Antimicrobial Activity of Orange Extract on Selective Oral Biofilm Bacteria

Anjum Jabeen [1], Dr.Muhammad Rauf Tahir[2] , Uzma Ramzan[3], Sidra Nazeer[4], Zunnu Raen Akther[5], Dr iram liaqat[6]

[1] Department of Zoology, Government College University Lahore.
[2] Allama Iqbal Medical College, Lahore.
[3] Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad.
[4] National Institute of Food Science and Techonology, University of Agriculture, Faisalabad.
[5] Department of Entomology, University of Agriculture, Faisalabad.
[6] Department of Zoology, Government College University Lahore.

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Abstract- This study was planned to identifying the Microorganisms are present in oral cavity as normal microflora. However the fact that they become harmful due to some oral infections made us to investigate the inhibitory effect of orange juice extract on them. Total 20 samples were collected. Samples were taken from patients suffering from throat infection. On the basis of morphological variations 6 strains were selected and were tested for their sensitivity against orange juice extract. Some strains are resistant but 3 strains were sensitive against orange juice extract. Aqueous layer of juice extract proved to be more effective in their antibacterial activity on studied strains as compared to methanolic ones. Minimum inhibitory concentration (MIC) values of these extracts were also determined. Moreover, these fruit extracts also tested for their antibacterial activity against clinical isolates and proved to have significant inhibitory effect on growth of these isolated bacteria, providing an alternative to treat various infections caused by these antibiotic resistant strains. Hence this study also enhances the nutrition value of orange juice because orange juice provides the resistance against some kind of oral bacteria.

Index Terms- orange, nutrition value, oral bacteria

I. INTRODUCTION

Microorganisms have important effect on human health to environment and to our economy. Some of them have beneficial effects without which we could not exist but others have harmful effects. Our battle overcome their harmful effects our understanding and ingenuity to the limit. Saprophytic decomposers play an important role in breaking down dead organic matter in ecosystem but on the other hand these microorganisms also responsible for food spoilage and subsequent illness so microorganisms can be beneficial or harmful depending upon what we want from them. (Kroes *et al*., 1999).

Disease and decay are results from physical damage or being eaten by insects it is microorganism that bring about these changes; these are not the inherent properties of organic objects. Many microorganisms are capable of invading in our body and causing human disease and some of them cause diseases in farm animals. (Beighton et al., 2010)

New compounds from plants which have some kind of vegetables and fruits lead synthetic antimicrobial compounds which have ability toprevent the resistance of pathogenic bacteria to antibiotics. It is necessary to evaluate the potential of folk medicine on scientific basis if we want to use folk medicine for the treatment of common infectious diseases. (Fabricant and Farnworth, 2001).

The presence of a resident microflora is not essential for life, but is an important component if the host is to have a normal existence. Information on the beneficial role of the resident microbial flora has come from early studies comparing the physiology of germ-free and conventional laboratory animals, and from humans in whom the normal flora has been disrupted by long-term administration of antibiotics. Most studies have focused on the gut microflora but, in general, such findings are also relevant to the mouth (Lamont *et al*., 2000).

One of the most popular fruits around the world is oranges. It is their juice that is most associated with good health having a reputation for being an integral part of healthy breakfast, while they are as a snack or as a recipe ingredient for many Americans. Oranges are round citrus fruits with textured skins and their color just like pulpy flesh. They usually two to three inches in diameter. Oranges are classified into two general categories sweet and bitter with former being type most commonly consumed. Valencia Navel and Jaffa oranges as well as the blood oranges are popular varieties of the sweet oranges (*Citrus sinensis*), smaller size hybrid species are more aromatic in flavor and has red hues running throughout its flesh. Bitter oranges serve as flavoring for liqueurs such as Grand Marnier and Cointreau or used to make jam or marmalade also. (Guarnieri *et al*., 20017). Against pimples, wrinkles, acne, blackheads and blemishes are good antioxidants. In glowing of skin oranges along with other products are helpful.Oatmeal and honey mix with the paste of orange juice and apply on the face and leave it for 20 minutes then wash it Orange peel powder and milk also used to make skin beautiful. Gentleness of skin increased when orange juice rubs on face since they have

adequate amount of vitamin C. For blemishes and prevention of acne eating of oranges is also good.(Abeysinghe *et al*., 2010).
The aims of this study was to identify and characterize the microorganism mostly present in oral cavity, to test the antibiotic resistance profile of isolated clinical strains, to find out the antibacterial activity of orange juice against oral bacteria either the orange juice have any ability to resist the bacteria present in oral cavity.

