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Lactobacillus casei isolated from Human Milk and Three Natural Agents act as Antibacterial against Gram Negative Pathogenic Bacteria isolated from Infected Eye

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A B S T R A C T

One of the most common causes of eye infections is bacteria. The symptoms of these infections in humans can vary from mild to life-threatening complications. This study was conducted to determine the Gram negative bacterial pathogens that infect the eyes of people whether wearing lenses or not & investigate the inhibitory effect of some antibiotics and compare them with the antibacterial effect of black & green tea extracts, honey (at 10%) and CFS of *Lactobacillus casei* that isolated from milk of healthy mother against the pathogens isolated from the eye by using the agar diffusion technique. A total of 30 samples collected from infected eyes by swabs and transferred to the laboratory for microbial culturing. 20 different isolated species of Gram negative bacteria were identified by Vitek-2 system are (*Enterobacter cloacae, Pseudomonas luteola, Leclercia adecarboxylata & pantoea agglomerans).* All natural agents showed varying effectiveness against the bacteria isolated from the eye, while the most of isolates were resisted to antibiotics, Honey & CFS of *Lactobacillus casei* were most effective against growth of all the test organisms. Black and green tea showed less effect than honey & probiotic against bacteria. Our results showed that a lot of the commercial antibiotics were not effective on the tested organisms. Whereas the natural agents that used in this study had higher inhibitory effect against bacteria that isolates from ocular infections than the selected antibiotics.Further in vivo studies for the confirmation the antibacterial effect of natural agents that were used in this study.

Introduction

Eye is one of the sense organ which is important throughout our life (Dagnachew *et al*., 2014). Pathogenic microorganisms cause diseases to the eyes due to their virulence and host's reduced resistance from many factors such as personal hygiene, living conditions, socio-economic status, nutrition, genetics, physiology, and age (Ubani, 2009). The eye infected may be is carried out by intraocular invasion through of microorganisms that are the blood stream or external source. External bacterial infections of the eye are usually localized but may frequently spread to other tissues (Bharathi *et al*., 2015). Ophthalmic infections can cause damage to structures of the eye, which can lead to vision loss and even blindness if left untreated (Joseph *et al*., 2009). *Staphylococcus aureus*, *Streptococcus epidermis* and *Pseudomonas aeruginosa* are opportunistic pathogens which have clinical significance in ophthalmology to the fact that they are causes of most eye infections

(Kwapong *et al*., 2013). Bacterial colonization on contact lenses and its solutions may be also causes inflammation of eye.

The complications of contact lens include acute red eye, peripheral ulcers, infiltrative keratitis and asymptomatic keratitis (Shaharuddin *et al*., 2009). It is well known that the deposits on contact lenses consist of proteins, lipids and mucin as tear components which stimulate the growth of microorganisms on its surfaces. Protein deposits are natural deposits on contact lenses which are unavoidable as they are formed by the interaction of the protein in our natural tears and the contact lenses. Contact lens cleansing solutions have been prepared using plant (papain) and animal (pancreatin, trypsin and chemotrypsin) proteases (Ashwini *et al*., 2014).

The effective use of antibiotics to treat ophthalmic infections requires an understanding of the disease and the pharmacokinetics and pharmacodynamics of the drugs used for the treatment. The failure of these antibiotics has resulted for man to search for more effective sources of natural products from plants and some insects (Omoya *et al*., 2011); such as honey, tea, probiotic. a rekindled interest in the pharmaceutical importance of plants has led to the discovery and adaptation of plant extracts which were commonly used in traditional medicine as alternative source of remedy (Roopal *et al*., 2011).

Antimicrobial agents are the substances known to have therapeutic effect on microorganisms either as a control, prevention or cure of microbial and non-microbial disease origin. These antimicrobial agents are synthesized chemotherapeutic substances obtained majorly from microorganisms, plants and some animal products (Alkhyat *et al*., 2017). This study aimed to prove the fact traditional treatment uses which derived from natural sources and that popular in developing countries against isolates caused by ocular infections.

The objectives of current study includes to isolate and diagnosis only Gram negative bacteria among people are suffering from discharges from the eye (whether lenses users or not), and to study the inhibitory effect of certain antibiotics on isolates and compare it with the effect natural agents. Also to investigate the antibacterial activity of black and green tea, honey and CFS of *Lactobacillus casei* on Gram negative bacteria that isolated from the eye infections.

