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Study of Antibacterial Activities of Selected Fruit Peels against Gram-positive Bacteria

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ABSTRACT: The antibacterial activity in peels of Citrus fruits was examined by Agar well diffusion method. The effect of different solvents on the antibacterial activity of lemon, orange, and grapefruit collected from different cities of Punjab such as Lahore, Burewala, and Rahim Yar Khan was studied.The antibacterial activity of fruit peels was found to be significantly influenced by solvents. Methanol extracted samples showed more antibacterial activity than acetone extracted samples. Samples collected from Lahore showed greater antibacterial activity as compared to the other cities. The use of these bioactive-rich citrus residues could provide a cost-effective, environment-friendly forum for the development of new nutraceuticals or the enhancement of existing ones. The bioactive compounds of Citrus peels and their mechanism of actionare discussed in this paper.

Abbreviations

ATP	Adenosine triphosphate
B. subtilis	Bacillus Subtilis
DNA	Deoxyribonucleic Acid
FVW	Fruit and vegetable waste
GSE	Grapefruit seed extract
NADH	Nicotinamide adenine dinucleotide
nh	Neohesperidose
OEOs	Orange essential oils
PG	Peptidoglycan
ROS	Reactive oxygen species
rut	Rutinose
S. aureus	Staphylococcus aureus
SSTI	Skin and Soft TissuesInfection
ZOI	Zone of Inhibition

CHAPTER NO. 1

1.Introduction

Citrus fruits belong to the Rutaceae family. They usually compriseof 140 genera and 1,300 species. Vitamin C is an essential nutrient found abundantly in citrus fruits and vegetables. Antibacterial properties of the peel extract of Citrus limon, Citrus sinensis, Citrus. Aurantium, Citrus reticulata, and Citrus paradisiwere examined against certain Gram-positive bacteria (Bacillus spp, Staphylococcus aureus). Citrus fruits are mainly used to relieve high blood pressure, respiratory disorders, and rheumatism [1].

It is the world's largest fruit crop, with over 100 million tons produced every year. Some of the parts of Citrus fruits which is almost 34% are converted into juices, resulting in a huge number of residues every year. The most common residues are the Citrus peels which contain a wide range of bioactive compounds and are therefore considered potential sources of functional components. Citrus peels contain a huge amount of bioactive compounds like phenolic acids, flavonoids, and limonoids than juices [2].

Consumption of Citrus fruits is a good habit due to their nutrient and functional properties [3]. Citrus fruit peels have been used as food in general, and very small amounts of the peels' essential oils have been used as fragrances, flavors, or synthetic starting materials for pharmaceutical and agricultural chemicals, as well as other chemical products. A significant portion of them has been wasted even now [4].

Citrus plants are mainly valued for their edible fruit, but they also have medicinal uses from ancient times. For decades, the peel of citrus fruits

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has been used in traditional Asian medicine. Citrus fruit peels are high in flavanones and polymethoxylated flavones, which are uncommon in other plants [5].Bioactive compounds are present in citrus fruits especially vitamin C and flavonoids. Flavonoids are very effective to cure different diseases due to the presence of antitumor and anti-inflammatory compounds. Lemon extract is the most favorable extract [6].

1.1 Distribution of Citrus Fruits

Worldcitrus fruit production is 98.4 million metric tons annually, and about 34 percent of the fruit is refined into juices. In the dairy, medicine, and cosmetic markets, volatile oil and non-volatile orange peel oil are commonly used[7].Citrus, also known as agrumes. Limes, Oranges, grapefruit, lemons, and tangerines are very good examples of citrus fruits with great market value. Citrus fruits are cultivated in more than 140 countries. India, Brazil, China, Mexico, the U.S.A, and Spain are the major citrus-producing countries [8].The total annual citrus fruit production in Pakistan is estimated to be around 2.0 million metric tonnes.Pakistan produced 1.62 million tonnes of citrus in1991–1992, which increased to 2.1 million tonnes in 2008–2009 and 2.4 million tonnes in 2014–2015[9].

Table 1
Pakistan's major citrus-growing regions[9].

Province	Major Districts
Sindh	Khairpur, Nawabshah, Sukkur, Sanghar
Punjab	Sahiwal, Khanewal, Vehari, Sargodha, Toba Tek Singh, Bahawalpur, Kasur, Multan, Layyah, Bahawalnagar, Faisalabad.
Baluchistan	Sibi, Dolan, Nasirabad, Lasbela, Gwadar.
Khyber Pakhtunkhwa (KPK)	Dera Ismail Khan, Mardan, Haripur, Nowshera, Swat, Malakand, Lower Dir.

1.2Nutritional benefits of Citrus Fruits

The intake of citrus fruits is beneficial for health due to their nutrition power and distinct flavor. Lemons (Citrus limon), sweet oranges (Citrus sinensis), limes (Citrus aurantiifolia), and grapefruit (Citrus paradisi) are mainly consumed by North American and European customers. Fruits are also used as sweets such as lime flavors and are used in liquid refreshments, bakery, cakes, and sweets [10]. Limes are used in Asian countries in culinary, pickling, and medicinal applications [11].

The usage of lemon is very effective for health due to its anticancer and antibacterial activities against bacterial strains.Sweet lime is a remarkable source of free citric acid, vitamin C, phosphorous, natural sugar, and calcium. Citrus flavonoids have bactericidal, analgesic, and anticarcinogenic activities [12]. Antibacterial action against Gram-positive and Gram-negative bacteria is observed in lemon juice[13].Rutin isa flavonoid described in lime. In oxidizing bacteria, Rutin has shown important scavenging effects, such as peroxyl radicals, superoxide radicals, and hydroxyl radicals [11].Several bioactive compounds have been studied in recent years for their possible antioxidant, anticancer and anti-inflammatory effects [14].

Flavanones, flavonols, isoflavones, flavones, and anthocyanidins are important subgroups of flavonoids[15].Flavonoids can impart colors to fruits and flowers. These are present in different forms of food such as vegetables, beverages, tea, nuts, coffee, and wine [16].Dietary polyphenols, ingested in vast amounts in plant-based foods, such as flavonoids and phenolic acids, have a variety of beneficial effects and play an important part in combating chronic and degenerative diseases [17].

Table 2
Nutritional Characteristics of citrus fruits[18].

Characteristics	Orange	Grapefruit	Lemon
Energy (kcal)	47	42	29

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Carbohydrates (g)	11.75	10.66	9.32
Protein (g)	0.94	0.77	1.10
Total Fat (g)	0.12	0.14	0.30
Cholesterol (g)	0	0	0
Dietary Fiber (g)	2.40	1.60	2.80
Pyridoxine (mg)	0.060	0.053	0.080
Riboflavin (mg)	0.040	0.031	0.020
Thiamin (mg)	0.087	0.043	0.040
Vitamin C (mg)	53.20	31.20	53
Vitamin A (IU)	225	1150	22
Vitamin E (mg)	0.18	0.13	0.15

1.3Phytochemicals

Phytochemicals are bioactive nutrient plant chemicals that are responsible to cure different diseases because they possess strong antioxidant, anti-inflammatory, antitumor, and antibacterial abilities and theyare also used as an additive in the food industry [1].

The rich source of phytochemicals is Fruit and vegetable waste (FVW) and has been examined for the extraction of essential fibers, phenolic compounds, and other bioactive compounds. Only pulp or flesh is consumed in most fruits and vegetables but research has shown that massive volumes of phytochemicals and vital minerals are stored in peels, seeds, and other constituents of fruits and vegetables that are not usually consumed. For instance, seeds of jackfruits, longans, avocados, mangoes, and peels of lemons, oranges, and grapes contain phenolic content more than 15% higher than those contained fruit pulp [18].

1.4 Antimicrobial Activity

In the food industry, antimicrobial constituents reduce the growth of microbes or suppress micro-organisms production. Antimicrobials can kill bacteria and fungi but they do not completely preserve the food. Currently, the food industry has to face serious problems regarding the usage of chemical constituents to prevent microbial growth, the rising trend is to use natural medicinal plants and fruits that contain antimicrobial agents and save food from pathogens. Citrus fruit's fiber possesses bioactive compounds like vitamin C, polyphenols, and essential oils residues. EOs are present in vegetables and fruits in excess amounts and act as natural antimicrobials[19].

With time bacterial species are going to become resistant to antimicrobial agents.So, there is a need for such antimicrobials that can completely diminish or retard the growth of microorganisms. Antimicrobial agents retard bacterial growth from wounds but also damage the tissues. Researchers have concluded that an ideal antimicrobial substance must show two important properties: it should be bactericidal and harmless [20].

1.4.1 Antibacterial Activity of Citrus Peels

Lemon peels contain essential oils which are mainly composed of aldehydes, esters, alcohols, and active terpenes that enhance their antimicrobial effect. The antibacterial activity of lemon peels in various solvents like methanol, acetone, and ethanol was investigated. Experiments show that the extract of lemon peel in ethanol shows greater antimicrobial activity than in acetone and methanol [21].

Orange peel and pulp are used to treat various diseases such as skin diseases and to cure stomach and intestinal-related complaints.Its peel is used for medicinal purposes to kill fungi and seed extract is used for urinary infections in China [22].Orange oil shows an inhibitory effect against Escherichia coli, Staphylococcus aureus, Proteus

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Vulgaris, Candida albicans, and Pseudomonas aeruginosa. Limonene is a vital constituent for antimicrobial activity [23].It is described that OEOs exhibits antioxidant and antibacterial activities and it is also acceptable for customers who demand natural ingredients [24].