II. Material and Methods

Bacterial sample collection:

Total 20 samples were collected from patients including both male and female of Mayo hospital Lahore. Samples were collected from mouth specifically tong and buccal region of patients having throat infections with the help of sterilized swab. The swabs were immediately dipped in 1ml of 0.85% saline solution. Within 2-3 hours of sample collection, 100µl of each sample was spread on nutrient ager plates (Prepared by 7g nutrient agar in 250ml distilled water).100µl of each sample was pour with the help of micropipette and spread on nutrient agar plates (Each plate containing 15µl nutrient agar) with sterilized glass spreader and the plates were incubated at 37^{o} C for 24 hours. After 24 hours of incubation, morphologically different bacterial colonies were selected and purified on agar plates after several hours of streaking and restreaking.

Table 1. Nutrient-Agar

Sr. No	Component	gL^{-1}
1	Nutrient broth	13
2	Agar	15

pH adjusted to 7.0

Morphological Characterization of Isolates

Following Gerhardt *et al.* (1994), purified colonies were grown on nutrient agar and characterized them on the basis of size, shape, margins, elevation, colour, texture etc.

Gram staining (Gerhardt *et al*., 1994)

Bacteria react differently to a specific staining process because they different from one another physically and chemically. Staining depends on the fact that bacteria differ chemically from their surroundings and thus can be stained to contrast with their environment. The Gram stain is extensively used for this purpose. The procedure involve is taking a clean glass slide and adding a drop of water on it. With the help of sterilized inoculating loop added a small amount of bacterial sample on clean slide and making a thin smear. Smear was heat fixed by passing the slide 2-3 times over the flame. The smear was stained with the basic dye crystal violet for 1-2 minutes. This is referred as primary staining. The smear was then treated with iodine solution for 1 minute, iodine solution act as mordant (enhance the interaction between dye and bacterial cell). The smear then wash with the decolorizer the crystal violet-iodine complex was retained by gram positive bacteria while gram negative bacteria appeared colorless as they lose their crystal-violet iodine complex. The smear was counterstained with safranin for about 30 seconds. Safranin did not change the deep purple color of gram-positive bacteria, while colorless gram negative bacteria were stained pink with it. Thus, gram negative bacteria appeared pinkish to red in color while gram positive bacteria appeared dark purple under the microscope with an oil immersion lens.

Solution for Gram's staining
Solution I

Table 2.Crystal violet solution

S. No	Components	Quantity
1	Crystal violet	10g
2	Ammonium oxalate	4g
3	Ethanol 20%	500ml

Solution II

Table 3. Iodine Solution

S. No	Components	Quantity
1	Iodine	1.25g
2	Potassium iodide	2.5g
3	Ethanol	156.2g

Solution III

Table 4 Safranine solution

S. No	Components	Quantity
1	Safranin	4g
2	Ethanol 95%	500ml

Acid-Fast staining (Benson, 2002)

It is the differential staining techniques which was first developed by Ziehl and later on modified by Neelsen. So this method is also called ***Ziehl-Neelsen staining*** techniques. Neelsen in 1883 used Ziehl'scarbol-fuchsin and heat then decolorized with an acid alcohol, and counter stained with methylene blue. Thus Ziehl-Neelsen staining techniques were developed. The main aim of this staining is to differentiate bacteria into acid fast group and non-acid fast groups. This method is used for those microorganisms which are not staining by simple or Gram staining method, particularly the member of genus *Mycobacterium*, are resistant and can only be visualized by acid-fast staining.

