RESEARCH ARTICLE

Detection of the Inhibition Activity of Alcoholic Extract of Green Tea Leaves (*Camellia sinensis*) and Zinc Oxide Nanoparticles Against Few Bacteria and Fungi Species Isolated from Eyes Infection

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ABSTRACT

A 80 samples were collected from patients who were suffering from eyes infections in Samarra hospitals, Samarra city during the period from December 20, 2020 to January 20, 2021. All the samples were from male and female aged between 1–75 years. They were cultivated on selective and differential media in the laboratory and stained by gram stain. Some biochemical tests were done as confirmation diagnostic tests. There were 71 (88.75%) samples yielded positive growth that includes *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus pneumonia* 24 (30%), 16 (20%) and 9 (11.25%), respectively. The results show that 7 isolates of *Haemophilus Influenzae,* 6 isolates of *Pseudomonas aeruginosa,* 5 isolates of *Escherichia coli* with percentages (11.25%), (7.5%) and (6.25%) respectively. Four isolates with 5% of *Candida albicans* were isolated from eyes infections. The sensitivity of bacterial isolates was tested using 12 antibiotics that included quinolones, aminoglycosides, beta- lactams and tetracyclines antibiotics. Imipenem and levofloxacin were more effective than the rest of the antibiotics which range from medium to inactive.

The antibacterial impact of alcoholic extracts of *Camellia sinensis* leaves and zinc oxide nanoparticles (ZnO NPs) on certain microbial infections were investigated. Results show that 100% concentration of alcoholic extract gives a high inhibitory effect on all isolated microbes in compares with NPs. The highest inhibition zone is *S. aureus* with a diameter of 35 mm and the less is 10 mm against *S. pneumoniae*. The alcoholic extract shows an effective result against *C. albicans* with a diameter of 15mm. At concentrations of 100% and 150%, zinc oxide nanoparticles inhibit *S. aureus*, *E. coli*, and *C. albicans*, respectively.

Keywords: Alcoholic extract of Green Tea, Antibiotics, Bacteria and fungi, Eyes infections, Zinc oxide nanoparticles.

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INTRODUCTION

The eyes are the most exposed portion of the body to dust and microorganisms such as bacteria, fungus, and viruses that cause eye infections, especially when the tissues of the lining of the eyes are scratched or damaged, as these germs easily settle on the eyes. Tissue breakdown, resulting in substantial eye damage,[1] includes conjunctivitis, sometimes known as "red eye" or "pink eye," which is an inflammation of the sclera's delicate layer. This condition might affect just one eye or spread to both.[2] These infections increase despite the eye's resistance to external effects, which is mediated by a variety of processes including hydration with the bactericidal enzyme lysozyme, mucous membrane motility, and eyelid motion.[3] Infections like conjunctivitis, keratitis, eyelid infections, and other types are responsible for increasing rates of morbidity and blindness over the world.[4] The virulence factors of pathogenic microorganisms that cause eye disorders are reduced by the hosts' resistance basing on variety of factors, including personal cleanliness, livelihood condition, nutrition, heredity, physiology, and age.[5] The eyes can be infected with a variety of viruses, fungi and bacteria, causing different diseases that their symptoms are signs of irritation, redness or inflammation.[6] *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis, Pseudomonas aeruginosa*, *Neisseria gonorrhoeae*, *Moraxella spp. Haemophilus influenzae* and *Streptococcus pneumoniae* are the bacteria that can cause eyes infections.[7]

