Determination of Antimicrobial Activity of Some Commercial Fruit (Apple, Papaya, Lemon and Strawberry) against Bacteria Causing Urinary Tract Infection

A DISSERTATION SUBMITTED TO THE DEPARTMENT OF MATHEMATICS AND NATURAL SCIENCES, BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF BACHELOR OF SCIENCE IN BIOTECHNOLOGY

Submitted by

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May, 2018

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To my loving parents, my brother and my sister

Declaration

I hereby declare that this thesis entitled **"Determination of antimicrobial activity of some commercial fruit (apple, papaya, lemon and strawberry) extracts against bacteria causing urinary tract infection"** is submitted by me, Sabiha Jahan Liya, to the Department of Mathematics and Natural Sciences under the supervision and guidance of Ms. Romana Siddique, Senior Lecturer, Biotechnology Programme, Department of Mathematics and Natural Sciences, BRAC University. I also declare that the thesis work presented here is original, and has not been submitted elsewhere for any degree or diploma.

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Acknowledgement

First and foremost, I would like to begin by thanking the Almighty Allah for giving me the strength and patience to complete my thesis work successfully.

I would like to express my sincere gratitude to **Professor A.F.M. Yusuf Haider**, Chairperson and **Professor A.A Ziauddin Ahmad**, Former Chairperson of the Department of Mathematics and Natural Sciences, BRAC University for providing me with the opportunity and the resources to conduct my thesis.

I am very much obliged and forever grateful to my supervisor, **Ms. Romana Siddique**, Senior Lecturer, Department of Mathematics and Natural Sciences, BRAC University, who constantly supported me through and through. Her office door was always open whenever I ran into a trouble spot or had a question about my research or writing. She consistently steered me in the right the direction whenever I needed it and not even for once let me lose hope.

I would like to show my appreciation towards Asma Binte Afzal, Nahreen Mirza and Salman Khan Promon for assisting me throughout my thesis work which would not have been possible without them. I would also like to thank Furkan bhai and Mamun bhai for making sure I do my lab work properly.

Really special thanks go to Sunayna Hossain, Shabnam Sayeed, Salma, Rabeya Tafsire Rudhy and Samia Afroz for always motivating me and encouraging me to do better and to be better.

Lastly and most importantly, my gratitude knows no bounds to my parents who are the sole reason I exist and whose contribution and love for me can never be expressed in words.

Sabiha Jahan Liya

May, 2018

Abstract

Urinary Tract Infection (UTI) is a worldwide phenomenon in modern times which is increasing the dependency on antibiotics for its treatment. The current study was conducted in order to find alternatives to antibiotics by investigating some commercial fruits for their antimicrobial activity. The fruits in this study includes **Green Apple (*Malus domestica*), Papaya (*Carica papaya*), Lemon (*Citrus limon*) and Strawberry (*Fragaria ananassa*)** which were used to prepare methanolic and ethanolic extracts through soxhlet extraction technique. The extracts were used against bacteria that causes UTI and five different strains were selected: ***E. coli ATCC: 15922, E. coli ATCC: 25922, Pseudomonas aeruginosa ATCC: 27853, Enterococcus faecalis ATCC: 29212*** and ***Klebsiella pneumoniae*.** The fruits were bought from the local market, cut into small slices and were subjected to sun drying to reduce their moisture content. The dried fruit slices were grounded to fine powder and then run through soxhlet extractor to obtain methanolic and ethanolic extracts by using respective solvents. Antimicrobial test of the extracts were conducted by following agar well diffusion method, where ciprofloxacin was used as a positive control and distilled water was used as a negative control. Among the fruits, Apple and Papaya did not show any zone of inhibition against any of the tested bacteria. But both Lemon and Strawberry showed inhibition zone against all of the mentioned bacteria. The ethanolic extracts of Lemon and Strawberry were more potent than the methanolic extracts. Lemon ethanolic extract showed the highest zone of inhibition against ***Pseudomonas aeruginosa ATCC: 27853*** (18.34 ± 0.58) and lowest against ***Klebsiella pneumoniae*** (16.00 ± 1.00). Strawberry ethanolic extracts were also more potent than their methanolic extracts. The highest zone of inhibition was seen against ***Pseudomonas aeruginosa ATCC: 27853*** (16.33 ± 0.58) and the lowest one against ***Klebsiella pneumoniae*** (13.33 ± 0.58). The results of Lemon and Strawberry can be considered to be used as an antimicrobial agent in treating UTI.

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Chapter One

Introduction

Introduction

1.1 Overview

Urinary tract infections (UTIs) are some of the most common bacterial infections, affecting 150 million people each year worldwide (Flores-Mireles et al., 2016; Stamm, 2001). UTIs refer to the presence of microbial pathogens within the urinary tract and it is usually classified by the site of infection as bladder (cystitis) and kidney (pyelonephritis) (Nerurkar et al., 2012). In comparison with men, UTI is reported more in women (Angoti et al., 2016). UTIs are a significant cause of morbidity in infant boys, older men and females of all ages. Serious sequelae include frequent recurrences, pyelonephritis with sepsis, renal damage in young children, pre-term birth and complications caused by frequent antimicrobial use, such as high-level antibiotic resistance and *Clostridium difficile* colitis (Flores-Mireles et al., 2016).

UTIs are caused by both Gram-negative and Gram-positive bacteria, as well as by certain fungi. The most common causative agent for UTIs is uropathogenic *Escherichia coli* (UPEC), followed in prevalence by *Klebsiella pneumoniae*, *Staphylococcus saprophyticus*, *Enterococcus faecalis*, group B *Streptococcus*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida* spp. (Flores-Mireles et al., 2016; Foxman, 2014; Nielubowicz and Mobley, 2010).

UTIs result in considerable economic and public health burdens and substantially affect the life quality of afflicted individuals (Kostakioti, 2012). Currently, antibiotics such as trimethoprim sulfamethoxazole, ciprofloxacin and ampicillin — are the most commonly recommended therapeutics for UTIs (Foxman, 2010). However, increasing rates of antibiotic resistance and high recurrence rates threaten to greatly enhance the burden that these common infections place on society (Flores-Mireles et al., 2016). If any infection in a patient is not controlled, infecting microbes get resistance to the applied antibiotics intrinsically and a drug resistant cell survives and predominates with concomitant bacterial genetic exchanges mechanisms (McMurry and Levy, 2011; Mishra et al., 2017). In such situations, alternatives has to be found and turning to nature for help is not unusual. For Centuries plants have been used throughout the world as drugs and remedies for various diseases and infections (Sharma et al., 2009). Many plant materials used as traditional medicine have been proven to be more effective, and relatively cheaper than their modern counterparts (Mann et al., 2008). Hence, researches are ongoing to evaluate different plants and fruits for their antimicrobial ability.