Table 5 Ingredient for acid fast staining

Application of	Reagent	Cell colour	
		Acid fast	Non-acid fast
Primary dye	Carbolfuchsin	Red	Red
Decolorizer	Acid alcohol	Red	Colorless
Counter stain	Methylene blue	Red	Blue

Procedure involve is that Prepare bacterial smear on clean and grease free slide, using sterile technique. Allow smear to air dry and then heat fix. Cover the smear with carbolfuchsin stain and placed over boiling water or steam for 5 minutes. Allow the heated stain to remain on the slide for 5 minutes .Afterward the slide was cooled and rinsed with decolorizing agent such as acid alcohol for about 5 minutes. well with clean water. Few drops of malachite green on the smear stain for 1–2 minutes using the longer time when the smear is thin. Wash off the stain with clean water. Wipe the back of the slide clean, and place it in a draining rack for the smear to air-dry. Examine the smear microscopically, using the 100 X oil immersion objective.

Solution for Acid-Fast Staining

Table 6 Ziehl-NeelsenCarbol-Fuchsin Solution

S. No	Components	Quantity
1	Basic Fuchsin	2.5g
2	Distilled water	250ml
3	100% alcohol	25ml
4	Phenol crystals, melted	12.5ml

Table 7. 2.1% Acid Alcohol

S. No	Components	L^{-1}
1	Hydrochloric Acid	10ml
2	70% Alcohol	990ml

Table 8 Methylene Blue Working Solution

S.No	Components	Quantity
1	Methylene blue stock	5ml
2	Distilled water	45ml

Motility test (Benson, 2002)

The motility of bacteria was tested by inoculating tubes containing semi solid agar medium. The bacterial culture was stabbed into medium tubes with inoculating needle and the tubes were incubated at 37^0 C for 24 hours. The growth of non-motile bacteria was confined to the stab line while the growth of motile bacteria was not restricted to the stab line of inoculation.

Table 9 Semi solid medium for motility (Benson)

S. No	Components	gL¯1
1	Tryptone	10
2	Yeast extract	5
3	Sodium	5
4	Agar	10

Fruit Material

Oranges (Citrus sinences) was used for the preparation of fruit extract.

Preparation of fruit extract

Methanolic extracts

Oranges were washed with distilled water and then their weight was determined by using weight balance. Then their juice was taken. After this fresh juice was filtered by using Whatsman Filter Paper (1), then again their weight was determined. Methanolic extract was prepared by mixing equal amount of orange juice and methanol 1:1. After mixing this, mixture was poured into a separating funnel. After vigorously shaking, three layers were separated. Collected three layers separately into three different clean beakers. The first layer called as aqueous layer which contains only polar compounds. Second layer called as central layer which contains both polar and non-polar compounds. Third layer called as Non polar layer. All three layers were subjected to dry in sunlight or to air dry for the collection of extract powder.

After getting powder, aqueous layer was used for making stock solution. Stock solution was made by adding 1g of extract powder into 5ml of distilled water. From this stock solution, 4 dilutions 100%, 50%, 25%, 12.5% were made by adding 0.5ml, 0.25ml, 0.125ml and 0.0625ml of juice into 5ml of distilled water respectively.

ANTIMICROBIAL ACTIVITY OF FRUIT EXTRACTS

Antimicrobial activity of fruit extract was determined by agar well diffusion method. To check bacterial susceptibility on different dilutions (100%, 50%, 25%, and 12.5%) of methanol fruit extracts were made. 10µl of bacterial culture was spread on labeled nutrient agar plate with the help of sterilized glass spreader. Well were made by using sterilized borer. Each well labeled for different dilutions was filled with 50µl fruit extract. Then plates were incubated at 37°C for 24 hours. After incubation the zones of inhibition were measured by measuring three values and taking mean value.

MINIMUM INHIBATORY CONCENTRATION

MIC is defined as the lowest concentration of fruit extract which inhibit the growth of microorganism. MIC of extracts was determined by broth dilution method. The methanolic extract of orange was diluted to the concentration

ranging from 6mg to 46mg ml^{-1} in 3ml nutrient broth. 150µl of overnight bacterial suspension with turbidity of 0.5 was inoculated into the test tubes. The tubes ere incubated for 24 hours at 37°C. After incubation O.D was determined at 590nm. MIC was recorded as the lowest concentration which showed no visible growth.

III. STATISTICAL ANALYSIS

Results obtained in these experiments were analysis statistically following. Means and standard errors of the means were calculated. The difference between the means of minimum inhibitory concentrations were collected using‘t’ test.