Materials and Methods

Bacterial sample

Eye samples collection

A total of 30 eye swabs were collected between November and December 2014 from persons who sufferers eyes mucous discharge (patients who wearing lenses and not wearing lenses previously). Fifteen samples taken from Al-Kadhimiya Teaching Hospital in Baghdad from patients have eye inflammation but do not use lenses and another 15 samples from students wearing soft contact lenses from Al-Mustansiriyha University.

Inoculation of samples

By using appropriate sterile moistened swabs, the discharges from eyes were taken carefully; and placed in sterile saline test tubes, then transported to the laboratory within an hour's. Each swab obtained was inoculated into separate tubes with brain heart infusion (BHI) incubated at 37°C for24 hours.

Identification of the Bacterial isolates

The isolates gently culturing on MacConkey's agar then incubated at 37°C for 24 hrs (Figure

1). According to the manufacturer's instructions, single colonies performed through streaking for identification by Vitek-2 system for final identification (Table 1 and Figure 1).

Antibiotic sensitivity test

The susceptibility of isolates to antimicrobial agents was examined by an agar diffusion method using paper disks containing the following antibiotic concentrations: cefixime (5µg), meropenem (10 µg), Gentamicin (10 µg), chloramphenicol (30 µg), oxytetracyciine (30 µg) and erythromycin (15 µg) Isolates were categorized as sensitive (S), moderately sensitive (I), and resistant (R), based upon the interpretive criteria developed by the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2013).

Assay of antibacterial activity by using agar well diffusion method

After solidification of Petri plates containing sterile Mueller Hinton agar 20 ml, few colonies (2 - 4) from overnight culture were transferred to 2 ml of normal saline to prepare the bacterial suspension of each isolate and were adjusted to (0.5 McFarland standard turbidity) equal to 1.5×10^{8} CFU/ml. Then the plates were swabbed of each bacterium isolate suspension. Thus were left at room temperature for 15 minutes allowing the absorption of the inoculums into the agar. Wells of 5 mm size were made with sterile cork borer into agar plates containing the bacterial inoculums.

Using the micropipette 50 µl volume of the extract was added into a well of inoculated plates. Sterilized distilled water was used as a negative control which was introduced into a well instead of extract. Plates incubated at 37 C^{o} for overnight. The diameter of the zones of inhibition was measured with scale (Kumar *et al*., 2010).

Cell free supernatants of LAB

Isolation of lactic acid bacteria

1 ml of milk sample was collected during lactation from healthy mother in a sterile tube by using sterile gloves. Previously, nipples and mammary areola were cleaned with soap and sterile water. The first drops were discarded. The sample was kept until delivery to the laboratory (Soto *et al*., 2014). Appropriate dilutions of the collected milk sample was made in normal saline and pour plated on MRS agar and incubated at 37°C an aerobically for 24 to 48 hours. At the end of 48 hours, when the colonies became predominant, morphologically distinct and well isolated colonies were picked and transferred to new MRS agar plates by streaking. Colonies showing typical characteristics of lactobacilli on agar surface were picked up randomly and transferred into MRS broth for further enrichment. Further, their purity was checked on MRS agar (Mithun *et al*., 2015).

Identification of *Lactobacillus* species

The growing *Lactobacillus casei* colonies were subjected to the following identification according to their morphological, cultural, and physiological and biochemical characteristics by the procedures as described in Bergey's Manual of Systematic Bacteriology. Colonies differ in morphology, pigmentation; shape and size.

Initially all of the isolates were examined for Gram staining and catalase production. Only the Gram positive, catalase-negative and rod shape isolates were then purified by streak plating using the same medium. After several subcultures, finally the single colonies of *Lactobacillus* was isolated by observing their colony morphology and some biochemical tests (carbohydrate fermentations, arginine

hydrolysis, CO_2 production and growth at different temperatures 15°C, 45°C) (Bhardwaj *et al*., 2012; Davoodabadi *et al*., 2015; Serrano *et al*., 2016).

Preparation of cell- free supernatants

CFS of Lactobacillus were obtained by centrifugation (5000 rpm for 20 min) of 20 ml MRS broth that containing growth *Lactobacillus casei*, the supernatant was filtered through a 0.22 mm filter to remove cells (Al-Fraji, 2013).