1.2 Green Apple (*Malus Domestica*)

Apples are fruits consumed worldwide in different forms *i.e.* fresh, in juices and cider. Their beneficial properties to human health are related to the high content of phenol compounds (Alberto et al., 2006). Apples contain many types of phenolic derivates and flavonoids (flavan-3-ols, flavonols, procyanidins, chalcones, and anthocyanins) (Mangas et al. 1999; Podsedek et al. 2000; Shoji et al. 2003; Alberto et al., 2006). Apple pulp contains catechin, procyanidin, caffeic acid and chlorogenic acid among other components. The skin contains all the aforementioned substances as well as flavonoids, not present in pulp, such as quercetin glycosides and cyanidin glycosides (Escarpa and Gonzalez, 1998; Vander Sluis et al. 200; Alberto et al., 2006). Apples have been found to have very strong antioxidant activity and antimicrobial properties (Boyer and Liu, 2004). Extracts from the organically grown apple exhibited a clear antimicrobial activity, against the *B. cereus* and *E. coli*. In this case, peel was found to possess a discrete activity, where the inhibition zones increased from 6 mm to 13mm of diameter (Fratianni et al., 2007).

Table 1: Scientific classification of *Malus domestica* (USDA, n.d)

Rank	Scientific Name
Domain	*Eukarya*
Kingdom	*Plantae*
Phylum	*Magnoliphyta*
Class	*Magnoliopsida*
Order	*Rosales*
Family	*Rosaceae*
Genus	*Malus*
Species	*Malus domestica*

Figure 1: *Malus domestica*

1.3 Papaya (*Carica papaya*)

Papaya is an economically important fruit that is extensively cultivated and marketed worldwide. Papaya is relished for its pleasant flavor, mouthfeel, and texture. Apart from the benefits of nutrition, the fruit is recognized to possess high therapeutic value also (Annegowda and Bhat, 2016). . Papaya contains a rich source of antioxidants, phytochemicals, nutrients such as; carotenes, vitamin C, and flavonoids, the B vitamins including folate and panthothenic acid, minerals such as potassium and magnesium, and dietary fiber (Murcia et al., 2001; Leong and Shui, 2002, Gopalan et al., 2004; Devaki et al., 2015). Papaya contains some specific antimicrobial substrates including Carpaine and Aglycons (Bandsode and Chavan, 2013). In case of antibacterial assay of dried leaf, green leaf, ripe pulp, ripe peel, root, stem, unripe pulp, unripe peel, seeds maximum zone of inhibition of 15mm, 12mm, 12mm, 13mm, 13mm, 28mm, 15mm, 13mm, 14mm against *Staphylococcus aureus, E. coli, Pseudomonas aeruginosa, E. coli, Pseudomonas aeruginosa, Staphylococcus aureus, Pseudomonas aeruginosa, Staphylococcus aureus, E. coli* were seen respectively (Khan et al., 2012).

Table 2: Scientific classification of *Carica papaya* (USDA, n.d)

Rank	Scientific name
Kingdom	*Plantae*
Division	*Magnoliophyta*
Class	*Magnoliopsida*
Order	*Violales*
Family	*Cacicaceae*
Genus	*Carica L.*
Species	*Carica papaya L.*

Figure 2: *Carica papaya L.*

1.4 Lemon (*Citrus limon)*

Citrus fruits are one of the world's most important fruit crops, and are known for their nutritive values and special aroma (Shie and Lay, 2013). Citrus fruits as a group of fruits which are in high demand in the world have remarkable economic, social and cultural impacts in our society (Iglesias et al., 2007; Motie et al., 2014). Among these fruits, lemon (*Citrus limon*) is the third most important citrus species after orange and mandarin (Perez-Perez et al., 2005; Motie et al., 2014).Medicinal plants have an important role for the health of individuals and communities and Citrus fruit is an important medicinal plant of the family Rutaceae. It is used mainly for its alkaloids, which are having anticancer activities and the antibacterial potential in crude extracts of different parts (leaves, stem, root, juice, peel and flower) of lemon against various bacterial strains. Citrus fruits have a broad spectrum of biological activity including antibacterial, antifungal, antidiabetic, anticancer and antiviral activities due to alkaloids (Akhilesh et al., 2012; Ali et al., 2017).The acetone extract of C. limon used against *E. faecalis* and *B. subtilis* gave inhibition zone diameters of 23 and 20 mm respectively (Otang and Afolayan, 2015). Methanolic extract of lemon peels exhibited the maximum zone of inhibition (23 mm) against *Pseudomonas aeruginosa* (Pandey et al., 2011).

Table 3: Scientific classification of *Citrus limon* (USDA, n.d)

Rank	Scientific name
Kingdom	*Plantae*
Division	*Magnoliophyta*
Class	*Magnoliopsida*
Order	*Sapindales*
Family	*Rutaceae*
Genus	*Citrus L.*
Species	*Citrus limon L.*

Figure 3: *Citrus limon*

1.5 Strawberry (*Fragaria ananassa*)

Fruits and berries contain a variety of phenolic compounds located in plant tissues, often in the surface layer of the plant, fruit or berry (Nohynek et al., 2006). One of the widely consumed fruits, Strawberries (*Fragaria x ananassa*) are cultivated worldwide for its aroma, bright red color, and the typical sweetness (Sitorus et al., 2012). Compared with other non-berry fruits, the strawberry is a rich source of folate, vitamin C several phytochemicals and phenolic compounds such as anthocyanins, flavonols, flavanols, condensed tannins (proanthocyanidins, ellagitannins, and gallotannins), hydroxybenzoic and hydroxycinnamic acid derivatives, and hydrolyzable tannins (Giampieri et al., 2012; Amatori et al., 2016). Strawberries have antibacterial and antioxidant effects. Previous studies state that the ingredients in strawberry juice showed antibacterial effects on *Streptococcus mutans*, which can help inhibit biofilm formation (Schaart et al., 2013; widyarman et al., 2017). According to Puupponen-Pimiae et al., 2001, strawberry extracts showed a zone of inhibition of up to 18 mm through the method of agar well diffusion.