Grapefruit juice has great medical importance and its daily use is suggested to gradually resist wound infection and usual colds. Crucial oils are being extracted from the peel of grapefruit and their manufacturing is 200 tons in the world.Demand for non-toxic natural preservatives has grown, with increasing concern about the existence of chemical residues in foods.Many compounds found in citrus fruits have antioxidant, allopathic, and bioregulatory effects. Grapefruit is a natural preserving agent due to its antibacterial activity [25]. The characteristic of a perfect antimicrobial agent and effectiveness are present in GSE. In contrast with other antibacterial agents, GSE contains tremendous antibacterial characteristics [20].

1.5 Structure of Anti-bacterial Compounds

Vitamin A

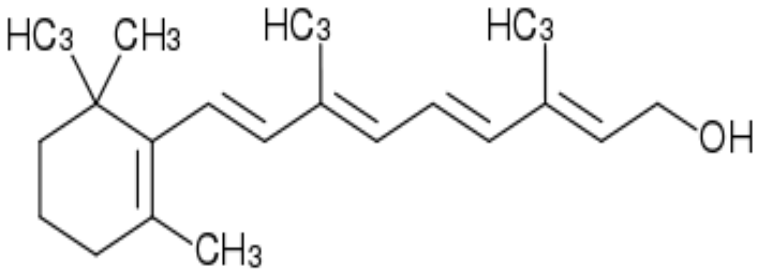

Figure 1. Chemical structure of vitamin A

Vitamin E

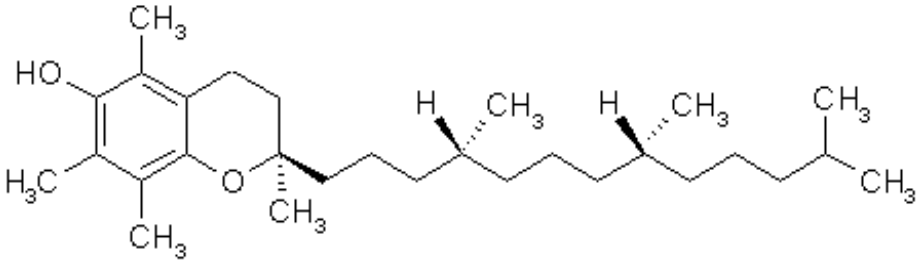

Figure 2. Chemical structure of vitamin E

Vitamin C

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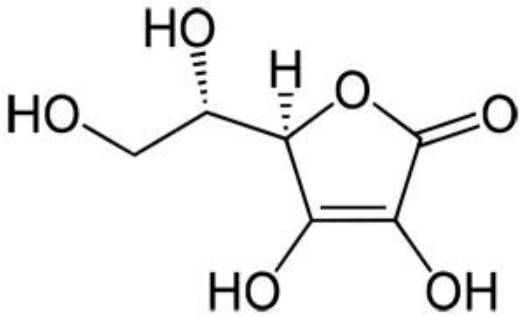

Figure 3. Chemical structure of vitamin C

Flavonoids

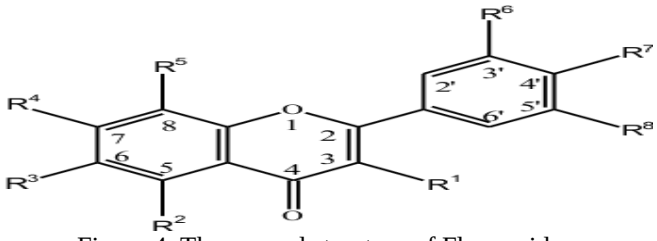

Figure 4. The general structure of Flavonoids

Table 3

Substitution in the general structure of flavonoids[27].

	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8
Flavanones								
Neohesperidin	H	OH	H	O-nh	H	OH	OCH_3	H
Narirutin	H	OH	H	O-rut	H	H	OH	H
Hesperidin	H	OH	H	O-rut	H	OH	OCH_3	H
Naringin	H	OH	H	O-nh	H	H	OH	H
Flavones								
Naringenin	H	OH	H	OH	H	H	OH	H
Hesperetin	H	OH	H	OH	H	OH	OCH_3	H
Polymethoxyflavones								
Tangeretin	H	OCH_3	OCH_3	OCH_3	OCH_3	H	OCH_3	H
Nobiletin	H	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	H

rut: rutinose; nh: neohesperidose.

Flavonols

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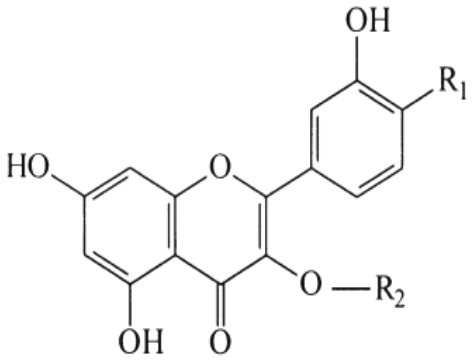

Figure 5. Chemical structure of flavonols

Table 4
Chemical structure of selected flavonols [26].

Flavonols	R_1	R_2
Quercetin	OH	H
Kaempferol	H	H
Isorhamnetin	OCH_3	H

Phenolic acids

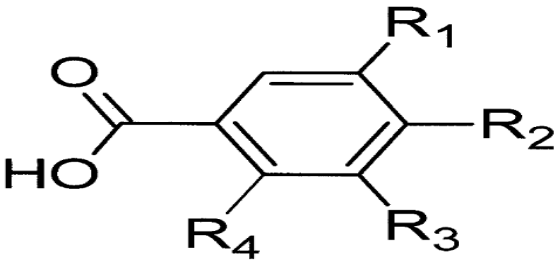

Figure 6. The general structure of phenolic acids

Table 5
Substitution in the general structure of phenolic acids[27].

	R_1	R_2	R_3	R_4
Salicylic acid	H	H	H	OH
p-Hydroxybenzoic acid	H	OH	H	H
Gentisic acid	OH	H	OH	H
Protocatechuic acid	OH	OH	H	H
Gallic acid	OH	OH	OH	H
Syringic acid	OCH_3	OH	OCH_3	H
Vanillic acid	OCH_3	OH	H	H

Tannic Acid

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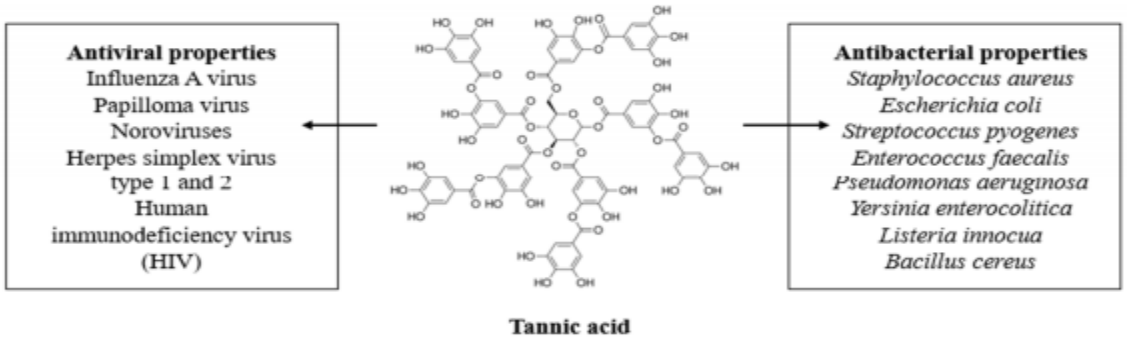

Figure 7. Chemical Structure of Tannic acid[28].

1.6 Mechanism of Antibacterial Action

1.6.1 Vitamin C

The antibacterial activity of vitamin C is based on its pro-oxidant activity and production of reactive oxygen species (ROS). Excess levels of vitamin C cause the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}), resulting in an excess intracellular load of ferrous ions. Through Fenton's reaction, excess ferrous ions generate ROS species (hydroxyl radicals, hydrogen peroxide, and superoxide), which destroy various intracellular targets and cause cell death.

The production of reactive oxygen species by Fenton's reaction is the natural mechanism behind the bactericidal action of vitamin C. The increase in Vitamin C's pro-oxidant activity induces an influx of iron into the cell, which contributes to the generation of ROS through Fenton's reaction. Free radical generation is implicated in membrane disintegration, DNA damage, disturbance of the iron-sulfur cluster, the influx of iron, and disrupting biosynthesis pathways, both of which lead to the death of microbes such as E. coli, S. aureus, S. epidermis, and P. aeruginosa[29].

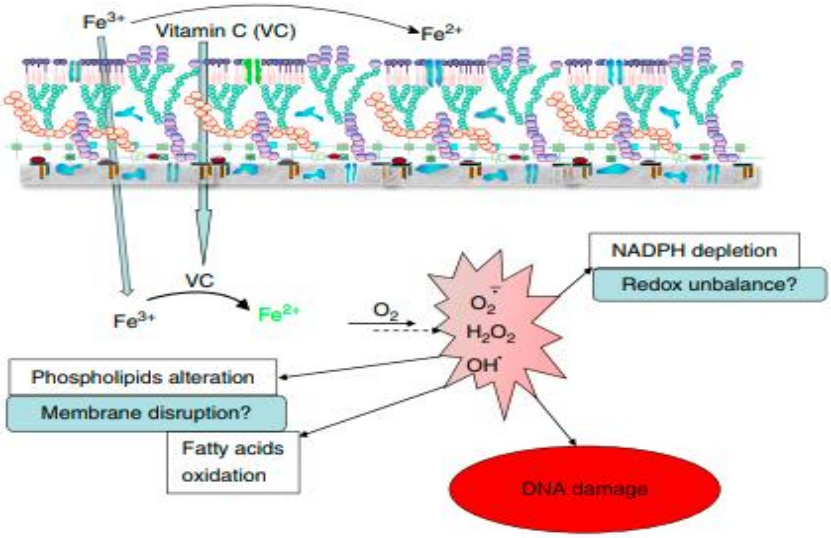

Figure 8. Mechanism of action of vitamin C[30].