Collection of plants

Tea

The dried leaves of SILANI black tea and green tea (*Camellia sinensis*), the granules form was purchased from market in Baghdad, Iraq.

Aqueous extracts preparation

Ten grams of leaves of green and black tea was soaked in 100 ml boiling distilled water for 10 min. Then the extract was soaking for 2 day, filtered from the leaves by using Whatman filter paper No 1. The filtrate was then centrifuged at the highest speed (3000 rpm) for 15 min. Only supernatant was used (Basam *et al*., 2016; Al-Fraji *et al*., 2016).

Collection of honey

Honey

Raw honey samples were collected form Al-Zafaraniyah rural areas in the Baghdad, Iraq. During the collection of honey we used sterile container for transferred to the college laboratory.

Preparation of dilution

Honey was diluted by using sterile distilled water; 10 g of honey was placed in 100 ml of D.W and then mixed well by vortex (Ramalivhana *et al*., 2014).

Results and Discussion

Out of 30 samples collected, 20 were showed positive results. *Enterobacter cloacae* (8) was the main isolated gram-negative bacteria from all ocular infections, followed by *Pseudomonas luteola* (7), *Leclercia adecarboxylata* (3) and (2) *Pantoea agglomerans;* identification by Vitek-2 system are shown in tables 1 and 2 and figure 2. The results of antibiotic susceptibility test of the eye isolates indicated that high proportion of test organisms were resistant to chloramphenicol, erythromycin, cefixime, while showed few isolates sensitivity to oxytetracycline, gentamicin, meropenem (Table 3 and Figure 3). Antibacterial activity of natural agents (Black tea, green tea, honey, CFS of *Lactobacillus casei*) were screened against isolates from different bacterial groups of infected eye, using agar well diffusion method (Figure 4, 5, 6, 7).

Black tea extract which recorded inhibition zone from (10 to 24 mm) (Table 4), these results provide evidence for the presence of antimicrobial tannic acid is an important inhibitor of bacterial growth and phenolic compounds in tea which are useful in the control of common bacterial infections, These compounds can degrade the cell wall, disrupt the cytoplasmic membrane, damage membrane proteins and interfere with membrane-integrated enzymes, which may eventually lead to cell death. Polyphenolic compounds including catechins and flavonoids (quercetin, kaempferol, myricetin and theirglycosides). Green tea extract which recorded inhibition zones ranged from (11 to 25mm) (Table 4). The properties of green tea which inhibit bacterial growth are mainly related to their flavonoids components including epicatechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate.

Table.1 Some general characteristics of isolated bacteria from infected eye in this study

Microorganism	Description and Significance	Classification	Phenotypic Characteristics
Enterobacter cloacae	is a rod-shaped, gram-negative bacteria, normal gut flora of many human, Nosocomial pathogens that can cause a range of infections such as bacteremia, lower respiratory tract infection, Urinary tract infections.	Enterobacteriaceae Family	Non-lactose fermentation on macconkey agar
Pseudomonas luteola	is a Gram-negative, motile by multitrichous flagella, found in damp environments, aerobe. They grow as rods, It is an opportunistic pathogen that can cause bacteremia, meningitis, prosthetietic.	Pseudomonadaceae Family	Non-lactose fermentation on macconkey agar
Leclercia adecarboxylata	is Gram-negative, facultative-anaerobic, peritrich-flagellated bacilli, isolated from food, water and other environmental sources. is a pathogen associated with water environments can causes bacteremia, wound infections.	Enterobacteriacae Family	lactose fermentation on macconkey agar
Pantoea agglomerans	is a facultative anaerobic, rod-shaped bacterium is a Gram-negative bacterium causing wound, blood, respiratory tract infection and urinary-tract infections. It is commonly isolated from animal or human feces. It is reported as both commensal and opportunistic pathogen. The main transmission path is direct or indirect contact with contaminated persons or objects.	Enterobacteriacae Family	lactose fermentation on macconkey agar