Table 4: Scientific classification of *Fragaria annanasa* (USDA, n.d)

Rank	Scientific name
Kingdom	*Plantae*
Division	*Magnoliophyta*
Class	*Magnoliopsida*
Order	*Rosales*
Family	*Rosaceae*
Genus	*Fragaria*
Species	*Fragaria ananassa L.*

Figure 4: *Fragaria ananassa*

1.6 *Escherichia coli*

Escherichia coli occur in diverse forms in nature, ranging from commensal strains to those pathogenic on human or animal hosts (Elsas et al., 2011). Urinary tract infection (UTI) is one of the most common bacterial infections with approximately 60% of all women being diagnosed with a UTI at least once during their lifetime (Foxman et al., 2000), and 20–30% of women with a first UTI will have recurring infection (Foxman, 1990). The most common cause of UTI is the rod-shaped Gram negative bacteria, *Escherichia coli* that belong to the family Enterobacteriaceae (Al-jiffri et al., 2011). The frequency of *E. coli* in urine samples varies in different studies from 32%-40%. During 1999, a total 13774 non hospital urine samples were analyzed and a total of 2798 strains were isolated, among which half of them were *E. coli* (Rosa et al., 2001; Al-Jiffri et al., 2011).

1.7 *Klebsiella pneumoniae*

K. pneumoniae is a gram negative, rod-shaped, facultative anaerobe (Podschun and Ullman, 1998). The reason for its pathogenicity is the thick capsule layer surrounding the bacterium (Amako et l., 1988). *Klebsiella pneumoniae* has been shown to be the second most common uropathogenic agent causing UTI (Behzadi et al., 2010). *K. pneumoniae* represented 22% of all the urinary *Enterobacteriaceae* isolated during a study (El Bouamri et al., 2014).

1.8 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a gram-negative, rod-shaped bacterium, which is the third most common pathogen associated with hospital-acquired catheter-associated UTIs (Jarvis et al., 1992; Mittal et al., 2009). Within the hospital setting, 7–10% of urinary tract infections are caused by *Pseudomonas aeruginosa* (Ferreiro et al., 2017). *P. aeruginosa* is a non-fermenter bacteria with a large intrinsic resistance to multiple antibiotics (Mittal et al., 2009).

1.9 *Enterococcus faecalis*

During the last decades Enterococcus species have become of increasing medical interest as human pathogens causing an increasing number of infections in worldwide (Pinholt et al., 2013; Wisplinghoff et al., 2004; Hansen et al., 2015). *Enterococcus spp.* are facultatively anaerobic, catalase-negative Gram-positive cocci. Optimal temperature for growth of *E. faecalis* is 35°C. They are commensal inhabitants of the human gastrointestinal tract and rated as one of the most common cause of urinary tract infections (UTI) and the third most common cause of bacteremia. *Enterococcus faecalis* accounts for 65-90% of clinical *Enterococcus spp.* isolates (Hansen et al., 2015).

1.10 Fruit Extraction Technique

There are several types of plant extraction techniques in practice today to separate the active compounds from any plant material. In any experiment regarding antimicrobial activities of plant extracts, the extraction process used holds a very important place for they deliver us with the components that further influences and establishes our experiment.

Various plant extraction methods include maceration, infusion, digestion, decoction, percolation, soxhlet extraction, aqueous alcoholic extraction by fermentation, ultrasound extraction, superficial fluid extraction etc. The products from plants are relatively liquids or semisolids. The purpose of any standardized extraction procedures is to attain the therapeutically desired portion and to eliminate the inert material by treatment with a selective solvent known as menstruum (Handa et al., 2008).

The extraction techniques followed in the current study is the soxhlet extraction procedure. In this method, finely ground sample is placed in a porous bag or "thimble" made from a strong filter paper or cellulose, which is placed in the thimble chamber of the Soxhlet apparatus. Extraction solvent is heated to a boiling point of the solvent used in the bottom flask, vaporizes into the sample thimble, condenses in the condenser and drips back. When the liquid content reaches the siphon arm, the liquids are emptied into the bottom flask again and the process is continued (Azwanida, 2015). The advantage of this method is that large amounts of plant material can be extracted with a much smaller quantity of solvent. This affects tremendous economy in terms of time, energy and consequently financial inputs (Handa et al., 2008).

1.11 Objective

Urinary tract infection is becoming a worldwide phenomenon in modern times. With such increase, dependency on antibiotics is also rising, which, might not always work in our favor in order to cure such infection. In such cases, nature could provide us with simple and easy answers.

Plant materials have a vast potential to be used as an alternative to antibiotics. Because, if we look back in ancient times when antibiotics was still not discovered, people relied on the natural and herbal remedies to be cured of any diseases. So, having kept that in mind, the aim of the current study is to find out if the commercial fruits available in our country like: apple, papaya, lemon and strawberry have the potency to exhibit antimicrobial activity against UTI causing bacteria.

The specific objective of this study is to obtain the above mentioned fruits extracts through soxhlet extraction technique and test those extracts against bacteria that causes UTI by the agar well diffusion method.

Chapter Two

Materials & Methods

Materials and Methods

2.1 Materials:

1. Fruits: Fruits were bought from the local market.
    - Green Apple (*Malus domestica*)
    - Papaya (*Carica papaya*)
    - Lemon (*Citrus limon*)
    - Strawberry (*Fragaria ananassa*)

2. Bacterial strains: The bacterial strains from the laboratory stocks were used in this research which were previously collected from clinical samples and have been properly stored in the lab.
    - *E. coli ATCC: 15922*
    - *E. coli ATCC: 25922*
    - *Pseudomonas aeruginosa ATCC: 27853*
    - *Enterococcus faecalis ATCC: 29212*
    - *Klebsiella pneumoniae*

3. Media and Reagents:
    - Nutrient agar: The bacterial strains were weekly subcultured on freshly prepared nutrient agar plates.
    - Mueller Hinton agar: Disk diffusion and agar well diffusion tests were conducted on MHA plates.
    - Ethanol: Absolute ethanol was used for preparing ethanolic crude extracts of the selected fruits.
    - Methanol: Methanol was used for preparing methanolic crude extracts of the selected fruits.
    - Saline (0.9% NaCl): Bacterial suspensions were made using saline.
    - 0.5% McFarland solution: Standard used for checking the turbidity of bacterial suspensions.
    - Water: Used as a negative control.
    - Ciprofloxacin: Antibiotic disk used as a positive control.

4. Equipment:
    - Soxhlet apparatus
    - Rotary evaporator
    - Fume hood
    - Laminar hood
    - Autoclave
    - Incubator
    - Vortex machine
    - Weighing machine
    - Micropipette
    - Test tubes, conical flasks, cotton swabs, cork borer, petri dishes etc.

