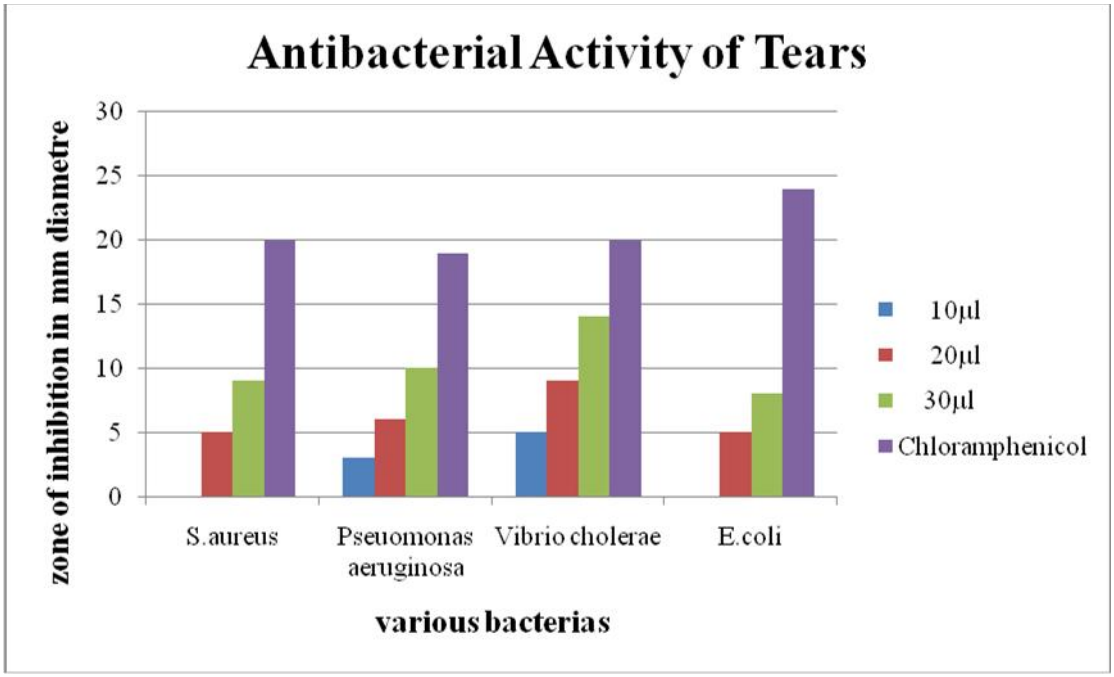


Fig. 7 Antibacterial Activities of Tears with Bar Graph

Fig. 7 Anti-bacterial activity of tears against four different bacterial and their zone of inhibition is plotted in bar graph. From the above study, the tears was found to be more effective *Vibrio cholerae* 14mm, ,*Staphylococcus aureus* ,*Pseudomonas aeruginosa* and *Escherichia coli* 9mm,10mm and 8mm, in 30µl concentration respectively. In the present work, the tears used have significant anti-bacterial effect on all the bacterial at lower concentration also, which implies that the tears sample had more efficient antibacterial effects against the different bacteria. Lysozyme (Innate and antimicrobial proteins) is the most alkaline protein in tears and makes up 20-40% of whole tear proteins. Lysosomes secrete higher levels of this enzyme in the PTF than in any other body fluid. The lysosomes that secrete lysozyme in the tear fluid are situated within the lacrimal glands. Lysozyme plays a role in innate immunity against bacteria and is thus also called an antibacterial enzyme. The role of lysozyme in innate immunity is to break down the peptidoglycan within bacterial cell walls thus destroying bacteria. Gram positive bacteria invading the ocular surface are most likely to be destroyed by lysozyme. It has been reported that lysozyme levels decrease with age. As discussed here analytical studies have shown that tears contain a variety of components with antimicrobial activity that can frankly kill or stop

the growth of a range of pathogenic organisms. While there is some redundancy in that there is overlie in spectrum of activity, having a big number of antimicrobials is a general feature of all biological fluids and reflects the complexity of the flora to which the body is regularly exposed. Having many antimicrobials with differing mechanisms of action helps to guarantee abolition of a pathogen, which may just happen to be defiant to a exact compound. Also it permits synergistic/additive interactions between two or perhaps more molecules, which can reduce the amounts needed and lower the risk of toxic effects to ocular surface cells. In addition to straight action on microbial growth and survival, reflex tearing and some tear antimicrobials such as mucins and sIgA which join pathogens ease their removal via the lachrymal drainage system.

Conclusion

The present study shows the presence of major basic components, each component having specific functions in the eyes. The human tears sample was first analyzed for the basic components like Salty nature of the tears shows the presence of electrolytes (Nacl). The study reveals the presence of urea, glucose, protein and amino acids. The presence of urea is representing the proper excretory function of tears glands. Urea is the end product of protein metabolism. Result shows the normal function of protein metabolism occur in the tears. Glucose is the only carbohydrate source present in the tears for healthy corneal functions of eyes. Because it does not have any blood supply in it. So all the nutrients are only supplied through the tears. The presence of glucose also shows that, the tear ducts are in proper function. Lipids are the most essential compound to prevent the evaporation of tears. If lipids are not present the concentration of tear components will be higher than normal level. Because of the evaporation of water content present in the tears. The human tear contains both enzymatic and non enzymatic antioxidants. So it prevents oxidative damage occurs in the cornea. In that vitamin C is present in high concentration so it represents the importance of diet. Because non enzymatic antioxidants are not produced by the body but only through the diet. Human tears against Gram-positive and Gram-negative strains tested based on agar well diffusion method shows the antibacterial activity of tears. Presence of many proteins like, lysozyme, lactoferin and IgA are reason for its antibacterial activity. Crying is the natural innate immunity present in the eyes to protect from foreign particles. We all take little care about our eyes but the tears make the eyes healthier. So we have to take care about the tears and its components.

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