1.6.2 Flavonoids

It is reported that flavonoids show antibacterial activity in different ways. It destroysthe cytoplasmic membrane by reducing membrane fluidity, inhibits synthesis of nucleic acid by inhibiting topoisomerase, and inhibits energy metabolism through NADH-cytochrome c reductase inhibition. It is investigated that the flavonol, flavan-3-ol, and flavolan classes cause destruction of cytoplasmic membrane by producing hydrogen peroxide, and flavan-3-ols and isoflavones inhibit the

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nucleic acid synthesis by inhibition of topoisomerase [31].

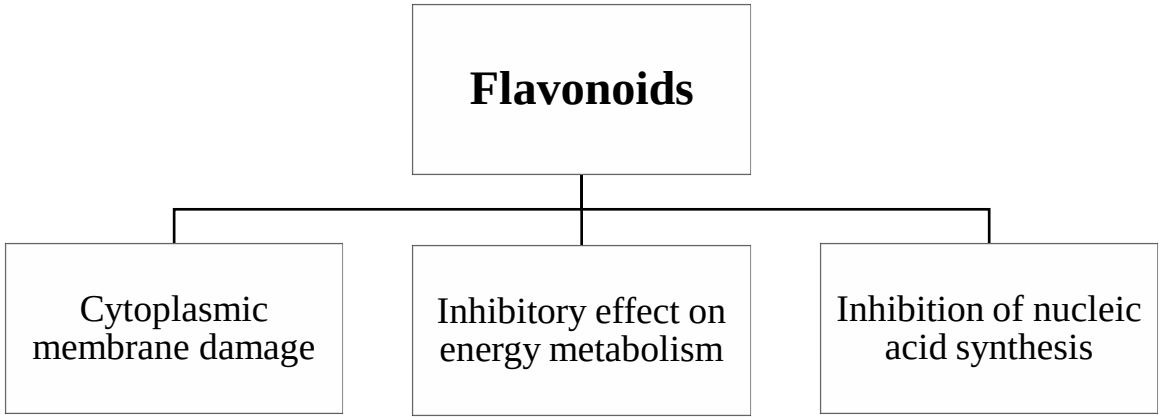

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Figure 9: Mode of action of flavonoids.

1.6.3 Flavonols

Flavonols, flavan-3-ols, and isoflavones cause inhibition of energy metabolism by inhibiting ATP synthase. They are also responsible to inhibit the synthesis of the cell wall by the inhibition of d-alanine-d-alanine ligase and also inhibits the synthesis of the cell membrane [31].

1.6.4 Tannins

The tannin's astringent property can cause complexation with enzymes or substrate. The toxicity of tannins is associated withtheir effect on the membranes of microorganisms. Tannin toxicity may be due to metal ion complexation by tannins. Iron is required for various purposes in aerobic microorganisms, it is investigated that the tannic acid's inhibitory effect on the growth of intestinal bacteria may be due to its high iron-binding ability [32].

Theantibacterial activity of tannins is influenced by various factors such as pH, temperature, matrix form, and time of action. Tannins are a class of multi-dentate ligands that bind to proteins primarily by hydrophobic interactions and hydrogen bonds. As a result, bacterial synthesis is inhibited. It is examined that the activity of tannic acid against Staphylococcus aureus and Escherichia coli was due to the presence of a phenolic hydroxyl group.

The antibacterial activity of tannins is due to their ability to move through the bacterial cell wall up to the internal membrane, interfering with the cell's metabolism and as a result their degradation. Tannin's activity is rapid in Gram-positive bacteria but in the case of Gram-Negative bacteria, its activity is slower due to the presence of a bi-layered membrane. Tannic acid prevents the attachment of bacteria to the surfaces which causes bacterial cell death. Furthermore, tannic acid inhibited the uptake of sugar and amino acids which limits the growth of bacteria[33].

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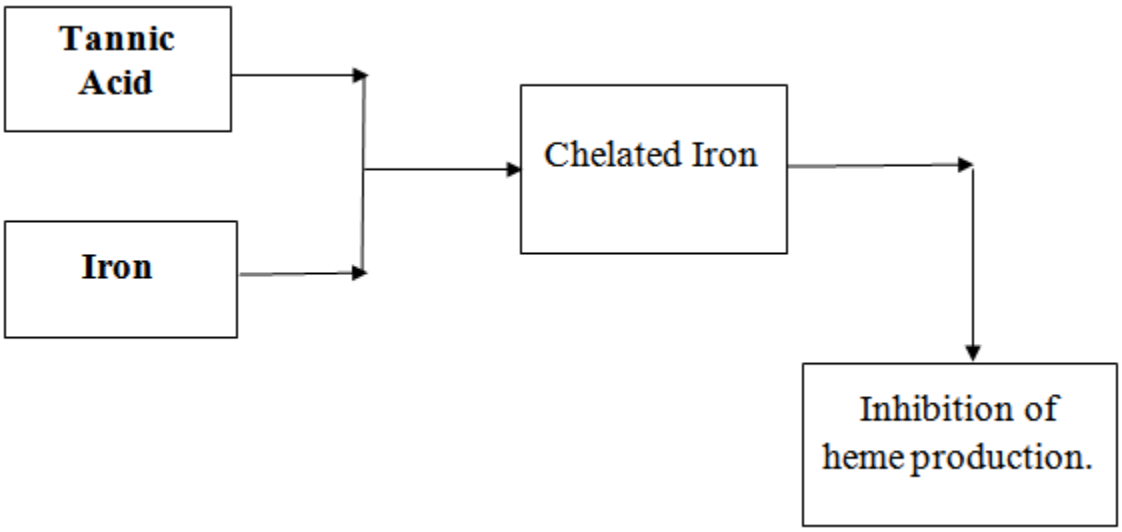

Figure 10: Mode of action of tannins.

Table 6

Mechanism of action of antibacterial compounds

	Mechanism of action	References
Vitamin C	Through Fenton's reaction, excess ferrous ions generate ROS species (hydroxyl radicals, hydrogen peroxide, and superoxide), which destroy various intracellular targets and cause cell death.	[29]
Flavonoids	It destroys the cytoplasmic membrane by reducing membrane fluidity, inhibits synthesis of nucleic acid by inhibiting topoisomerase, and inhibits energy metabolism through NADH-cytochrome c reductase inhibition.	[31]
Flavonols	It causes inhibition of energy metabolism by inhibiting ATP synthase.	[31, 34]
Tannins	Tannic acid's inhibitory effect on the growth of bacteria is due to its high iron-binding ability. Tannins can move through the bacterial cell walls, interfering with the cell's metabolism and as a result their degradation.	[32]

1.7 Types of Bacteria

Most bacteria are classified into two types Gram-positive and Gram-negative. The cell walls of Gram-positive bacteria are made up of a thick layer of peptidoglycan. On Gram staining, Gram-positive cells turned purple. The cell walls of Gram-negative bacteria consist of a thin layer of peptidoglycan. Gram-positive bacteria are easier to destroy than Gram-negative bacteria[33].

1.7.1 Gram-positive Bacteria

In Gram-positive bacteria, a protective outer membrane is absent, but peptidoglycan (PG) layers are many times thicker than in Gram-negative organisms[35].

A large fraction of the biodiversity of the human microbiome and many significant pathogens, such as Staphylococcus aureus, Streptococcus pneumoniae, Listeria monocytogenes, or Clostridium

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difficile are Gram-positive bacteria. Also, this broad community of bacteria contains several organisms that are used in biotechnology and the everyday industry, such as Bacillus licheniformis or lactic acid bacteria [36].

1.7.2Staphylococcus Aureus

S. aureus is a member of the genus Micrococcaceae. The species occur in groups on microscopic examination as gram-positive cocci.S. aureus is distinct from other staphylococcal species.The staphylococcal genome comprises a circular chromosome (about 2800bp), with plasmids, prophages, and transposons. On the chromosome along with extrachromosomal elements, genes regulating antibiotic resistance and virulence are located[37].

Staphylococcus aureus is both a commensal and pathogenic human bacterium.About 30 percent of the human population is colonized byS. aureus[38].

Staphylococcus.aureusis a disease-causing agent, including measles, cellulitis, bacteremia, and osteomyelitis, with the bulk of community-associated illnesses in the United States affecting the skin and soft tissues. Of all military personnel, 4 to 6 percent eventually develop Skin and Soft Tissues Infection (SSTI) and 91 percent of these infections were due to S. aureus[39].

S. aureus is present in the atmosphere and is often found in typical human flora, distributed on mostly healthy individual's skin and mucous membranes (most commonly the nasal area). Normally,S. aureus may not cause infection on healthy skin; but, once it is allowed to penetrate the bloodstream or internal organs, a range of potentially dangerous infections can be caused by these bacteria. Transmission is usually by close contact [40].

The gram-positive bacterium Staphylococcus aureus colonizes many species. In poultry, S. aureus infections are most widely reported in dairy-producing animals and "bumblefoot" in chickens as a source of mastitis, as well as being recognized as a farmed rabbit pathogen [41].

1.7.3Bacillus Subtilis

There are 337 species of Gram-positive, rod-shaped bacteria in the genus Bacillus [42].It is a surprisingly distinct microbial species of bacteria that can thrive in many habitats.However, like all members of the Bacillus family, Bacillus subtilis can form highly resistant dormant endospores in reaction to nutrient deficiency and other environmental stresses.Such spores are airborne and wind-dispersed readily[43].

Species of Bacillus are normally round-ended [44].The only obligatory pathogen of Bacillus in vertebrates is Bacillus anthracis, the anthrax agent.A variety of other species are occasional human and livestock pathogens, particularly B. cereus, but the vast majority of species of Bacillus are harmless saprophytes [45].The Bacillus genus is, in general, identified as a category of soil residents [46].