2.2 Methods:

1. Fruit preparation:
    - Fresh fruits were bought from the local market and washed with water.
    - Fruits were cut into thin and small slices and spread onto clean plates.
    - The plates were kept under direct sunlight and was covered with a clean white cloth to protect from dusts.
    - The fruits were dried for 5-6 days (depending on the water quantity of the fruits) until all the moisture content was lost and they became crisp.
    - Then the slices were grinded using a grinder to turn them into powdery form.

2. Preparation of fruit ethanolic extract:
    - 75gms of grounded fruits were measured and placed into a thimble which was run through a soxhlet apparatus at 78˚C temperature.
    - The flask of the soxhlet apparatus was filled with 250ml absolute ethanol as solvent.
    - Three cycles were observed for about 3-4 hours until the cotton inside turned white.
    - The extract, which also contained the solvent (ethanol), was then subjected to rotary evaporator at 78˚C.
    - The solvent was evaporated until a concentrated ethanolic crude extract was obtained.
    - Then this extract was stored in an autoclaved MacCartney bottles in the refrigerator (4˚C)
    - The crude extracts were later diluted to a concentration of 0.2 mg/µl, 0.4 mg/µl and 0.6 mg/µl using ethanol as the diluent.

3. Preparation of fruit methanolic extract:
    - 75gms of grounded fruits were measured and placed into a thimble which was run through a soxhlet apparatus at 64˚C temperature.
    - The flask of the soxhlet apparatus was filled with 250ml methanol as solvent.
    - Three cycles were observed for about 3-4 hours until the cotton inside turned white.
    - The extract, which also contained the solvent (methanol), was then subjected to rotary evaporator at 64˚C.
    - The solvent was evaporated until a concentrated methanolic crude extract was obtained.
    - Then this extract was stored in an autoclaved MacCartney bottles in the refrigerator (4˚C)
    - The crude extracts were later diluted to a concentration of 0.2 mg/µl, 0.4 mg/µl and 0.6 mg/µl using methanol as the diluent.

4. Preparation of Nutrient Agar:
    - The required amount of nutrient agar was weighed in a weighing machine and added with distilled water in a conical flask.
    - The solution was then boiled, while being continuously stirred, until it became transparent and bubbles were formed.
    - The flask was covered with aluminium foil, labeled and subjected to autoclave at 121˚C in order to rid of any impurities inside the media.
    - After autoclave, the media was poured onto small petri dishes inside the laminar hood and was kept to solidify before they were used for subculture of the bacterial strains.

5. Preparation of Mueller Hinton Agar:
    - The required amount of Mueller Hinton agar was weighed in a weighing machine and added with distilled water in a conical flask.
    - The solution was then boiled, while being continuously stirred, until it became transparent and bubbles were formed.
    - The flask was covered with aluminium foil, labeled and subjected to autoclave at 121˚C in order to rid of any impurities inside the media.
    - After autoclave, the media was poured onto large petri dishes inside the laminar hood and was kept to solidify before they were used for agar well diffusion test.

6. Preparation of saline:
    - NaCl (0.9g) was measured in the weighing machine and added with 100ml distilled water in a conical flask
    - Then the saline solution was pipetted into 5 test tubes, each containing 9ml of NaCl.
    - The test tubes were then subjected to autoclave to rid of any impurites
    - After autoclaving, the tubes were labeled and stored in room temperature for further use.

7. Subculture of bacterial strains in Nutrient agar:
    - Stock cultures were placed inside the laminar hood along with freshly prepared NA plates.
    - A loop was heated in the Bunsen flame until red hot and then dipped into one side of an NA plate for cooling
    - A loopful of bacteria was scooped up from the stock and streaked onto the fresh NA plates
    - The streaked NA plates were then incubated for 24h at 37˚C after which they were stored in 4˚C

8. Agar Well Diffusion:
    - 24h subcultured plates of bacterial strains were placed in the laminar hood.
    - A loop was sterilized in the Bunsen flame and was used to scoop of appropriate amount of bacteria and then dipped into the test tubes containing saline solution to make a suspension.
    - The test tubes were vortexed and the turbidity of the suspension was compared with the 0.5% MacFarland standard solution.
    - Then, an autoclaved cotton swab was dipped into the suspension and pressed against the inner walls of test tubes to remove excess liquid before taking them out
    - The cotton swab was rubbed horizontally across the surface of the labelled MHA plates to conduct lawn culture of the bacterial strains
    - Then, a cork borer was heated to sterilize, cooled and then pressed onto the MHA plates to create 3 wells on 3 different quadrant of the agar.
    - After that, each well was labelled and filled accordingly with 60 microlitres of respective diluted methanolic and ethanolic fruit extracts and distilled water.
    - Ciprofloxacin antibiotic disk was used as a positive control and placed onto one quadrant.
    - Then the MHA plates were kept in the incubator for 24h at 37˚C and the results were recorded the next day.

- All the tests were conducted 3 times to obtain the average value of zones of inhibition.
- The mean value of zones of inhibition was afterwards used to calculate the standard deviation value for the respective fruit extracts and the activity index for measuring the relative efficacy.
- The following formula for activity index was used:

Activity Index = zone of inhibition of fruit extract / zone of inhibition of ciprofloxacin

Chapter Three

Results

Results

3.1 Green Apple

Green apple extracts did not show any zone of inhibition against the selected bacterial strains.

Table 5: Antimicrobial test results of Apple methanolic and ethanolic extracts

	Quadrant (mm)	*E.coli ATCC: 15922*	*E.coli ATCC: 25922*	*Pseudomonas aeruginosa ATCC:27853*	*Enterococcus faecalis ATCC: 29212*	*Klebsiella pneumoniae*
Average Diameter of Zone of Inhibition (mm)	Antibiotic	32.67	31.55	36.66	37.00	30.25
	Methanol	0.00	0.00	0.00	0.00	0.00
	Ethanol	0.00	0.00	0.00	0.00	0.00
	Distilled water	0.00	0.00	0.00	0.00	0.00
Activity Index	Methanol	0.00	0.00	0.00	0.00	0.00
	Ethanol	0.00	0.00	0.00	0.00	0.00

3.2 Papaya

Papaya extracts did not show any zone of inhibition against the selected bacterial strains.