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Some antibiotics are underused due to the resistance of microorganisms to these drugs and therefore new drugs must be sought. New resistance mechanisms emerge and spread internationally as antibiotics resistance reaches alarming levels threatening to our capacity to treat common infectious diseases. Due to the ineffectiveness of antibiotics, a number of infections are more difficult, if not impossible, to be treated. Antibiotic resistance is more likely to occur and spread when antibiotics are obtained without a prescription for human use. In addition, in countries where normal treatment standards are rarely followed, health workers and doctors frequently prescribe antibiotics that individuals use frequently. And unless we act soon, we will be approaching a post-antibiotic era, in which even small diseases can become lethal. As a result, herbal therapy is one option for treating infections caused by multidrug-resistant bacteria. The utilization of antibacterial plant extracts and phytochemicals could be very beneficial in the future. A number of studies have been undertaken in various nations in recent years to demonstrate this efficacy.[8] *Camellia sinensis* (green tea) is a shrub or evergreen tree that grows up to 16 meters and belongs to the Theaceous family. Green tea is widely consumed due to its numerous health benefits, including anticancer, anti-oxidant, and antibacterial properties, as well as its ability to help people lose weight. It is also regarded by Chinese people as an effective traditional drink that is required for the prophylaxis of a variety of health problems. This is due to green tea's complex chemical composition, which includes polyphenols, alkaloids, proteins, minerals, vitamins, amino acids, and other types of chemical components.[9]

Green tea also has antibacterial activity against gram-positive and gram-negative bacteria, according to an *in-vitro* investigation.[9] Nanostructured materials contain a higher percentage of atoms on their surface resulting in a high level of surface reactivity. As a result, nanomaterials have recently gained prominence in basic and applied sciences as well as bio nanotechnology. Zinc oxide nanoparticles (ZnO NPs) come in a variety of shapes and sizes and they have been shown to have strong antibacterial activity against a wide range of bacteria by a number of researchers.[10]

When the particle size of ZnO is lowered to the nanoscale scale, it can interact with the bacterial surface when it enters the cell and exhibits distinct bactericidal processes.[11] So, isolating and diagnosing the microbes that cause eyes inflammation, determining the susceptibility of antibiotics towards different bacterial isolates, studying the effect of alcoholic extract of *C. sinensis* as antimicrobial agent and comparing its effect with the effectiveness of ZnO NPs are the main concern of this study.

MATERIAL AND METHOD

The research was conducted in Samarra city where 80 clinical samples were collected from patients who visited eyes clinics. The samples were taken from male and female patients under the age of 75 from three areas of the eye (Conjunctiva, Cornea, and Eyelids). Specialist physicians diagnosed all of the patients clinically. The samples were collected using sterile cotton swabs and sent to the laboratory of pathological analysis at the College of Applied Sciences, University of Samarra. All samples were cultivated in primary media (nutrient agar, mannitol salt agar, MacConkey agar, blood agar base, and sabouraud dextrose agar, Himedia-India, Oxoid-Germany) for 24 hours aerobically at 37°C.

Table 1: Represents the positive isolates, their number and percentage.

No.	*Samples*	*Number*	*Percentage (%)*
1	*S. aureus*	24	30
2	*S. epidermidis*	16	20
3	*S. pneumoniae*	9	11.25
4	*H. influenzae*	7	8.75
5	*P. aeruginosa*	6	7.5
6	*E. coli*	5	6.25
7	*C. albicans*	4	5
	Total	71	88.75

Morphological characteristics (colony size, color, rim and height) were apparently checked. Then, gram stain was performed in order to monitor their shapes and arrangement. To confirm the diagnosis, biochemical tests and API 20E and API Staphylococcus were performed.[12]

Candida Diagnosis

Cultural Characteristics

On sabouraud dextrose agar medium, the shape, color, and texture of *Candida* colonies are evaluated.[13]

Microscopic Test

After being dyed with lactophenol-cotton blue stain,[14] the form of yeast cells was studied microscopically and then checked under 40X.[15,16]

Urea Hydrolysis Test

This test was performed to determine whether yeast could create urea or not.

Growth on ChromAgar Culture

A sterile loop to inoculum ChromAgar with the isolated yeast colonies, then incubated at 37°C for 48 hours to assess colony development and color.[17]

Germ Tube Production Test

A small, clean test tubes containing 0.5 mL of human blood serum were inoculum with yeast colonies. The tubes were incubated for 3–4 hours at 37°C, then a drop of the liquid was placed on a clean glass slide, covered with cover slide and checked at 40X for germination tube detection.[18]

Antimicrobial Susceptibility Test

The disc diffusion method was used to assess antibiotics susceptibility patterns against microbial isolates as specified by the Clinical and Laboratory Standards Institute (CLSI, 2021). For each antimicrobial disc, the diameter of the inhibition zone (mm) was determined, and the isolates were categorized as resistant or susceptible.