IV. RESULTS

ISOLATION OF BACTERIAL STRAINS

Out of 20 samples taken from patients suffering from throat infections 6 bacterial strains were isolated and then purified on nutrient agar. Through Morphological characterization of 6 strains isolated from throat 6 clearly different strains were selected out of which 1 strain was yellow, 2 were light yellow, 1 was white, 2 were transparent. Three strains showed smooth surface, 1 had wrinkled and 2 had shiny surface. Among the 2 strains, 1 exhibits the circular shape, entire margin and raised elevation. Moreover out of six strains 3 were moist, 2 were buttery and 1 was dry in their texture.

Table 10: Analysis of colony morphology of oral microbial isolates

Strains	Form	Color	Texture	Margins	Elevation	Optical character	Surface
A1	Circular	Yellow	Buttery	Entire	Raised	Yellow	Shiny
A2	Circular	Light yellow	Buttery	Entire	Raised	Light yellow	Smooth
A3	Circular	Light yellow	Moist	Entire	Raised	White	Smooth
A4	Circular	white	Moist	Undulate	Raised	Light yellow	Wrinkled
A5	Circular	transparent	Moist	Entire	Raised	Transparent	Shiny
A6	Circular	transparent	Dry	Entire	Raised	Transparent	Smooth

MORPHOLOGICAL CHARACTERIZATION OF ISOLATES

Gram staining

In gram staining , 2 strains appeared dark purple under the microscope with an oil immersion lens indicating that they were gram positive (Figure4.1) while the remaining 4 strains appeared pinkish to red in color showing that they were gram negative (Table-4.3).

Acid Fast Staining

All 6 selected strains showed blue color under 40x light microscope indicating negative results and thus confirming absence of Mycobacterium among tested isolates (Table-4.3).

Motility test

In motility test, growth of 3 strains [A1, A2 and A4] was not confined to the stab line of inoculation showing that these strains were motile, while the rest of 3 strains were found to be non-motile (Table-4.2).

Table 11: Morphological characterization

S. No	Strains	Gram staining	Acid fast staining	Motility
1	A1	-	Blue	Non motile
2	A2	-	Blue	Non motile
3	A3	-	Blue	-
4	A4	-	Blue	Motile
5	A5	+	Blue	-

ANTIBACTERIAL ACTIVITY OF PLANT EXTRACTS

Antibacterial activity of fruit extract was determined against all the 6 strains. Among four dilutions that were made, 100% dilution of all extract was found to be most effective. In case of orange juice extract the diameter of zone of inhibition 1.5-6.25mm.When we take 500µl juice extract in wells maximum zone of inhibition is shown by *Klebsillaspp* that is 6.25±0.5mm and smallest zone of inhibition is shown by Lactobacillus *spp.* which is 3±0.816mm *Salmonella spp* shows in between zone of inhibition which was 4.25±0.5mm. When we take the amount of juice extract is 250µl then maximum zone of inhibition was shown by *Klebsillaspp* which is 5.5±1.290mm and smallest zone of inhibition is shown by *Lactobacillus spp* which is 2.25±0.957mm and salmonella shows zone of inhibition 3.25±0.957mm which is intermediate zone of inhibition between first two bacterial species. When we use the amount of juice extract is 125µl in wells then again maximum zone of inhibition is shown by *Klebsilla spp*. Diameter of zone of inhibition is 3.5±0.577mm and *Salmonella spp.* was shown the intermediate zone of inhibition whose diameter is 3±0.186mm. When we use

the 62.5µl concentration of juice extract in wells then smallest zone of inhibition is shown by *Lactobacillus* 1.25±0.5mm and *Salmonella spp* shows the diameter of zone of inhibition is 1.25+0.5mm and *klebsilla spp*shows the diameter of zone of inhibition is 3±0.816.