Table.2 Source and number the isolates from infected eye

Isolate Source.	NO.
Contact lenses users.	**10**
Non-users.	**10**

Table.3 Antibiotic susceptibility test of the bacterial isolates

Ocular Bacterial Isolates	C	G	E	CXM	MEM	T
Enterobacter cloacae1	R	R	R	R	R	S
Enterobacter cloacae2	R	S	R	R	S	S
Enterobacter cloacae 3	R	S	R	R	R	R
Enterobacter cloacae 4	R	R	R	R	R	R
Enterobacter cloacae 5	R	R	R	R	R	S
Enterobacter cloacae 6	R	R	R	R	R	S
Enterobacter cloacae 7	R	R	R	R	R	R
Enterobacter cloacae 8	R	R	R	R	R	R
Leclercia adecarboxylata 1	R	R	R	R	S	R
Leclercia adecarboxylata 2	R	S	R	R	S	S
Leclercia adecarboxylata 3	R	R	R	R	R	R
Pantoea1	R	S	R	R	S	R
Pantoea 2	R	R	S	R	S	R
***Pseudomonas luteola* 1**	R	R	R	R	R	S
***Pseudomonas luteola* 2**	R	R	R	R	S	S
***Pseudomonas luteola* 3**	R	S	R	R	R	R
***Pseudomonas luteola* 4**	R	R	R	R	R	R
***Pseudomonas luteola* 5**	R	R	R	R	R	R
***Pseudomonas luteola* 6**	R	S	R	R	S	R
***Pseudomonas luteola* 7**	R	R	R	R	R	R

Chloramphenicol, C; gentamicin, G; erythromycin, E; Cefixime, CXM; meropenem, MEM; oxytetracycline, T; Sensitive S; Resistance R

Figure.1 *Enterobacter cloacae, Leclercia adecarboxylata, Pseudomonas luteola, pantoea agglomerans* on MacConkey agar