Table 2: The susceptibility of bacterial isolates to antibiotics

No	*Antibiotic disc*	*E. coli*	*H. influenza*	*P. aeruginosa*	*S. pneumoniae*	*S. epidermidis*	*S. aureus*
1	Amikacin	S	S	R	S	R	S
2	Augmentin	S	S	S	S	S	S
3	Azithromycin	R	R	R	R	S	S
4	Carbenicillin	R	R	R	R	R	R
5	Ceftazidime	R	R	R	R	R	R
6	Clarithromycin	R	R	R	R	R	R
7	Cloxacillin	R	R	R	R	R	R
8	Imipenem	S	S	S	S	S	S
9	Levofloxacin	S	S	S	S	S	S
10	Norfloxacin	S	S	S	S	S	S
11	Tigecycline	R	S	R	S	S	S
12	Tobramycin	R	R	R	R	R	R

Table 3: The susceptibility of fungi isolates to antibiotic

No.	*Antibiotic disc*	*C. albicans*
1	Nystatin	S
2	Fluconazole	S
3	Ketoconazole	S

Preparation of Alcoholic Extract of *C. sinensis* Leaves

One hundred 100 grams of dry *C. sinensis* leaves were weighed and put in a beaker, then 500 mL of ethanolic alcohol was added to it and the mixture was left for 24 hours with continuous shaking every half hour. The next day the solution was filtered by filter papers, after which the solution was placed in a rotary evaporator under vacuum. A viscous semi-liquid extract weighing about 20 g was obtained and the extract was placed in containers with sealed caps under freezing.[19,20]

Preparation of Zinc Oxide Nanoparticles

The weight of (0.1 g) of Zinc Oxide nanoparticles (ZnO NP/10–30 nm/nano skyspring nanoparticles, Inc. 2935 Westhollow Drive • Houston, TX • 77082 • USA) was dissolved in 10 mL (D/W) and placed in a water bath for 5 minutes, then placed in an ultrasonic device for 20 minutes, and the solution was kept in sealed tubes in the refrigerator until later use.

RESULTS AND DISCUSSION

Depending on cultural characteristics of growing microbial on basic, enrichment, selective and differential media, 71 (88.75%) samples yield positive culture, whereas 9 (11.25%) show no microbial growth. The absence of growth may be due to the fact that infectious agents belong to species of anaerobic bacteria that are excluded from the present study or other microorganisms that cannot be diagnosed by routine laboratory methods. Furthermore, the possibility that some patients had doses of antibiotics before taking the sample. The results are in accordance with other researchers[21] who found that 86% of the collected samples were positive culture.

Table 1 shows that 30% of the isolates is due to *S. aureus*. The results of this study are consistent with others[7] who found that the higher percentage of isolates are *S. aureus*. The reason for behind the increasing of the percentage of *S. aureus* may be attributed to its presence as a normal flora on the skin and being opportunistic bacteria that causes infection in immunocompromised patients as well as having many virulence factors. As shown in the same table the percentage of *H. influenzae* is 8.75% while a pervious study[22] revealed that the higher percentage was due to *H. influenza*. This inconsistency may be due to the different geographical areas in addition to the difference in the number of samples. A 9 samples were diagnosed as *S. pneumonia*e. This microorganism is still considered as a major pathogen of human because of its importance as a causative agent of meningitis pneumonia and otitis media. The findings of this study are similar to others[21] which evaluated the study of the antibacterial activity of green tea against bacterial eye infection in Shendi City, showed that *S. aureus*, *S. pneumoniae,* and *P. aeruginosa* bacteria cause eye infection, and that *S. aureus* was the highest among 20 isolates with a percentage of 64.5%. The percentage of isolation of *P. aeruginosa* and *E. coli* is 7.5, and 6.25%, respectively. These two types of gram-negative bacteria are the main causes of many infections, including eye infections. The results of this study disagree with the findings of,[23] who found that the isolates rate was 4.92, and 1.1%, respectively. The same study above showed that the percentage of *C. albicans* isolates are 5.13% which is identical with the current study. Keratitis produced by yeasts such as *Candida spp.* often occur in eyes that have previously been abnormal, such as those with dry eye, persistent corneal ulceration, or corneal scarring.[23]