In many biomedical, medicinal, industrial, and agricultural processes, Bacillus species are used to take advantage of their wide range of physiological properties and their capacity to generate a variety ofantibiotics, enzymes, and other metabolites.Two well-known antibiotics derived from the bacillus genes are bacitracin and polymyxin [45]. Several hundred strains of wild-type B. subtilis have been obtained, with the ability to produce more than two dozen antibiotics with an impressive number of structures. The potency of Bacillus subtilis has been known to develop antibiotics for 50 years [47].

Compared to other micro-organisms, gram-positive Bacillus subtilis species are well known for their superior protein secretion capacity. Besides,Bacillus species have become the selected organisms for industrial production of several goods, including biopolymers, platform additives, and proteins, because of their strong growth in cheap carbon supplies, their distinct endogenous metabolism, and their robustness in industrial fermentation [48].

1.8 Purpose of Study

The main objective of this study is as follows:

- Analysis of antibacterial potential in citrus fruit wastes by Agar well diffusion method.
- Effects of climate changeon Antibacterialactivity.
- Effects of different solvents on antibacterial activity of citrus peels.
- Development of cheap antibacterial source from fruit wastes.
- Uses of fruit wastes in beneficial ways.

1.9 Literature Review

Saleem and Saeed, 2020 investigated the antibacterial potential of peel extract of three different fruits and have been compared using three different solvents in their study. They observed that

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distilled water was the best solvent for extracting antibacterial fractions as compared to methanol, ethanol, and acetone. They also found that Klebsiella pneumoniae (gram-negative) bacteria showed a maximum zone of inhibition among the studied microorganisms. They examined that yellow lemon peels were more effective than orange and banana peels and the reason behind their effectiveness was may be due to the high concentration of magnesium, zinc, and total phenolic content [49].

Jayaprakasha et al., 2000 investigated the antibacterial activity of citrus peels using different solvents. The EtOH-soluble fraction was found to be more effective against all the studied microorganisms. The acetone extract was least effective than all other fractions. The MIC value of EtOH soluble fraction was 1200 and 600 µg/ml for E. coli and Pseudomonas aeruginosa. Citrus reticulata peel extracts in hexane, chloroform, and acetone were reported to have antibacterial activity. The EtOH soluble fraction was discovered to have a significant level of antibacterial activity, suggesting that it could be employed as a bio preservative. [7]

Egbuonu and Osuji, 2016 reported the antibacterial activity of orange peels and seeds by agar disc diffusion method. It was noted that the antibacterial activity of sweet orange peels was greater than the of sweet orange seeds. The results showed that the water and ethanol extracts of sweet orange peels were more effective than the seed extracts against both gram-positive and gram-negative bacteria. [50]

Gopal et al., 2012 examined the antibacterial activity of C. aurantium against both gram-positive and negative bacteria. Ethanol extract, chloroform extract, water extract, and petroleum ether extract had the lowest MIC values against the studied bacteria. The findings showed that extracts from C. aurantium leaves were efficient against Gram-positive and Gram-negative bacteria. [51]

Adham et al., 2015 demonstrated the antibacterial activity of juice and peel extract of Citrus medica. The antibacterial properties of peel extracts and juice differed depending on the test organism. When compared to gram-positive bacteria, peel extracts were more effective against gram-negative bacteria, whereas juice extracts were more effective against gram-positive bacteria. Citrus medica peel and juice have antibacterial properties due to the presence of bioactive components. [52]

CHAPTER 2.

2. Materialsand Methods

2.1 Collection and Preparation of Sample

Fresh fruits of Citrus Limon (Lemon), Citrus Sinensis (Orange), and Citrus paradisi (Grapefruit) used in this study were collected from different regions of Pakistan i.e.,Lahore, Burewala, and Rahim Yar Khan. The fruits were washed under tap water. Allthe Citrus fruits were peeled off after collection as soon as possible. The fruit peels were washed thoroughly with distilled water to remove any impurity, and then peels were air-dried. After this, the fruits were dried in a hot air oven at 80^{o}C for the removal of moisture. A grinder was used to pulverize the dried peels. The pulverized samples were enclosed in airtight bags and held at 4 degrees Celsius until further study.

2.2 Preparation of sample extract

In the flask, the powder sample was mixed with the extraction solvents to make a volume of 100ml.The flask was then subjected to ultrasonic treatment following the procedure of ultrasound-assisted extraction method and the extracts were then filtered.

2.3 Ultrasound-Assisted Extraction method

To extract the sample, weigh 5g in an amber flask and add 40ml methanol, acetone, and 50ml distilled water in three separate flasks. Place these amber flasks in an ultrasonic bath for about 15 minutes. After the specified time, the resultant suspension was centrifuged for almost 3 minutes at 3000 rpm, and the supernatant layer was transferred to a volumetric flask of 25ml and filled with ultra-pure distilled water.

2.4Preparation of inoculum

In this analysis, we usedtwo bacterial strains for the assay. Staphylococcus aureus and Bacillus Subtilisboth were gram-positive bacteria. Nutrient broth medium was prepared by dissolving 1.3g of nutrient broth powder in 100ml of distilled water and mixed it gently by shaking the flask.In a 500ml flask 100ml of media was dispensed and then autoclaved at 121°C for 15 minutes. The medium was then poured into the Petri plates. Bacterial cultures were inoculated with the help of inoculating loop and the loop was sterilized by flame sterilization. The Petri plates were stored in an incubator at 37°C overnight.

2.5 Preparation of Agar Plates

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For the preparation of nutrient agar medium, 14g of nutrient agar powder was weighed at a Digital weighing balance. The powder was dissolved in 500ml of distilled water.Then shacked and dissolved it properly with the help of a glass spatula. Then media was dispensed in a 500ml flask and then autoclaved at 121°C for 15 minutes. The Petri plates were labeled by using a permanent marker. The medium was pouredonto labeledPetri plates and then allowed to solidify.

2.6Agar well diffusion method

The prepared inoculum was spread to the Agar plates uniformly with the help of disposable sterile swab sticks. Bacterial colonies were formed at 37°C for about 12 hours.In the inoculated media the required number of wells were made with the help of a sterile cork-borer. Each well was sealed by adding 20µl of liquid nutrient agar medium.The peel extract of 100µl was poured intothe respective wells with the help of a micropipette. The plates were then incubated at 37°C.The diameter of the zone of inhibition around each well was measured in mm after a 24-hour incubation time. This experiment was done three times for each extract. The standard deviation method was used to calculate the mean zone of inhibition.

CHAPTER NO 3

3 Results and Discussion

3.1 Antibacterial activity of Citrus Sinensis

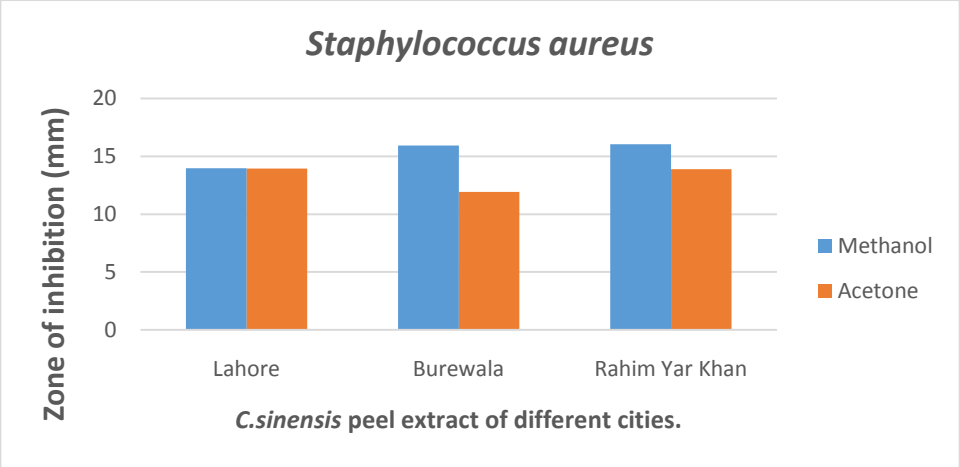

Figure 11. Antibacterial activity of Citrus Sinensis.

When we used methanol extract, the Zone of Inhibitionof the peel extract of Citrus Sinensis collected from Lahore was (13.96±0.124mm),Burewala was (15.93±0.169mm), and Rahim Yar Khan was (16.03±0.205mm). In the case of acetone extract, the Zone of Inhibition of the peel extract of Citrus Sinensis collected from Lahore was (13.93±0.329mm), Burewala was (11.93±0.169mm), andRahim Yar Khan was (13.9±0.374mm). Inmethanol extract, the peel extract of C. Sinensis collected from Rahim Yar Khan (16.03±0.205mm) and Burewala (15.93±0.169mm) showed almost similar zone of inhibition with maximum valuesbut the peel extract of Lahore (13.96±0.124mm) showed minimum ZOI as compared to other two cities. Inacetone extract, the peel extract of Citrus Sinensis collected from Lahore (13.93±0.329mm) and Rahim Yar Khan (13.9±0.374mm) showed almost similar zone of inhibition with maximum values but the peel extract of Burewala (11.93±0.169mm) showed minimum ZOI as compared to other two cities. Figure 21 represents that methanol extract of Citrus Sinensis is more efficient than acetone extractagainst Staphylococcus aureus. The peel extract collected from Rahim Yar Khan showed the highest antibacterial activity in the case of both acetone and methanol extract.