Table 6: Antimicrobial test results of Papaya methanolic and ethanolic extracts

	Quadrant (mm)	*E.coli ATCC: 15922*	*E.coli ATCC: 25922*	*Pseudomonas aeruginosa ATCC:27853*	*Enterococcus faecalis ATCC: 29212*	*Klebsiella pneumoniae*
Average Diameter of Zone of Inhibition (mm)	Antibiotic	30.55	31.34	34.00	32.67	29.34
	Methanol	0.00	0.00	0.00	0.00	0.00
	Ethanol	0.00	0.00	0.00	0.00	0.00
	Distilled water	0.00	0.00	0.00	0.00	0.00
Activity Index	Methanol	0.00	0.00	0.00	0.00	0.00
	Ethanol	0.00	0.00	0.00	0.00	0.00

3.3 Lemon

Lemon showed inhibition zones against the selected bacterial strains, which were used to measure the mean value, standard deviation and the activity index as followed:

Table 7: Antimicrobial test results of Lemon methanolic and ethanolic extracts

No. of Trials	Quadrant (mm)	*E.coli ATCC:15922*	*E.coli ATCC:25922*	*Pseudomonas aeruginosa ATCC:27853*	*Enterococcus faecalis ATCC:29212*	*Klebsiella pneumoniae*
1	Antibiotic	30.00	34.00	36.00	37.00	30.00
	Methanol	16.00	16.00	18.00	16.00	15.00
	Ethanol	17.00	16.00	19.00	17.00	16.00
	Distilled water	0.00	0.00	0.00	0.00	0.00
2	Antibiotic	32.00	33.00	37.00	36.00	31.00
	Methanol	17.00	16.00	17.00	17.00	15.00
	Ethanol	18.00	17.00	18.00	18.00	17.00
	Distilled water	0.00	0.00	0.00	0.00	0.00
3	Antibiotic	33.00	31.00	37.00	38.00	31.00
	Methanol	16.00	14.00	15.00	16.00	14.00
	Ethanol	17.00	16.00	18.00	18.00	15.00
	Distilled water	0.00	0.00	0.00	0.00	0.00
Average diameter of Zone of Inhibition (mm) ± SD	Antibiotic	31.67±1.52	32.67±1.53	36.66±0.58	37.00±1.00	30.66±0.58
	Methanol	16.34±0.58	15.32±1.15	16.67±1.53	16.32±0.58	14.67±0.58
	Ethanol	17.32±0.58	16.33±0.58	18.34±0.58	17.67±0.58	16.00±1.00
Activity Index	Methanol	0.5	0.4	0.4	0.4	0.4
	Ethanol	0.5	0.4	0.5	0.4	0.5

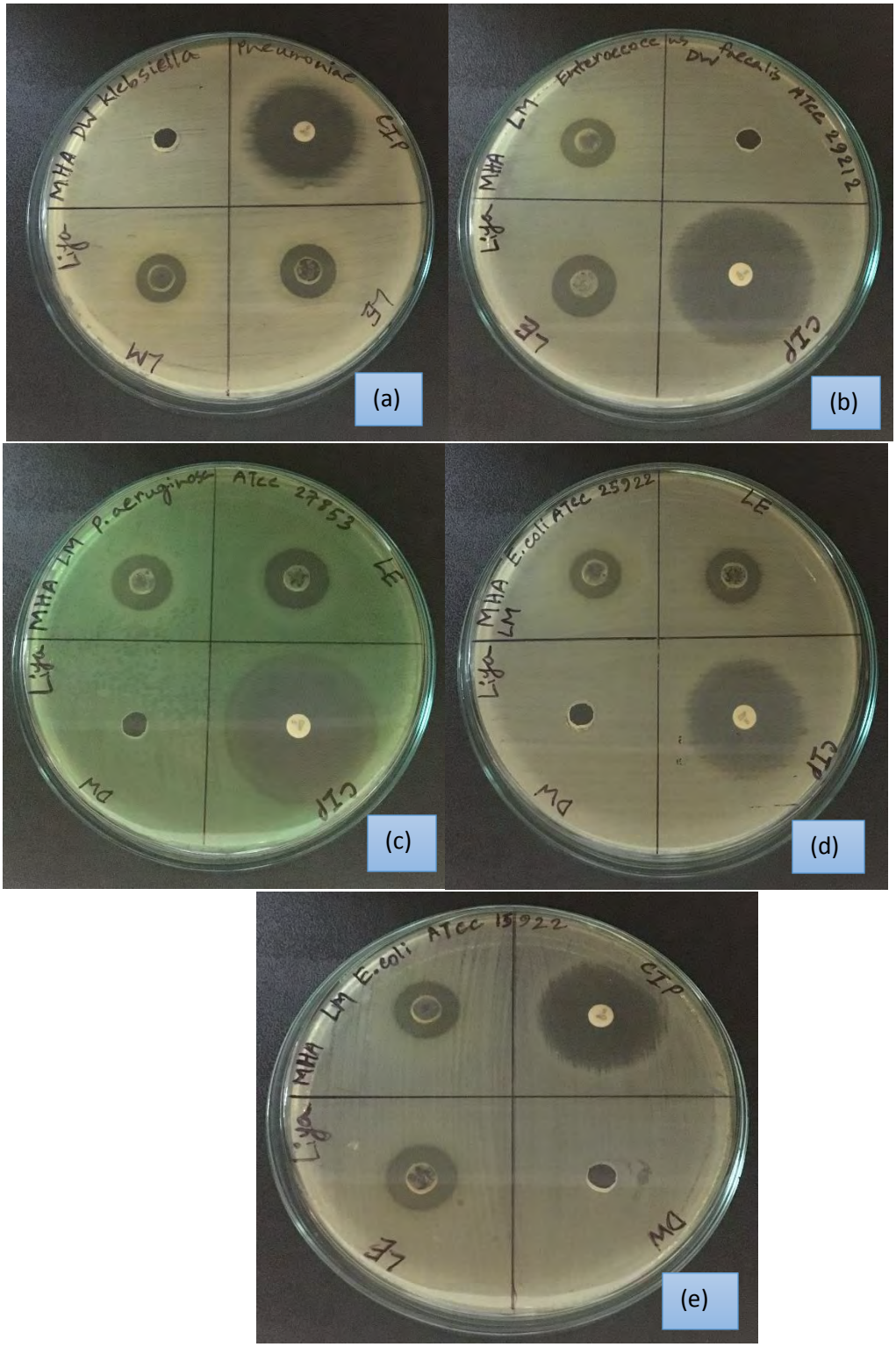

Figure 5: Antimicrobial effects of lemon methanol and ethanol extracts against (a) *K. pneumoniae* (b) *E. faecalis ATCC: 29212* (c) *P. aeruginosa ATCC: 27853* (d) *E. coli ATCC: 25922* and (e) *E. coli ATCC: 15922*