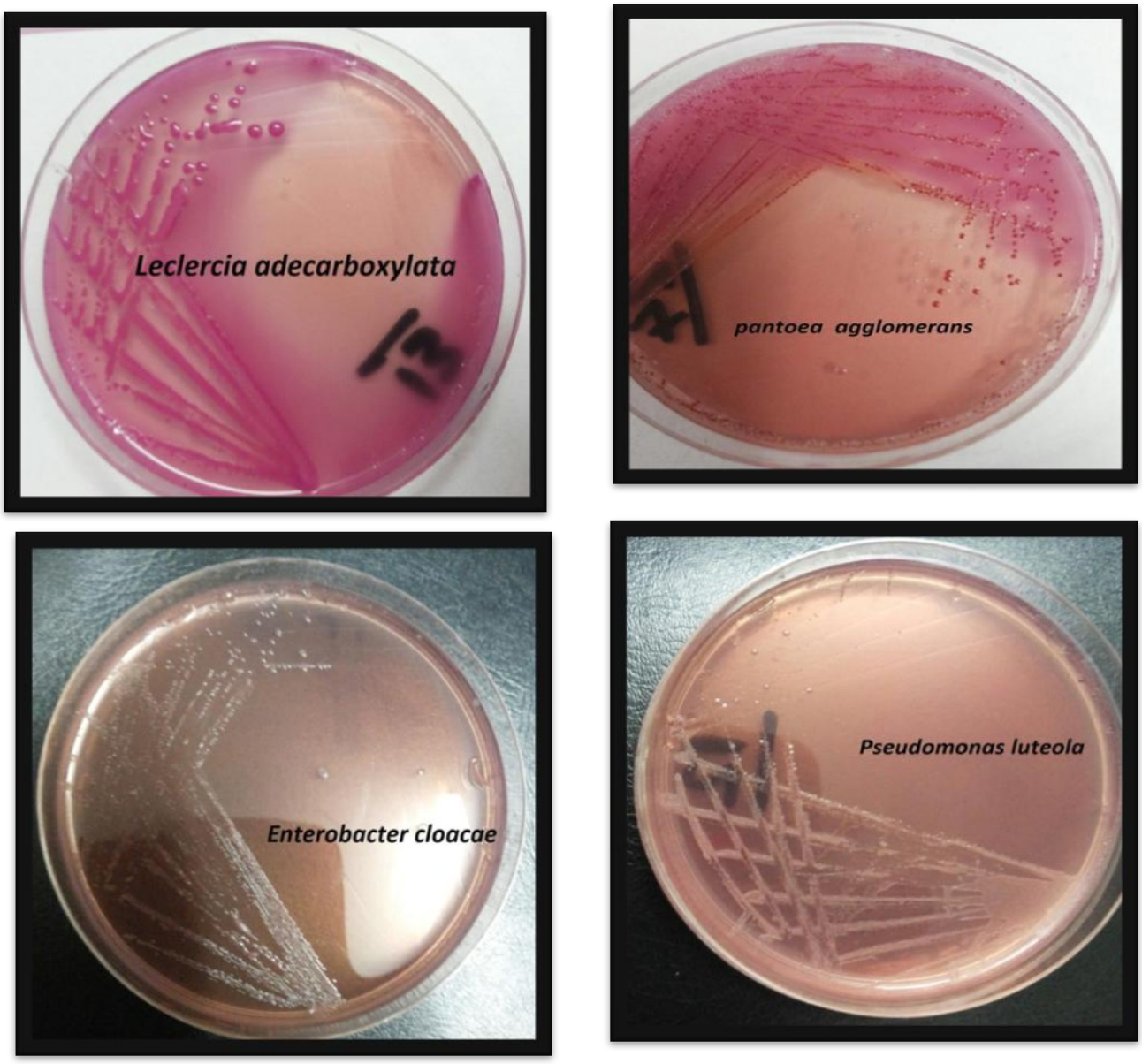


Table.4 The Antibacterial Activity of Natural agents Against Infected Eye Isolates

Isolates	Antibacterial activity			
	Black tea	Green tea	Honey	Probiotic
	Zone of inhibition (mm)			
Enterobacter cloacae 1	11	20	15	15
Enterobacter cloacae 2	12	18	15	16
Enterobacter cloacae 3	12	20	18	15
Enterobacter cloacae 4	10	25	22	23
Enterobacter cloacae 5	12	20	18	16
Enterobacter cloacae 6	10	20	18	13
Enterobacter cloacae 7	15	20	21	18
Enterobacter cloacae 8	13	20	22	20
Pseudomonas luteola 1	11	13	26	20
Pseudomonas luteola 2	11	20	20	15
Pseudomonas luteola 3	12	18	21	15
Pseudomonas luteola 4	14	25	23	15
Pseudomonas luteola5	11	18	20	16
Pseudomonas luteola 6	12	18	30	13
Pseudomonas luteola7	10	17	25	25
Leclercia adecarboxylata1	10	11	27	17
Leclercia adecarboxylata2	10	12	30	18
Leclercia adecarboxylata3	10	12	25	18
Pantoea 1	15	25	30	20
Pantoea 2	24	14	25	20

Figure.2 % the percentage distribution of bacteria in isolates eye

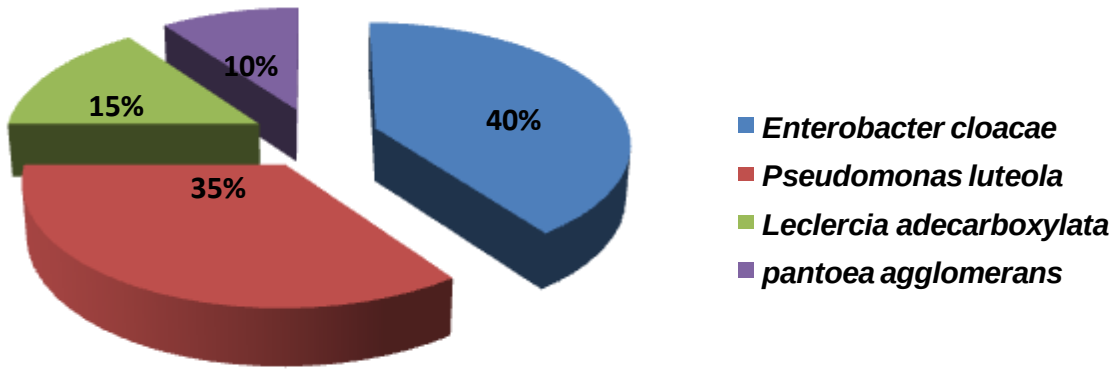
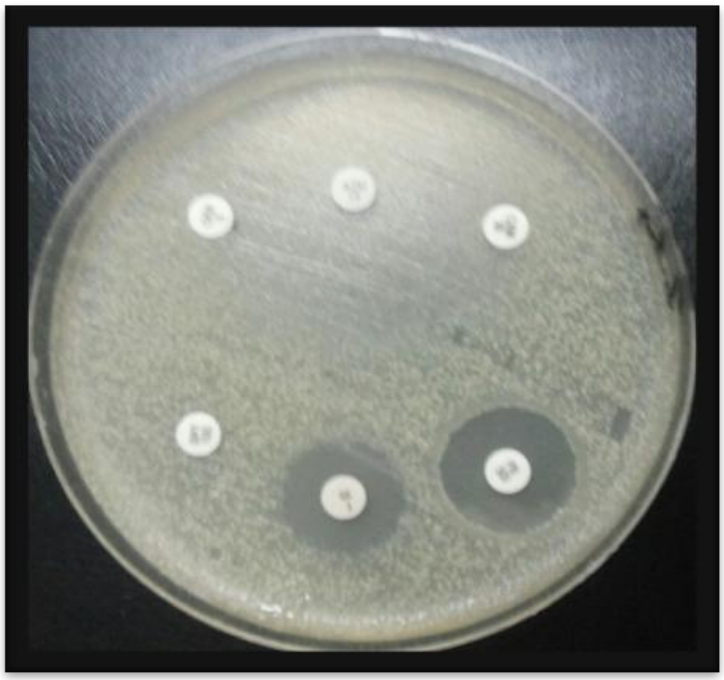