According to clinical and laboratory standards institute,[24] antibiotics sensitivity has been determined. The results in Table 2 showed that all isolates are resistant to tobramycin, cloxacillin, clarithromycin, ceftazidime, carbenicillin and azithromycin while they are sensitive to imipenem, levofloxacin, norfloxacin and augmentin and their resistance to tigecycline and amikacin are variable. In recent years, it has been highlighted that the incidence of antibiotic resistance bacterium infection has increased dramatically in many parts of the world. For example, some researchers[25] reported that *P. aeruginosa* infections are difficult to treat since this

Table 4: The results of microbial activity of Alcoholic extract and ZnO NPs

Treatment	*con. %*	*S. aureus*	*P. aeruginosa*	*C. albicans*
Alcoholic extract	100	35 mm	30 mm	15 mm
	75	30 mm	25 mm	12 mm
	50	10 mm	10 mm	10 mm
	25	0 mm	0 mm	0 mm
ZnO NPs	150	26 mm	22 mm	15 mm
	100	15 mm	12 mm	0 mm
	75	11mm	10 mm	0 mm
	50	8 mm	8 mm	0 mm
	25	7 mm	6 mm	0 mm

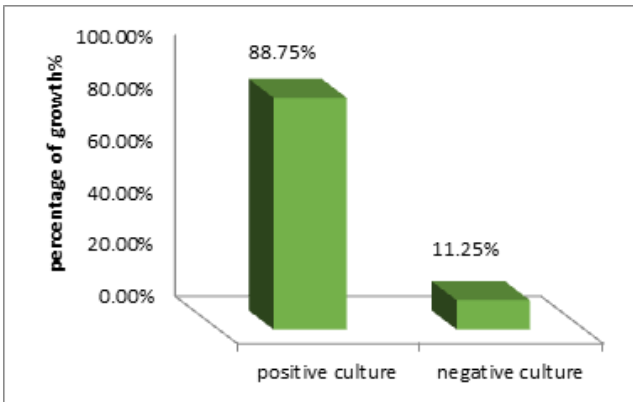

Figure 1: Percentage of the microbial growth.

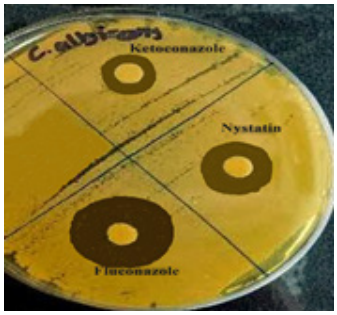

Figure 2: *C. albicans* with different antifungal discs in SDA.

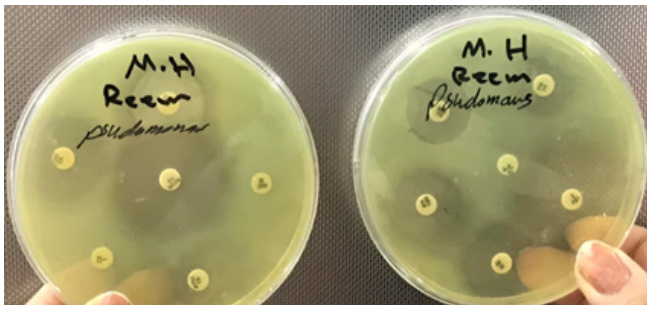

Figure 3: P. aeruginosa in Mueller Hinton agar with antibiotic disc.

bacterium has a high level of intrinsic antibiotic resistance. According to the study, he also discovered that fluoroquinolone antibiotic treatment resulted in substantial increases in the