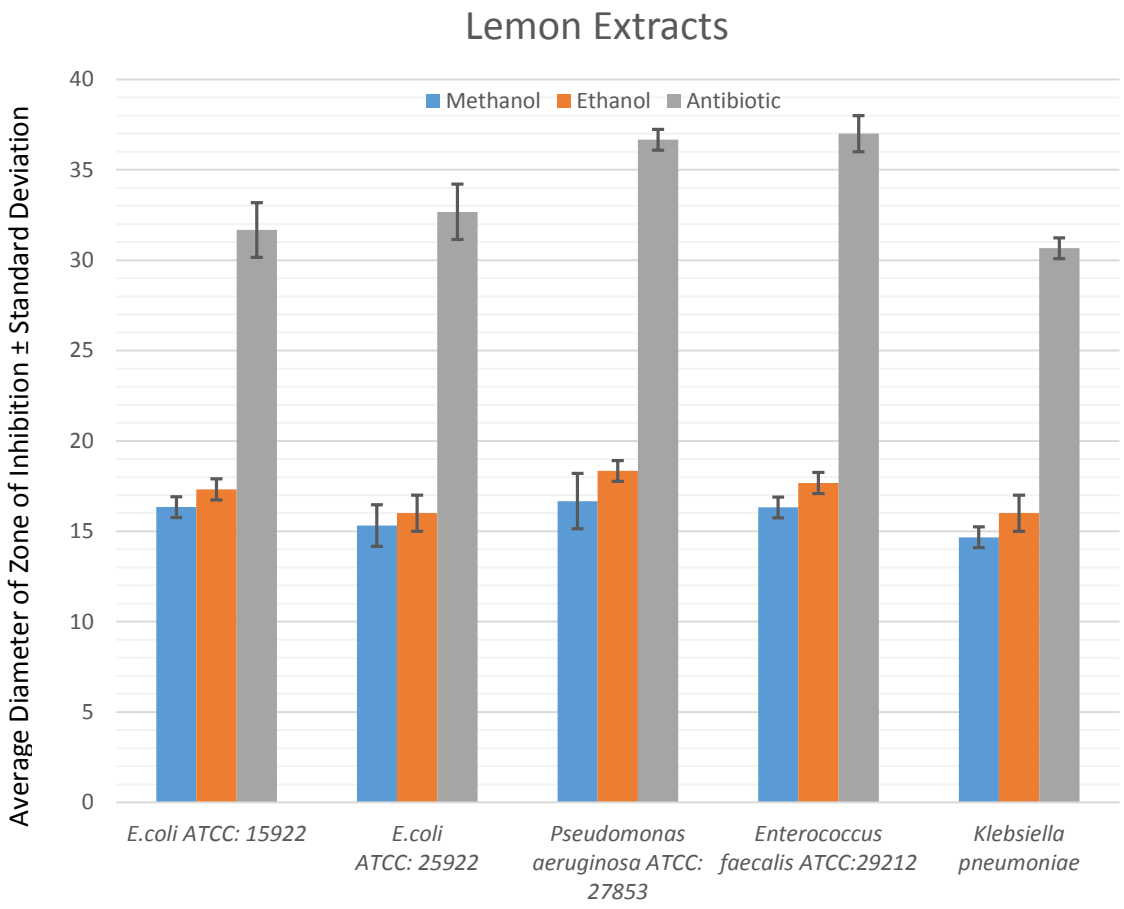

Figure 6: Antimicrobial test results of Lemon extracts are expressed as average diameter of zone of inhibition ± standard deviation of the three replicates

3.4 Strawberry

Strawberry showed inhibition zones against the selected bacterial strains, which were measured and recorded in the chart as followed:

Table 8: Antimicrobial test results of Strawberry methanolic and ethanol extracts

No. of Trials	Quadrant (mm)	*E.coli ATCC:15922*	*E.coli ATCC:25922*	*Pseudomonas aeruginosa ATCC: 27853*	*Enterococcus faecalis ATCC:29212*	*Klebsiella pneumoniae*
1	Antibiotic	31.00	30.00	32.00	31.00	30.00
	Methanol	13.00	13.00	15.00	14.00	11.00
	Ethanol	15.00	14.00	17.00	15.00	13.00
	Distilled water	0.00	0.00	0.00	0.00	0.00
2	Antibiotic	30.00	33.00	34.00	35.00	30.00
	Methanol	14.00	11.00	15.00	13.00	13.00
	Ethanol	16.00	14.00	17.00	15.00	14.00
	Distilled water	0.00	0.00	0.00	0.00	0.00
3	Antibiotic	29.00	31.00	35.00	32.00	28.00
	Methanol	12.00	12.00	14.00	12.00	11.00
	Ethanol	15.00	13.00	16.00	14.00	13.00
	Distilled water	0.00	0.00	0.00	0.00	0.00
Average diameter of Zone of Inhibition (mm)	Antibiotic	30.00±1.00	31.34±1.53	34.00±1.53	32.67±2.08	29.34±1.15
	Methanol	13.00±1.00	12.00±1.00	14.67±0.58	13.00±1.00	11.67±1.15
	Ethanol	15.33±0.58	13.67±0.58	16.33±0.58	14.67±0.58	13.33±0.58
Activity Index	Methanol	0.43	0.39	0.43	0.40	0.39
	Ethanol	0.51	0.43	0.48	0.44	0.45