Figure.3 Antibiotic susceptibility test of the bacterial isolate



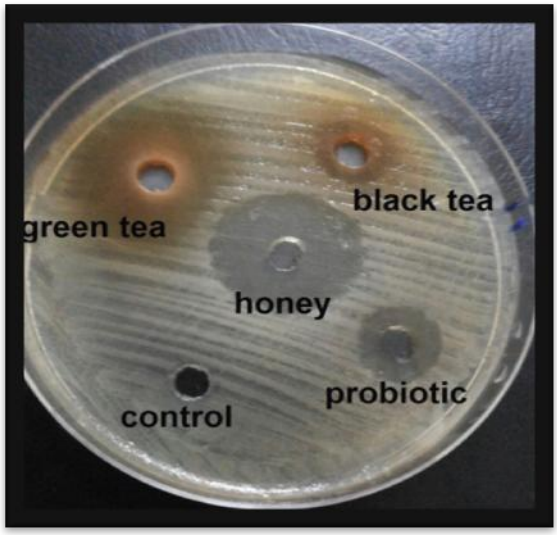


Figure.4 Antibacterial activity of 4 Natural agent against *Enterobacter cloacae*

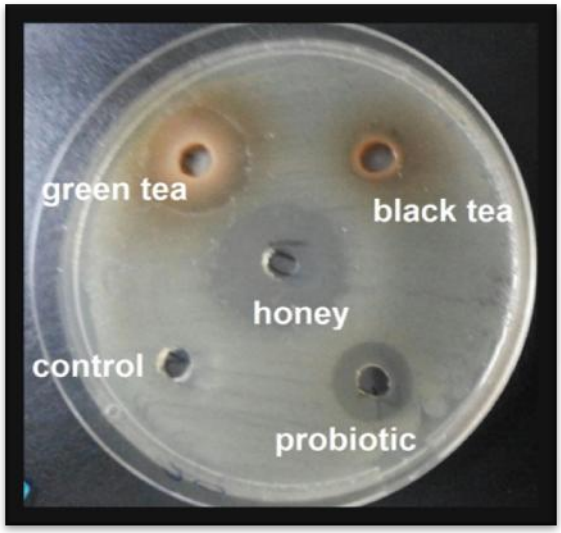


Figure.5 Antibacterial activity of 4 Natural agent against *Pseudomonas luteola*

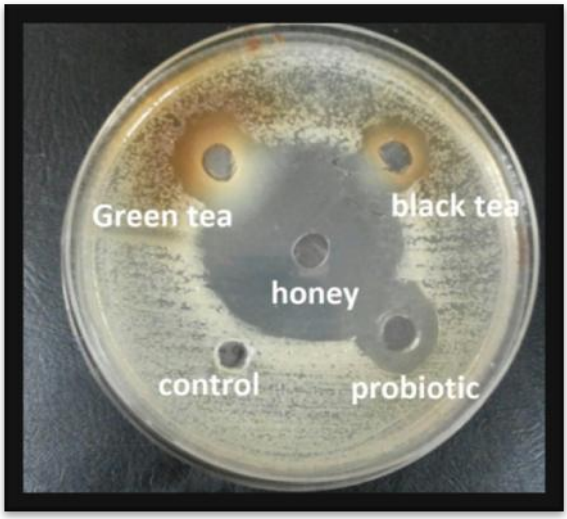


Figure.6 Antibacterial activity of 4 natural agent against *Leclercia adecarboxylata*