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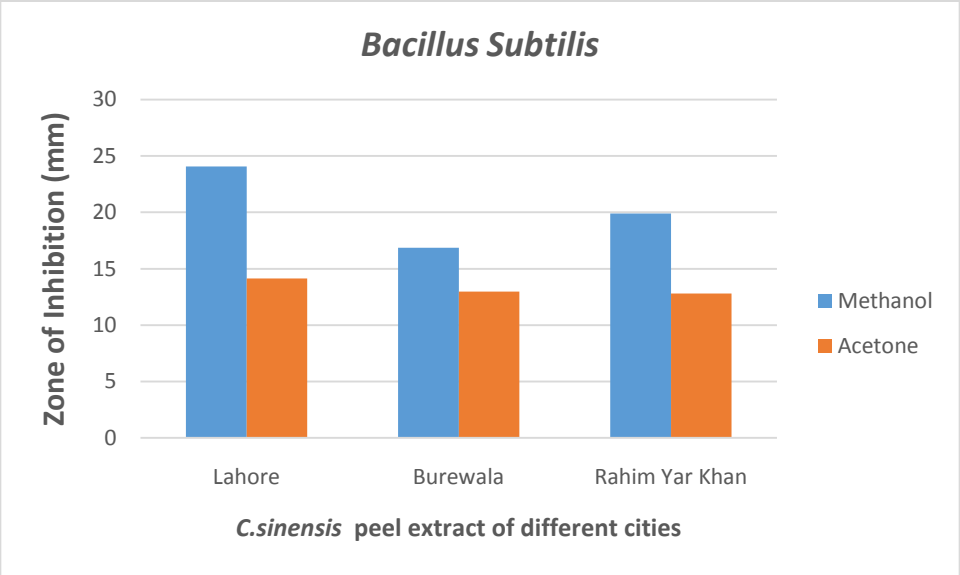

Figure 12. Antibacterial activity of Citrus Sinensis.

When we used methanol extract, the Zone of Inhibition of the peel extract of Citrus Sinensis collected from Lahore was (24.06±0.249mm),Burewala was (16.86±0.339mm), and Rahim Yar Khan was (19.9±0.454mm). In the case of acetone extract, the Zone of Inhibition of the peel extract of Citrus Sinensis collected from Lahore was (14.13±0.339mm), Burewala was (12.96±0.531mm), and Rahim Yar Khan was (12.8±0.432mm). Inmethanol extract, the peel extract of C. Sinensis collected from Lahore (24.06±0.249mm) showed the highest zone of inhibition.The peel extract of Rahim Yar Khan (19.9±0.454mm) showed the second-highest ZOI and the peel extract of Burewala (16.86±0.339mm) showed a minimum zone of inhibition as compared to the other two cities. Inacetone extract, the peel extract of Citrus Sinensis collected from Lahore (14.13±0.339mm) showed the highest zone of inhibition. The peel extract of Burewala (12.96±0.531mm)showed the second-highest ZOI and the peel extract of Rahim Yar Khan (12.8±0.432mm) showed third-highest ZOI. Figure 22 represents that the methanol extract of Citrus Sinensis is more efficient than acetone extract against Bacillus Subtilis. The peel extract collected from Lahore showed the highest antibacterial activity in the case of both acetone and methanol extract.

3.2 Agar plates after 24hour incubation

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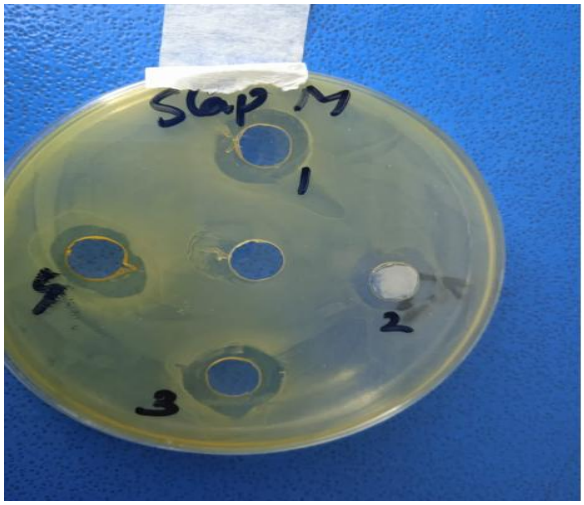

Figure 13. Inhibition zone by methanol extracts in S. aureus inoculated plate.

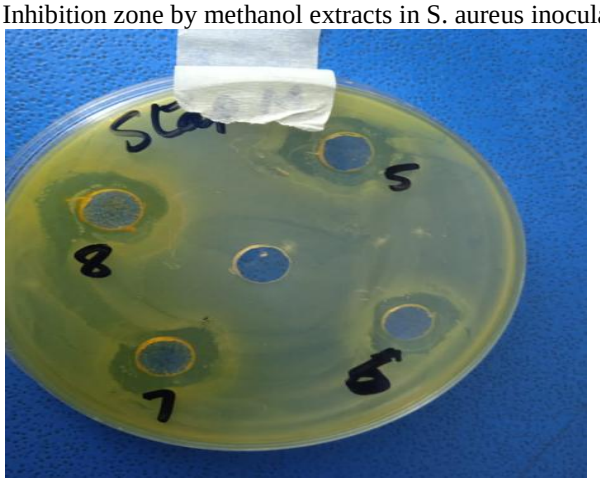

Figure 14. Inhibition zone by methanol extracts in S. aureus inoculated plate.

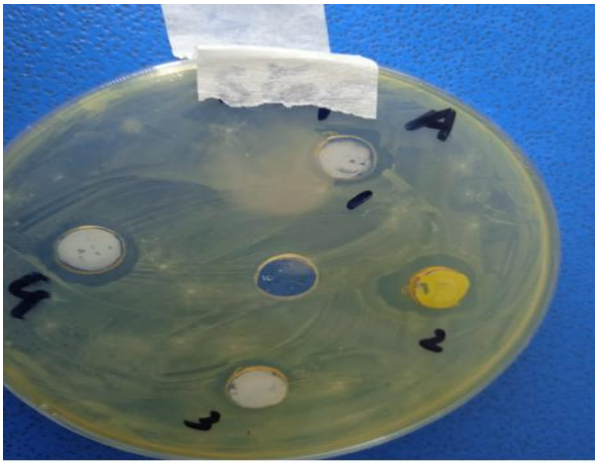

Figure 15. Inhibition zone by acetone extracts in S. aureus inoculated plate.

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Figure 16. Inhibition zone by acetone extracts in S. aureus inoculated plate.

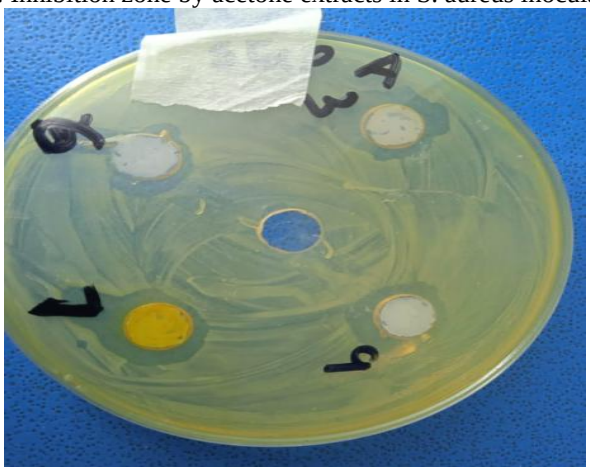

Figure 17. Inhibition zone by acetone extracts in S. aureus inoculated plate.

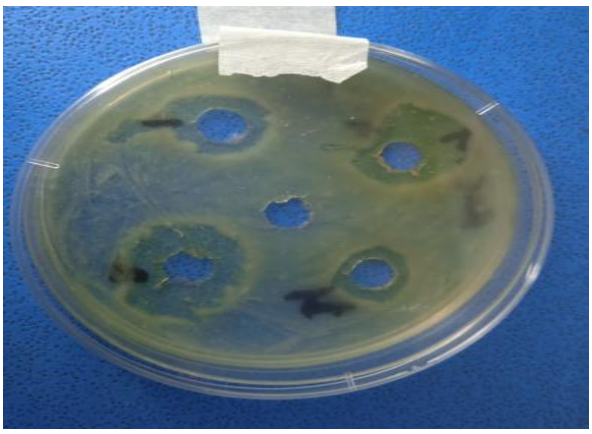

Figure 18. Inhibition zone by methanol extracts in B. subtilis inoculated plate.

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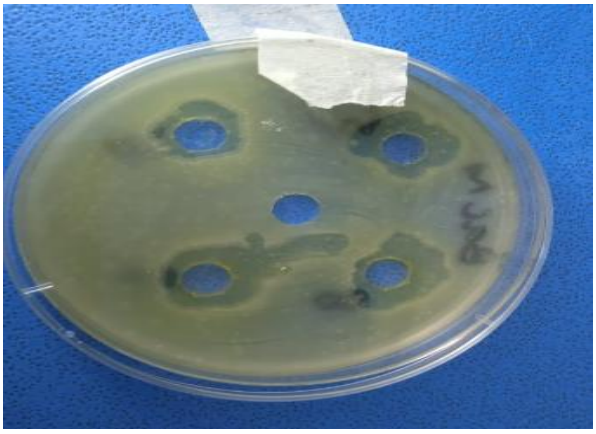

Figure 19. Inhibition zone by methanol extracts in B. subtilis inoculated plate.

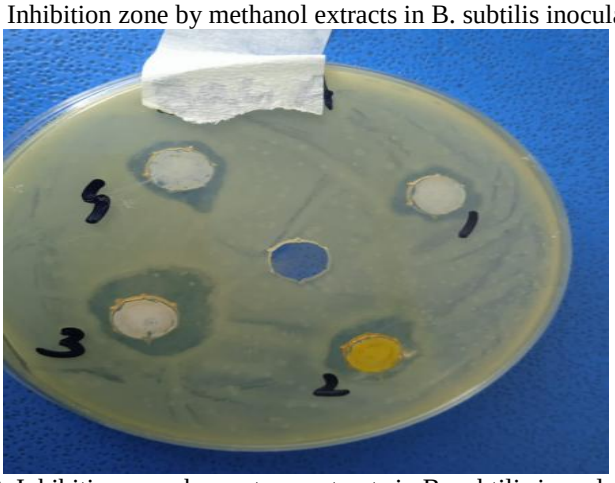

Figure 20. Inhibition zone by acetone extracts in B. subtilis inoculated plate.