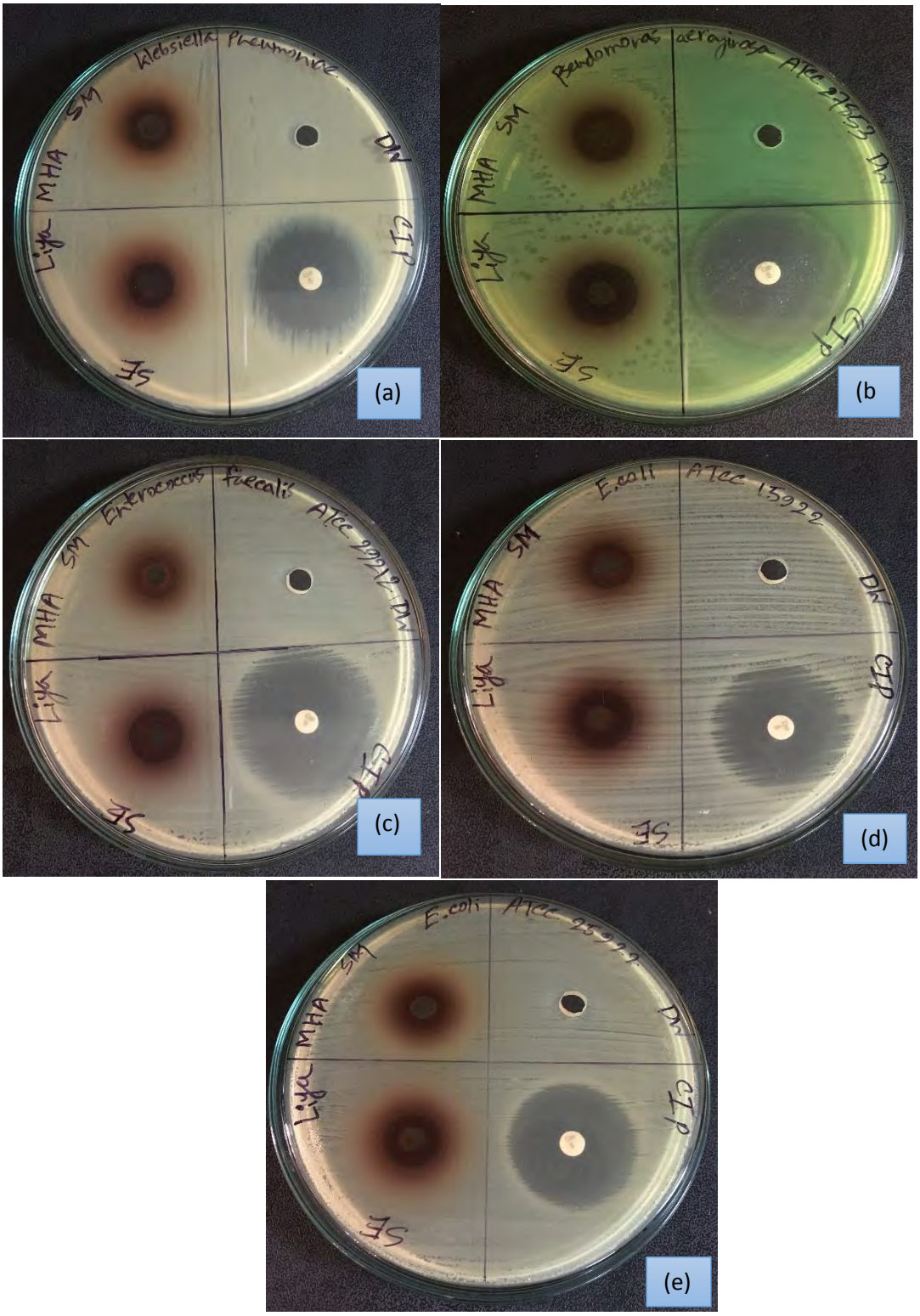

Figure 7: Antimicrobial effects of strawberry methanol and ethanol extracts against (a) *K. pneumoniae* (b) *P. aeruginosa ATCC: 27853* (c) *E. faecalis ATCC: 29212* (d) *E. coli ATCC: 15922* and (e) *E. coli ATCC: 25922*

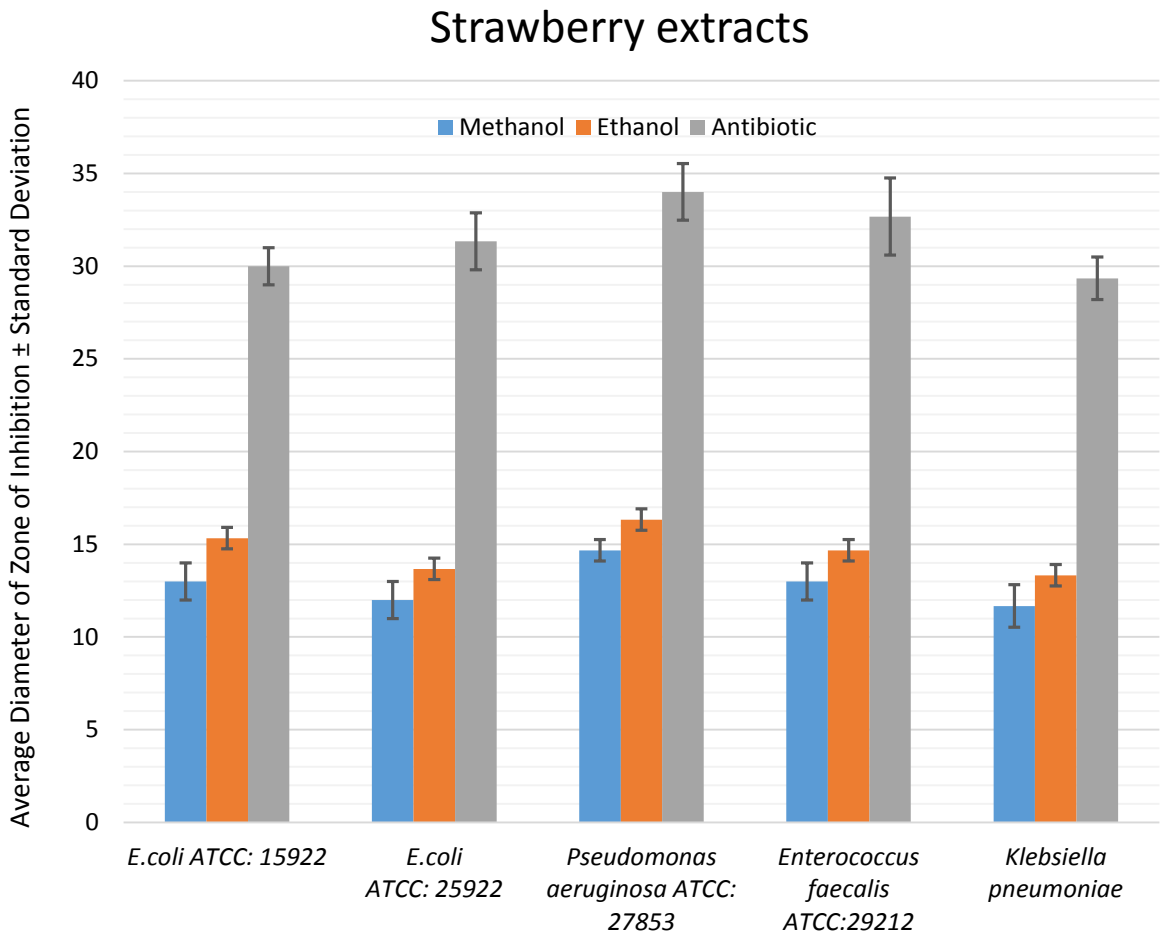

Figure 8: Antimicrobial test results of Strawberry extracts are expressed as average diameter of zone of inhibition ± standard deviation of the three replicates