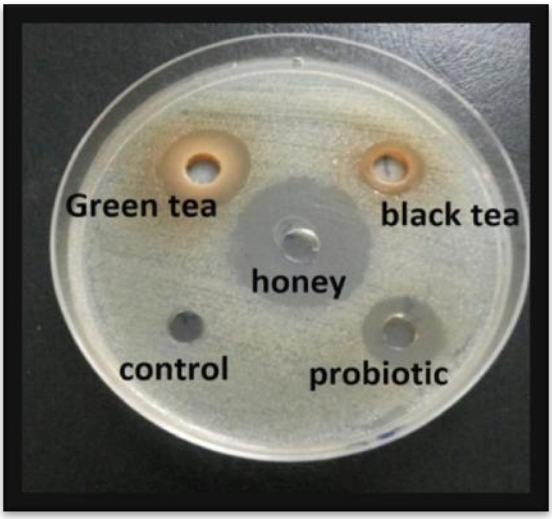


Figure.7 Antibacterial activity of 4 natural agent against *Pantoea*

Mechanism of action of green tea leaves extract has been proposed that green tea can prevent the attachment of pathogenic bacteria on the host cell membrane. Thus, green tea extract inhibits the adhesion of bacteria on host cell surface membranes and acts as a potential anti adhesive agent (Maksum *et al*., 2013). Tea intake is second to water in terms of worldwide popularity as a beverage, green and black tea comes from the leaves of the plant *Camellia sinensis*. The antimicrobial activity of green tea was recognized about 90 years ago (Mervat *et al*., 2007). The boiling water extract of green tea was effective to oral bacteria, especially periodontal pathogens, so its use as mouthwash for the treatment of periodontitis in Pregnant Women (Enas, 2014).

CFS of *Lactobacillus casei* give us from (13 - 25) mm (Table 4). Antimicrobial action of Lactobacilli is production of different antimicrobial metabolites such as organic acids, H_2O_2, bacteriocins [26]. LAB produce lactic acid and other organic acids thus lower the pH of the environment and inhibit the growth of the bacterial pathogens, *Lactobacillus* is penetrate of the bacterial outer membrane. Hydrogen peroxide inhibits both Gram-positive and Gram-negative organisms. While bacteriocin, is family includes a wide variety of peptides and proteins, microbial targets, it has important role in immunity (Pithva *et al*., 2011).

While honey which recorded high inhibition zones ranged from (15 – 30) mm (Table 4). The fact that inhibition of bacterial pathogens by honey was superior, over the most common antibiotics used to treat bacteria, makes them a novel source of anti microbial agents. Previous studies confirmed the antimicrobial activity of honey against a wide range of microbes like multidrug resistant pathogens (Uzma *et al*., 2014).

The antibacterial nature of honey depends on different factors acting singularly or synergistically, which are phenolic compounds, H_2O_2, complex carbohydrates, pH of honey and osmotic pressure exerted by the honey itself also. It has been documented that the pronounced antibacterial activity and bactericidal factors due to presence the methylglyoxal (MGO) in honey (José *et al*., 2014; Paulus *et al*., 2010). In Ghana, it is a

common practice to see traditionalists instilling few drops of the honey on the eyes of patients presenting with conjunctival redness.

In conclusion the results of this study showed:

Novel eye isolates as *Enterobacter cloacae, Pseudomonas luteola, Leclercia adecarboxylata and pantoea agglomerans*.

The isolates were resistant to most antibiotics that used.

Compared to selected antibiotics that used, the natural agents are more effectively on all isolated contrast to antibiotics.

Recommendations

Natural components and the ethnobotanicals have been used since the early days of humankind and are still used throughout the world for health promotion and treatment of disease. Most of drugs prescribed worldwide are derived from plants. For that we recommend Extraction and purification of active substances of natural materials substances that were used in this study and add in the pharmaceutical industry in different ways and forms, and they include the essential oils, eye ointments or even solutions for soft contact lenses under systematic approach to assess their safety and effectiveness. And also need more research studies are required about correlation eye infections with soft contact lenses.

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