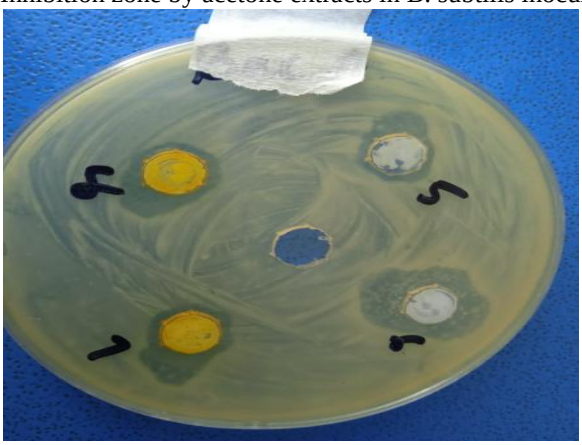

Figure 21.Inhibition zone by acetone extracts in B. subtilis inoculated plate.

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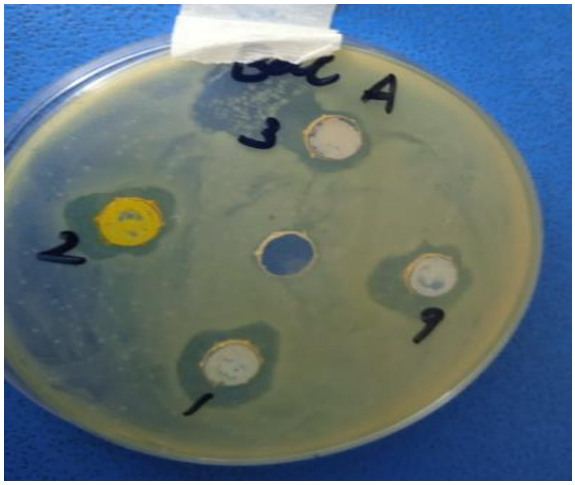

Figure 22. Inhibition zone by acetone extracts in B. subtilis inoculated plate.

3.3Antibacterial activity of Citrus Limon

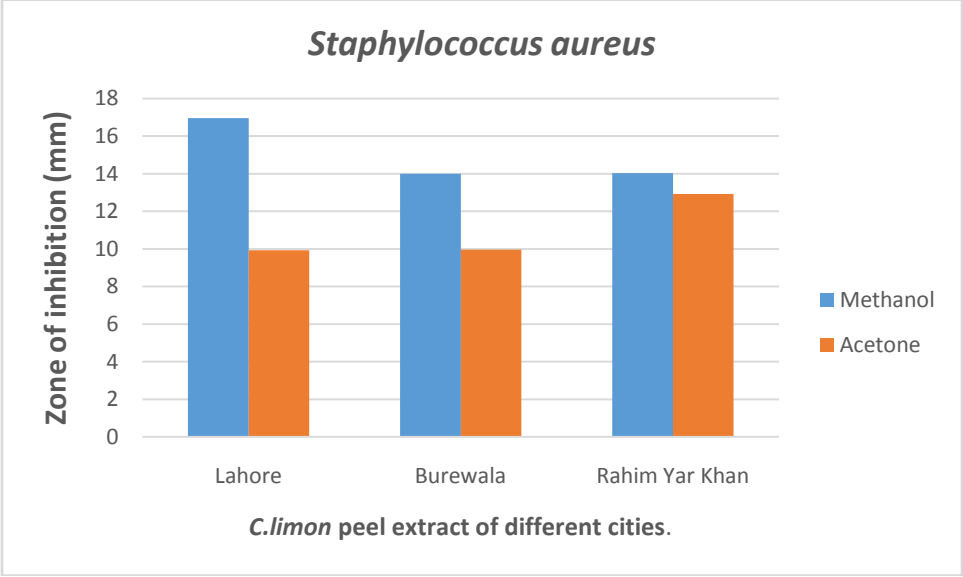

Figure 23.Antibacterial activity of Citrus Limon.

When we used methanol extract, the Zone of Inhibition of the peel extract of Citrus Limon collected from Lahore was (16.96±0.124mm),Burewala was (14±0.081mm), and Rahim Yar Khan was (14.03±0.124mm). In the case of acetone extract, the Zone of Inhibition of the peel extract of Citrus Limon collected from Lahore was (9.93±0.249mm), Burewala was (9.96±0.368mm), and Rahim Yar Khan was (12.93±0.329mm). Inmethanol extract, the peel extract of C. Limon collected from Lahore (16.96±0.124mm) showed the highest zone of inhibition. The peel extract of Burewala (14±0.081mm) and the peel extract of Rahim Yar Khan (14.03±0.124mm)showed an almost similar zone of inhibition. Inacetone extract, the peel extract of Citrus Limon collected from Rahim Yar Khan (12.93±0.329mm) showed the highest zone of inhibition. The peel extract collected from Lahore (9.93±0.249mm) and Burewala (9.96±0.368mm) showed an almost similar zone of inhibition. Figure 23 represents that methanol extract of Citrus Limon is more efficient than acetone extract against Staphylococcus aureus. Inthe case of methanol extract, the peel extract of Lahore showed the highest

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antibacterial activity but in the case of acetone extract, the peel extract of Rahim Yar Khan showed the highest antibacterial activity.

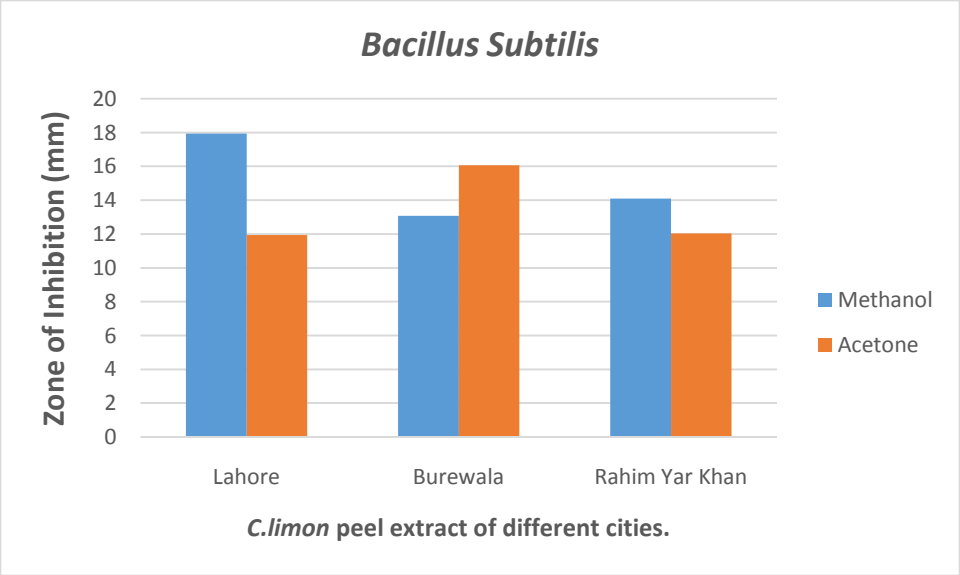

Figure 24. Antibacterial activity of Citrus Limon.

When we used methanol extract, the Zone of Inhibition of the peel extract of Citrus Limon collected from Lahore was (17.93±0.249mm), Burewala was (13.06±0.169mm), and Rahim Yar Khan was (14.1±0.216mm). In the case of acetone extract, the Zone of Inhibition of the peel extract of Citrus Limon collected from Lahore was (11.93±0.329mm), Burewala was (16.06±0.249mm), and Rahim Yar Khan was (12.03±0.449mm). In methanol extract, the peel extract of C. Limon collected from Lahore (17.93±0.249mm) showed the highest zone of inhibition. The peel extract of Rahim Yar Khan (14.1±0.216mm) showed the second-highest ZOI and the peel extract of Burewala (13.06±0.169mm)showedthe third-highest zone of inhibition. Inacetone extract, the peel extract of Citrus Limon collected from Burewala (16.06±0.249mm) showed the highest zone of inhibition. The peel extract collected from Rahim Yar Khan(12.03±0.449mm) showed the second-highest ZOI andthe peel extract of Lahore (11.93±0.329mm) showed the third-highest zone of inhibition. Figure 24 represents that methanol extract of Citrus Limonis more efficient than acetone extract against Bacillus Subtilis. In the case of methanol extract, the peel extract of Lahore showed the highest antibacterial activity but in the case of acetone extract, the peel extract of Burewala showed the highest antibacterial activity.

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3.4Antibacterial activity of Citrus paradisi

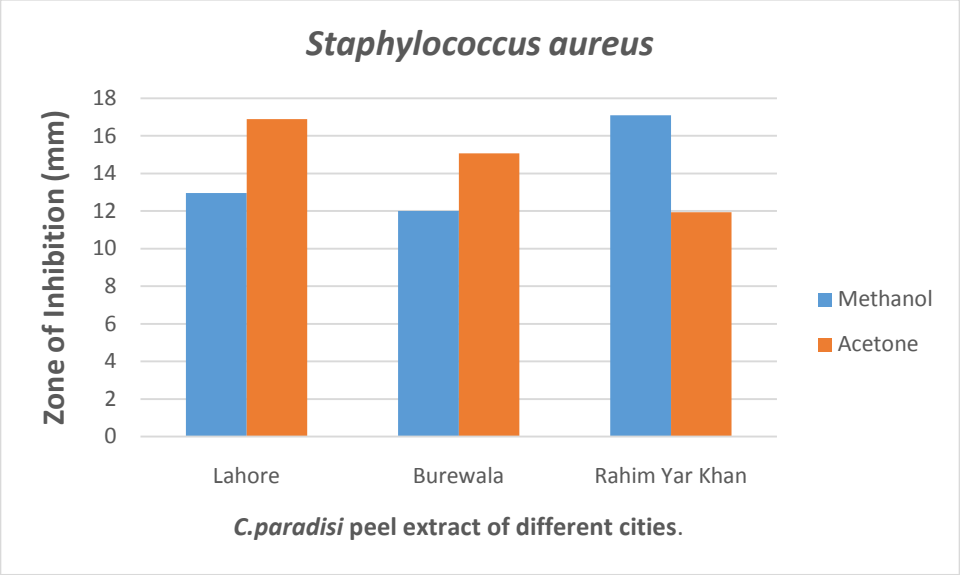

Figure 25.Antibacterial activity of Citrus paradisi.