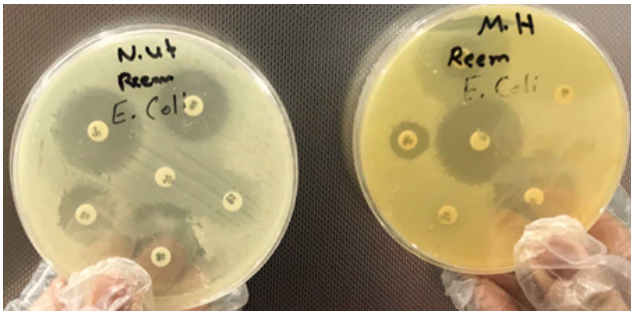

Figure 4: *E.coli* in mueller hinton and nutrient agar with antibiotic discs.

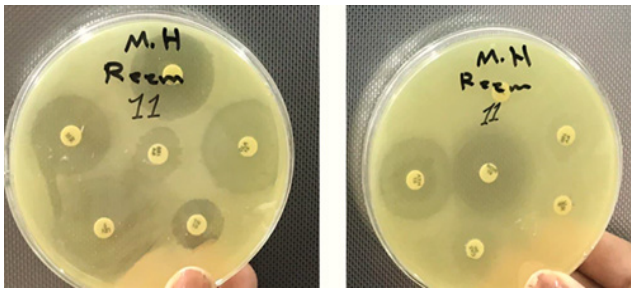

Figure 5: *S. epidermidis* in mueller hinton agar with antibiotic discs.

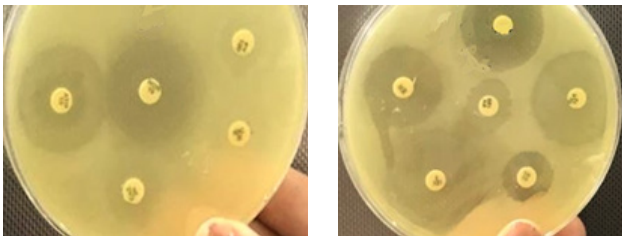

Figure 6: *S. aureus* in mueller hinton agar with antibiotic discs.

MIC, which could be linked to treatment failure. With the prevalence of resistance transfer factors (RTF), antibiotic resistance in enterobacteriaceae and some gram-positive cocci undergoes a notable shift in characteristics. This increasing resistance has motivated many researchers to find alternatives such as nanomaterials in order to reduce the spread of bacterial resistance to antibiotics.[26]

As for Table 3, it shows the activities of antifungals against *C. albicans*, that are illustrated by Figure 2, the anti-fungal fluconazole has more inhibitory ability than the others. This finding is comparable to other studies.[27]

Alcoholic Extract of *C. sinensis* Leaves Effect

S. aureus and *P. aeruginosa* have been selected as a model of gram positve and gram negative. *C. albicans* was also chosen as a yeast model to see how the alcoholic extract of *C. sinensis* leaves and ZnO NP affected it. A series of concentrations (100, 75, 50, 25)% were prepared from the previously prepared alcoholic extract of *C. sinensis* leaves and dissolved in the organic solvent DMSO. microbial isolates were activated using nutrient broth for 24 hours by incubating them at 37°C.

The results showed in Table 4 that the inhibitory action of alcoholic extract of *C. sinensis* leaves on *S. aureus* were (35, 30, 10, 0) mm in concentration of (100, 75, 50, 25)%, respectively.

While the effect of the same extract on *P. aerugenosa* were (30. 25. 10. 0) mm in concentration of (100, 75, 50, 25)%, respectively. These results are consistent with those of other styudies,[19] who discovered that the antibacterial activity of methanol and aqueous extracts of *C. sinensis* leaves on *L. monocytogenes* was 20 mm. Also these results of the current study agree with others.[28] Table 4 also reveals the antifungal activity of alcoholic extract of *C. sinensis* on the pathogenic yeast.