Table 12: Effects of methanolic extract of fruits on bacterial isolates

Fruit extract used	Strain	Zone of inhibition in mm(ZI)± S.D			
		12.5%	**25%**	**50%**	**100%**
Orange	*Klebsiella spp.*	3±0.816	3.5±0.577	5.5±1.290	6.25±0.5
	Salmonella spp	2.25±0.5	3±0.816	3.25±0.957	4.25±0.5
	Lactobacillus spp.	1.25±0.5	1.75±0.5	2.25±0.957	3±0.816

aq: aqueous; meth: methanolic
ZI: Zone of inhibition; SD: Standard Deviation

MINIMUM INHIBITORY CONCENTRATION (MIC)

MIC of fruit extract showing antibacterial activity were determined and broth dilution method was used to determine the MIC of fruit extract (Table-4.7). Result indicated that MIC values of aques layer of orange juice extract were in the range of 15-25mg ml^{-1} for different selected strain. The MIC value against *Klebsilla spp* was 20mg ml^{-1},15mg ml^{-1} for *Lactobacillus*and 25mg ml^{-1} for *salmonella spp.*

Table 13: Minimum Inhibitory concentration (MIC) of fruit extract

Fruit Extract	Strains		MIC Mg ml^{-1}
Orange	A1	*Klebsiella spp.*	20
	A4	*Salmonella spp*	25
	A5	*Lactobacillus spp.*	15

V. Discussion

Bacterial species were identified by using standard laboratory criteria (Colony morphology, Texture, Color, Catalase production, Hemolysis pattern etc.) as well as through biochemical testing. Isolates showed resemblance in morphology and biochemical characteristics but they also exhibited some diversity. They showed circular/irregular margin (Table-4.2). The selected strains also showed different hemolytic patterns on blood agar. All strains were good in growth on chocolate agar (Table-4.4). The bacterial ability to exhibit morphological variation may be an adaptation to thrive in a wide range of environmental conditionsJiang (2007), On the basis of biochemical testing, the strain isolated from clinical sample belong to the genus *Klebsiella*, and *Salmonella* (Table- 4.6). Similar results were reported byKeskin (2010), who found that gram negative bacteria belonging to the family Klebsiella, Enterobacter were the most common cause of oral infections. However gram positive bacteria such as *staphylococcus spp* and *Lactobacillus spp* also play their role in causing these infections. According to Noack (2001), the microorganism causing oral infections vary from time to time. These infections may be monomicrobial or polymicrobial.

Fruits extracts were effective in decreasing the capacity of biofilm formation showed by studied strains susceptibility of oral bacteria against fruit extract (Table- 4.6). According to study by Percival (2010) fewer fractions of terpineol and other terpene citrus oil appeared to have greater inhibitory effect on food-borne bacteria than other citrus oils or derivatives. As compare to gram negative bacteria gram positive bacteria were more sensitive to essential oils in general.

The purpose of this study was to identify bacteria present in oral cavity of those people who suffering from some kind of oral infections and monitoring the inhibitory effect of fruit extract against them. Oral infections causing clinical isolates usually cause serious infection and are very tough to eradicate mainly because of the development of high resistance against commonly used antibiotics. Hence, there is an immediate need to find out an alternative to treat these microbial infections. Fruit extract prove to have remarkable antibacterial activity against antibiotic resistant bacteria. In this study, three clinical isolates i.e. *Klebsialla spp, Salmonella spp*and *Lactobacillus* were found to have potential oral infection capacity. Alcoholic extract of orange juice was tested for their antibacterial properties against these isolates. This fruit extract proved to have significant antibacterial activity and thus provided a way use them as an alternative to treat different infection caused by antibiotic resistant, biofilm forming clinical isolates

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AUTHORS

First Author – Anjum Jabeen, Department of Zoology, Government College University Lahore, Pakistan. anjumjabeen36@yahoo.com
Second Author – Dr. Muhammad Rauf Tahir, Allama Iqbal Medical College, Lahore, Pakistan. rauftahir26@gmail.com
Third Author – Uzma Ramzan, Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad, Pakistan. Uzmaramzan99@gmail.com
Fourth Author – Sidra Nazeer, National Institute of Food Science and Techonology, University of Agriculture, Faisalabad. Sidranazeer719@gmail.com
Fifth Author – Zunnu Raen Akther, Department of Entomology, University of Agriculture, Faisalabad, Pakistan. Zunnuraen@gmail.com
Sixth Author – Dr iram liaqat, Department of Zoology, Government College University Lahore, Pakistan. Iramlqt@gmail.com