When we used methanol extract, the Zone of Inhibition of the peel extract of Citrus paradisi collected from Lahore was (12.96±0.286mm),Burewala was (12±0.163mm), and Rahim Yar Khan was (17.1±0.216mm). In the case of acetone extract, the Zone of Inhibition of the peel extract of Citrus paradisi collected from Lahore was (16.9±0.216mm), Burewala was (15.06±0.169mm), and Rahim Yar Khan was (11.93±0.329mm). Inmethanol extract, the peel extract of C.paradisi collected from Rahim Yar Khan (17.1±0.216mm) showed the highest zone of inhibition. The peel extract of Lahore (12.96±0.286mm) showed the second-highest ZOI and the peel extract of Burewala (12±0.163mm)showedthe third-highest zone of inhibition. Inacetone extract, the peel extract of Citrusparadisi collected fromLahore (16.9±0.216mm) showed the highest zone of inhibition. The peel extract collected from Burewala(15.06±0.169mm) showed the second-highest ZOI but the peel extract of Rahim Yar Khan (11.93±0.329mm) showeda minimum zone of inhibition as compared to the other two cities.Figure 25 represents that acetone extract of Citrus paradisiis more efficient than methanol extract againstStaphylococcus aureus. In the case of methanol extract, the peel extract of Rahim Yar Khan showed the highest antibacterial activity but in the case of acetone extract, the peel extract of Lahore showed the highest antibacterial activity.

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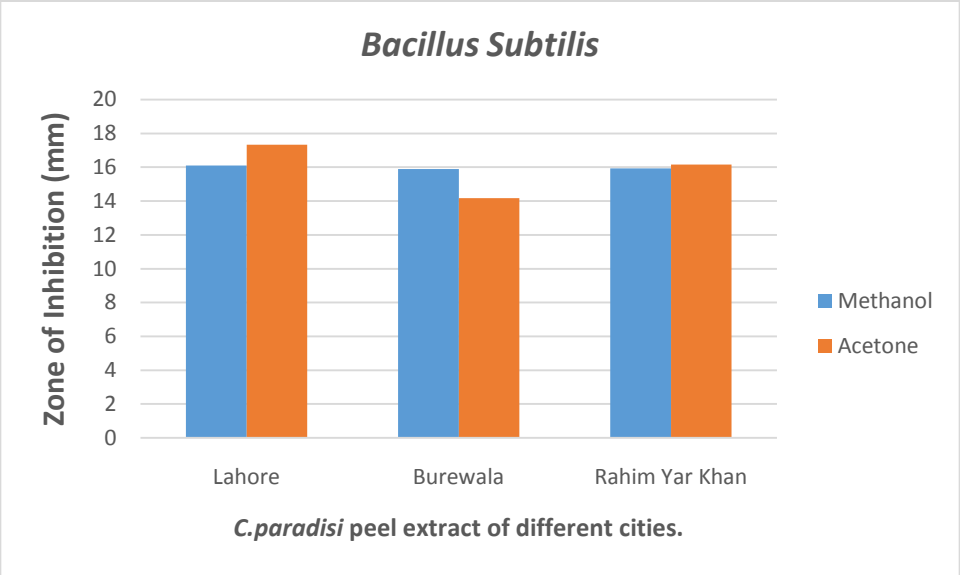

Figure 26. Antibacterial activity of Citrus paradisi.

When we used methanol extract, the Zone of Inhibition of the peel extract of Citrus paradisi collected from Lahore was (16.1±0.294mm), Burewala was (15.9±0.535mm), and Rahim Yar Khan was (15.93±0.492mm). In the case of acetone extract, the Zone of Inhibition of the peel extract of Citrus paradisi collected from Lahore was (17.33±0.339mm), Burewala was (14.16±0.309mm), and Rahim Yar Khan was (16.16±0.385mm). Inmethanol extract, the peel extract of C.paradisi collected from Lahore (16.1±0.294mm) showed the highest zone of inhibition. The peel extract of Burewala (15.9±0.535mm) and the peel extract of Rahim Yar Khan (15.93±0.492mm) showed an almost similar zone of inhibition with maximum values. Inacetone extract, the peel extract of Citrus paradisi collected from Lahore (17.33±0.339mm) showed the highest zone of inhibition. The peel extract collected from Rahim Yar Khan (16.16±0.385mm) showed the second-highest ZOI but the peel extract of Burewala (14.16±0.309mm) showed a minimum zone of inhibition as compared to the other two cities. Figure 26 represents that acetone extract of Citrus paradisiis more efficient than methanol extract againstBacillus subtilis. In the case of methanol extract, the peel extract of Lahore showed the highest antibacterial activity but in the case of acetone extract, the peel extract of Lahore again showed the highest antibacterial activity.

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Table 7Zone of inhibition of samples collected from different regions of Pakistan.

Diameter of inhibition zone (DIZ) in mm (Mean ±SD)										
Strain		Citrus Sinensis			Citrus Limon			Citrus paradisi		
		Lahore	Burewala	Rahim Yar Khan	Lahore	Burewala	Rahim Yar Khan	Lahore	Burewala	Rahim Yar Khan
S. aureus	Methanol	13.96±0.124	15.93±0.169	16.03±0.205	16.96±0.124	14±0.081	14.03±0.124	12.96±0.286	12±0.163	17.1±0.216
B. subtilis		24.06±0.249	16.86±0.339	19.9±0.454	17.93±0.249	13.06±0.169	14.1±0.216	16.1±0.294	15.9±0.535	15.93±0.492
S. aureus	Acetone	13.93±0.329	11.93±0.169	13.9±0.374	9.93±0.249	9.96±0.368	12.93±0.329	16.9±0.216	15.06±0.169	11.93±0.329
B. subtilis		14.13±0.339	12.96±0.531	12.8±0.432	11.93±0.329	16.06±0.249	12.03±0.449	17.33±0.339	14.16±0.309	16.16±0.385

In this study,methanol extract of Citrus paradisi collected from Rahim Yar Khan was found to be more effective against Staphylococcus aureus with the maximum value of zone of inhibition than all other samples collected from all the selected cities of Pakistan. The methanol extract of Citrus paradisi of Burewala showed a minimum zone of inhibition than all other samples. In the case of acetone extract, the peel extract of Citrus paradisiobtained from Lahore showedthe highest zone of inhibition towards Staphylococcus aureus than other samples and the peel extract of Citrus Limon of Lahore showed minimum inhibition towards S. aureus as compared to all other samples.

The methanol extract of Citrus Sinensis collected from Lahore was found to be more effective against Bacillus subtilis with the maximum value of zone of inhibition than other samples. The methanol extract of Citrus Limon of Burewala showed a minimum zone of inhibition than other samples. In the case of acetone extract, the peel extract of Citrus paradisiobtained from Lahore showed the highest zone of inhibition towards Bacillus subtilis than other samples and the peel extract of Citrus Limon of Lahore showed minimum inhibition towards B. subtilis as compared to all other samples.

Saleem and Saeed, 2020 reported that the ZOI of Orange peel extract in the case of methanol solvent against gram-positive (S. aureus) bacteria was (13±0.4)which is related to our sample collected from Lahore (13.96±0.124) and the ZOI of Lemon peel extract in methanol solvent was (13±0.3), which is relevant to the sample collected from Burewala (13.06±0.169)[49].

Okunowo et al., 2013 examined the ZOI of grapefruit peel extract in the case of methanol solvent was (17.00±1.00) [53] which is relevant to the sample collected from Rahim Yar Khan (17.1±0.216). When we compared our results to the past studies, it was shown that our values were related to the literature studies.

3.5Conclusion

In this study, the peels of CitrusLimon, Citrusparadisi, and CitrusSinensis of all the selected cities of Punjab showed good antibacterial activity but the acetone extract of the peels of Citrus paradisi of Lahore showed the highest antibacterial activity against both bacterial strains and the peel extract of Citrus Limon of Lahore showed minimum inhibition towards both Staphylococcus aureus and Bacillus Subtilis. Fluctuation in antibacterial activity of citrus fruit peels of all remaining cities was due to environmental factors which may be water, temperature, light, and soil composition.

Citrus peels are used as flavorings, food additives, and antimicrobial agents. It was investigated that the folklore information about the

therapeutic effects of citrus fruit peels was authentic.Phytochemicals are responsible to cure and prevent diseases mainly flavonoids are well known. The natural sources of flavonoids are fruits and vegetables.To minimize the ill effects of synthetic preservatives, Citrus peel essential oils should be employed as an alternative as they are natural and protect human health. Citrus peels are a low-cost source of nutraceuticals and a low-cost nutritional treatment for degenerative diseases.