Green tea contains polyhydroxyphenol chemicals called polyphenols. It is considered the primary active ingredient and has a number of pharmacological actions, including the elimination of free radicals and anticancer activity. However, the polyphenols' poor lipid solubility, limited bioavailability, and ease of oxidation, restrict their use in the food and pharmaceutical industries. Currently, innovative preparation methods including nano-emulsion and liposomes are used to increase the absorption of tea polyphenols into the cells of the human body.[29]

Epigallocatechin gallate (EGCG) has been demonstrated in numerous studies to be the most efficient antibacterial polyphenol at normal concentrations. Although the specific mechanisms of EGCG's antibacterial effect are unknown, it is thought that EGCG breaks the cell membrane and hinders DNA super coiling, resulting in the bacterial cell's demise. *In-vitro* studies show that EGCG has an effect on fungal pathogens, gram-positive bacteria, and gram-negative bacteria, with gram-positive bacteria being particularly sensitive. The cell wall distinguishes gram-positive bacteria from gram-negative bacteria. The gram-positive cell wall is made up of numerous layers of peptidoglycans that are connected together to form a thick, stiff wall, whereas the gram-negative cell wall is made up of a thinner peptidoglycan wall with an extra membrane covering it. Lipopolysaccharides (LPS) and lipoproteins, which are essential for the bacteria's survival under extreme bacterial pressure, are found in this outer membrane.[30] Green tea (catechin) has antifungal action against many strains of *C. albicans*.[31]

Zinc Oxide Nanoparticles Effect

The inhibitory effect of ZnO NPs was determined using the well diffusion method. The inhibitory action against isolated microbiological species *S. aureus* and *P. aeruginosa* at concentrations of (100, 75, 50, 25)% is shown in Table 4. While *C. albicans* shows no effect against the four concentrations mentioned above. The concentration of 150% shows a good activity of inhibition. The electrostatic attraction among negatively charged bacterial cells and positively charged nanomaterials is the main advantage of NPs as antibacterial inhibitors. In gram-positive and gram-negative bacteria, teichoic acid and lipoproteins are found.[32] The NPs can enter the cell after the affinity process and produce hydrogen peroxide H_2O_2, which kills the cell. While the resistance may be due to the basic differences between the bacterial strains in terms of the genetic components and the enzymes they possess, their acquisition of antibiotic resistance mechanisms especially when isolated from the hospital, the size and shape of the NPs utilized, the surface area of the molecules, their solubility, chemical composition, and particle permeability. All of these variables play a significant impact in deciding how bacteria respond to these compounds biologically.[33] In addition, a number of bacterial species are naturally adaptable to resist toxins or NPs in the environment that being adapted to such molecules.[34] The current findings are consistent with other studies.[35,36] which found that ZnO NPs had inhibitory activity against bacterial species. However, the concentrations used in each study differed, which could be due to the different types of bacteria or NPs used. The majority of researches, including,[37] found that raising the amount of ZnO NPs increased microorganism inhibition, which was confirmed in our investigation.

The findings are similar to those of others,[38] who found that nanoparticles have antifungal activity against *C. albicans* through causing damage to the cell membrane structure and inhibiting normal budding as a result of destroying the membrane's integrity, and that increases the concentration of ZnO NPs and consequently increases the inhibition. As well as the findings of others[39] who demonstrated the inhibitory activity of ZnO NPs against yeast *C. albicans*.

ZnO NPs dissolve cell membranes and accumulate in the cytoplasm, where they interact with biomolecules, inducing cell death by apoptosis.[40]

CONCLUSION

The most common bacteria that causes eyes infection is *S. aureus*. Gram-positive bacteria are more sensitive to *C. sinensis* leaves than gram-negative bacteria. The concentration of alcoholic extract in 100% is more effective than other concentration against bacteria and yeast. Increasing the concentration of ZnO NPs to 150% increases the inhibition of microorganisms. *C. albicans* showed a need for a higher concentration of ZnO NPs compared to bacteria. *C. sinensis* leaves is more effective comparing with ZnO NPs in the same concentrations.

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