REFERENCES

[1]. Shakya, A., et al. Comparative Study of Antibacterial Activity of Juice and Peel Extract of Citrus Fruits. 2019.
[2]. Ho, S.-C., C.-C.J.J.o.a. Lin, and f. chemistry, Investigation of heat treating conditions for enhancing the anti-inflammatory activity of citrus fruit (Citrus reticulata) peels. 2008. **56**(17): p. 7976-7982.
[3]. Agriopoulou, S., et al., Lactic Acid Bacteria as Antibacterial Agents to Extend the Shelf Life of Fresh and Minimally Processed Fruits and Vegetables: Quality and Safety Aspects. Microorganisms, 2020. **8**.
[4]. Sawabe, A. and Y. Matsubara, Bioactive glycosides in citrus fruit peels, in Studies in Plant Science. 1999, Elsevier. p. 261-274.
[5]. Choi, S.-Y., et al., Correlation between flavonoid content and the NO production inhibitory activity of peel extracts from various citrus fruits. 2007. **30**(4): p. 772-778.
[6]. 6. CONTE, A., et al., Effect of lemon extract on foodborne microorganisms. Journal of food protection, 2007. **70**(8): p. 1896-1900.
[7]. Jayaprakasha, G.K., et al., Antibacterial activity of Citrus reticulata peel extracts. Zeitschrift für Naturforschung C, 2000. **55**(11-12): p. 1030-1034.
[8]. Liu, Y., E. Heying, and S.A. Tanumihardjo, History, global distribution, and nutritional importance of citrus fruits. Comprehensive reviews in Food Science and Food safety, 2012. **11**(6): p. 530-545.
[9]. Siddique, M.I. and E.J.A.V.C. Garnevska, Citrus value chain (s): A Survey of Pakistan citrus Industry. 2018. **37**.
[10]. Guimarães, R., et al., Targeting excessive free radicals with peels and juices of citrus fruits: grapefruit, lemon, lime and orange. Food and Chemical Toxicology, 2010. **48**(1): p. 99-106.
[11]. Patil, J.R., et al., Bioactive compounds from Mexican lime (Citrus aurantifolia) juice induce apoptosis in human pancreatic cells. Journal of agricultural and food chemistry, 2009. **57**(22): p. 10933-10942.
[12]. Unnisa, N., et al., Evaluation of antibacterial activity of five selected fruits on bacterial wound isolates. Pomegranate (Punica granatum L.), 2012. **10**(11): p. 12.
[13]. Okeke, M.I., et al., Antibacterial activity of Citrus limonum fruit juice extract. Pak. J. Pharm. Sci, 2015. **28**(5): p. 1567-1571.
[14]. Patil, B.S., et al., Bioactive compounds: historical perspectives, opportunities, and challenges. Journal of agricultural and food chemistry, 2009. **57**(18): p. 8142-8160.
[15]. Wang, S., et al., Characterization and metabolic diversity of flavonoids in citrus species. Scientific Reports, 2017. **7**(1): p. 1-10.
[16]. Sharma, K., N. Mahato, and Y.R. Lee, Extraction, characterization and biological activity of citrus flavonoids. Reviews in Chemical Engineering, 2019. **35**(2): p. 265-284.
[17]. Adamczak, A., M. Ożarowski, and T.M. Karpiński, Antibacterial activity of some flavonoids and organic acids widely distributed in plants. Journal of Clinical Medicine, 2020. **9**(1): p. 109.
[18]. Sagar, N.A., et al., Fruit and vegetable waste: bioactive compounds, their extraction, and possible utilization. Comprehensive Reviews in Food Science and Food Safety, 2018. **17**(3): p. 512-531.
[19]. Viuda-Martos, M., et al., Antibacterial activity of lemon (Citrus lemon L.), mandarin (Citrus reticulata L.), grapefruit (Citrus paradisi L.) and orange (Citrus sinensis L.) essential oils. Journal of food safety, 2008. **28**(4): p. 567-576.
[20]. Heggers, J.P., et al., The effectiveness of processed grapefruit-seed extract as an antibacterial agent: II. Mechanism of action and in vitro toxicity. The Journal of Alternative & Complementary Medicine, 2002. **8**(3): p. 333-340.
[21]. Dhanavade, M.J., et al., Study antimicrobial activity of lemon (Citrus lemon L.) peel extract. British Journal of pharmacology and Toxicology, 2011. **2**(3): p. 119-122.

[22]. Ekwenye, U.N. and O.V. Edeha, The antibacterial activity of crude leaf extract of Citrus sinensis (sweet orange). Int J Pharm Bio Sci, 2010. **1**(4): p. 742-750.
[23]. Jantrawut, P., et al., Enhancement of antibacterial activity of orange oil in pectin thin film by microemulsion. Nanomaterials, 2018. **8**(7): p. 545.
[24]. do Evangelho, J.A., et al., Antibacterial activity, optical, mechanical, and barrier properties of corn starch films containing orange essential oil. Carbohydrate polymers, 2019. **222**: p. 114981.
[25]. Negi, P. and G. Jayaprakasha, Antibacterial activity of grapefruit (Citrus paradisi) peel extracts. European Food Research and Technology, 2001. **213**(6): p. 484-487.
[26]. Dubber, M.-J. and I.J.J.P.P.S. Kanfer, High-performance liquid chromatographic determination of selected flavonols in Ginkgo biloba solid oral dosage forms. 2004. **7**(3): p. 303-309.
[27]. Giada, M.J.O.s. and c.d.d.A.r.f.a. InTech, Food phenolic compounds: main classes, sources and their antioxidant power. 2013: p. 87-112.
[28]. Kaczmarek, B., Tannic acid with antiviral and antibacterial activity as a promising component of biomaterials—A minireview. Materials, 2020. **13**(14): p. 3224.
[29]. Shivaprasad, D., et al., In vitro and in situ abrogation of biofilm formation in E. coli by vitamin C through ROS generation, disruption of quorum sensing and exopolysaccharide production. 2021. **341**: p. 128171.
[30]. Vilchèze, C., et al., Mycobacterium tuberculosis is extraordinarily sensitive to killing by a vitamin C-induced Fenton reaction. Nature communications, 2013. **4**(1): p. 1-10.
[31]. Cushnie, T.T. and A.J.J.I.j.o.a.a. Lamb, Recent advances in understanding the antibacterial properties of flavonoids. 2011. **38**(2): p. 99-107.
[32]. Akiyama, H., et al., Antibacterial action of several tannins against Staphylococcus aureus. 2001. **48**(4): p. 487-491.
[33]. Kaczmarek, B.J.M., Tannic acid with antiviral and antibacterial activity as a promising component of biomaterials—A minireview. 2020. **13**(14): p. 3224.
[34]. Akiyama, H., et al., Antibacterial action of several tannins against Staphylococcus aureus. Journal of antimicrobial chemotherapy, 2001. **48**(4): p. 487-491.
[35]. Rajagopal, M. and S. Walker, Envelope structures of Gram-positive bacteria, in Protein and Sugar Export and Assembly in Gram-positive Bacteria. 2015, Springer. p. 1-44.
[36]. Michna, R.H., et al., Subti Wiki 2.0—an integrated database for the model organism Bacillus subtilis. Nucleic acids research, 2016. **44**(D1): p. D654-D662.
[37]. Lowy, F.D., Staphylococcus aureus infections. New England journal of medicine, 1998. **339**(8): p. 520-532.
[38]. Tong, S.Y., et al., Staphylococcus aureus infections: epidemiology, pathophysiology, clinical manifestations, and management. Clinical microbiology reviews, 2015. **28**(3): p. 603-661.
[39]. Kobayashi, S.D., N. Malachowa, and F.R. DeLeo, Pathogenesis of Staphylococcus aureus abscesses. The American journal of pathology, 2015. **185**(6): p. 1518-1527.
[40]. Taylor, T.A. and C.G. Unakal, Staphylococcus aureus. 2017.
[41]. Smith, T.C., Livestock-associated Staphylococcus aureus: the United States experience. PLoS Pathog, 2015. **11**(2): p. e1004564.
[42]. Caulier, S., et al., Overview of the antimicrobial compounds produced by members of the Bacillus subtilis group. Frontiers in microbiology, 2019. **10**: p. 302.
[43]. Earl, A.M., R. Losick, and R.J.T.i.m. Kolter, Ecology and genomics of Bacillus subtilis. 2008. **16**(6): p. 269-275.
[44]. Logan, N.A. and P.D. Vos, Bacillus. Bergey's manual of systematics of archaea and bacteria, 2015: p. 1-163.
[45]. Turnbull, P.C., J. Kramer, and J. Melling, Bacillus. Manual of clinical microbiology, 1991. **5**: p. 296-303.
[46]. Elshaghabee, F.M., et al., Bacillus as potential probiotics: status, concerns, and future perspectives. Frontiers in microbiology, 2017. **8**: p. 1490.
[47]. Stein, T.J.M.m., Bacillus subtilis antibiotics: structures, syntheses and specific functions. 2005. **56**(4): p. 845-857.

[48]. Gu, Y., et al., Advances and prospects of Bacillus subtilis cellular factories: from rational design to industrial applications. 2018. **50**: p. 109-121.
[49]. Saleem, M. and M.T. Saeed, Potential application of waste fruit peels (orange, yellow lemon and banana) as wide range natural antimicrobial agent. Journal of King Saud University-Science, 2020. **32**(1): p. 805-810.
[50]. Egbuonu, A.C.C. and C.A. Osuji, Proximate compositions and antibacterial activity of Citrus sinensis (sweet orange) peel and seed extracts. European Journal of Medicinal Plants, 2016: p. 1-7.
[51]. Gopal, P.V., Evaluation of antimicrobial activity of Citrus aurantium against some gram positive and negative bacterial strains. Pharmacia J, 2012. **1**(3): p. 107-109.
[52]. Adham, A., Phytochemical analysis and evaluation antibacterial activity of Citrus medica peel and juice growing in Kurdistan/Iraq. Journal of Applied Pharmaceutical Sciences, 2015. **5**(10): p. 136-141.
[53]. Okunowo, W.O., et al., Essential oil of grape fruit (Citrus paradisi) peels and its antimicrobial activities. 2013.