Chapter Four

Discussion

Discussion

4.1 Apple

According to this study, the golden apple did not show any zone of inhibition against any of the tested bacteria. Both the ethanol and methanol extracts of apple showed negative results. These findings were surprisingly discordant with several studies conducted earlier using apple extracts. In one study, apple skin extracts were produced through cold maceration technique, using petroleum ether, ethanol and water as the solvents. Their study showed that ethanol extracts of apple gave zone of inhibition against *Klebsiella sp.* (9.2 mm) and *Pseudomonas sp.* (16.3 mm) (Sunilson et al., 2016). In another study conducted by Jelodarian et al. (2013), 4 cultivars of apple were used to make extracts, among which, two cultivars gave zone of inhibition against *P. aeruginosa* (11 mm), *E. coli* (16 mm) and *K. pneumoniae* (12 mm).According a third study, both apple methanol and ethanol extracts showed inhibitory activity against *S. aureus* (14.3 mm and 13 mm respectively) and EAEC (16.3 mm and 14.7 mm respectively). But the extracts did not show any results against *E. faecalis, P. aeruginosa or Klebsiella sp.* (Kabir et al., 2017). Their study is somewhat similar to this study but still holds the potential for apple to have the antimicrobial ability against some microbes.

The unusual results in the current study may be due to the differences in the variety of apple used in this experiment compared to other studies. The genotype of the *Malus domestica* may not be so contributive to the inhibitory effects against the tested bacteria. Antimicrobial properties are related to the bioactive compounds of the fruits (Sunilson et al., 2016) and apples are an important source of bioactive compounds like flavonoids, phenolic compounds and anti-oxidants. Their concentrations and activity may vary with cultivar and variety (Gonzalez-Aguilar et al., 2008). Furthermore, the soxhlet extraction process might not have been that much efficient in producing the apple extracts with the desired components present. Hence, the golden apple extracts failed to give the desired results.

4.2 Papaya

In the current study, papaya extracts of methanol and ethanol did not show any zone of inhibition against any of the selected bacteria. According to the study of Aruljothi et al., 2014, methanol extracts of *Carica papaya* leaf showed zone of inhibition against *P. aeruginosa* (17 mm), *E. coli* (12 mm) and *Klebsiella pneumoniae* (11 mm). Papaya ethanol extracts from their leaf, peel and seeds showed antimicrobial activity against *P. aeruginosa* and *E. coli* (Orhue and Momoh, 2013).

However, the present study was conducted using the ripened pulp of the papaya fruit unlike other studies where its leaf or seed or peels were used. In one study, ripened papaya fruit pulp extracts

of methanol and ethanol were used. The ethanol extract did not give any zone of inhibition for *E. coli*and *P. aeruginosa.* But the methanol extract showed inhibition zone only against *P. aeruginosa* (11 mm) but not for *E. coli* (Khan et al., 2012).This slight variance in result may be due to the variety of the papaya used. And also the bacterial strains used in the experiments could be more resistant to the inhibitory effects of papaya fruit pulp extracts compared to the bacterial strains used in the above mentioned research.

4.3 Lemon

The methanolic and ethanolic extracts of lemon showed positive results for all of the bacterial strains tested. The results were shown as mean value ± the value of standard deviation. Lemon extract for methanol showed the highest antimicrobial activity against *P. aeruginosa ATCC: 27853*(16.67 ± 1.53) and lowest antimicrobial activity against *K. pneumoniae* (14.67 ± 0.58). Lemon extract for ethanol showed highest antimicrobial activity against *P. aeruginosa ATCC: 27853* (18.34 ± 0.58) and lowest for *K. pneumoniae* (16.00 ± 1.00).

Tumane et al., 2014 conducted a similar study, using only the lemon peel for ethanolic and methanolic extraction using soxhlet. Their study also showed antimicrobial activity against *E. coli, P. aeruginosa, Klebsiella sp. & Enterococcus sp.* The difference between their study and the current experiment is that they only used the peel, whereas in this study, the whole lemon fruit were used for methanolic and ethanolic extraction.

According to another study, lemon fruit juice extract were made and tested against locally isolated clinical strains of E. coli, P. aeruginosa and gave zone of inhibition of 14 mm and 20 mm respectively (Okeke et al., 2015). These comparisons of studies indicate that lemon holds a good potential as an alternative to commercial antibiotics used now-a-days.

4.4 Strawberry

The methanolic and ethanolic extracts of strawberry showed positive results for all of the bacterial strains tested. The results were shown as mean value ± the value of standard deviation. Methanolic strawberry extract showed the highest antimicrobial activity against *P. aeruginosa ATCC: 27853*(14.67 ± 0.58) and lowest antimicrobial activity against *K. pneumoniae* (11.67 ± 1.15). Ethanolic extract showed highest antimicrobial activity against *P. aeruginosa ATCC: 27853* (16.33 ± 0.58) and lowest for *K. pneumoniae* (13.33 ± 0.58).

Strawberries are rich in vitamins and phenolic compounds, have antibacterial and antioxidant effects. Inhibition effect of strawberry extract was studied on monospecies and multispecies *E.faecalis* and *P. gingivalis* bacteria grown as biofilms *invitro.* The 100% strawberry extract concentration inhibited the formation of both the monospecies and multispecies *E. faecalis* and *P. gingivalis* biofilms (Widyarman, 2017). On the contrary, in another study, strawberry fruit extracts were used to evaluate the antimicrobial activity against certain bacterial and fungal

strains by cup plate diffusion method. Surprisingly, it did not present any anti-bacterial activity but did show anti-fungal activity against *C. albicans* and *A. niger* (Debnath et al., 2011).

Compared to these mixed results of previous experiments, strawberry extracts showed much better results in our study. This difference may be due to the fact of different cultivar of strawberries used in the experiments. The commercially available cultivar of strawberry in our country proves to be a potent antimicrobial agent and therefore holds the potential to be studied and experimented further.

4.5 Limitations and Further scope

Even though soxhlet extraction technique is a traditional method for plant material extraction, it does come with a few limitations. To begin with, this technique is very time consuming and only one solvent can be used for plant extraction at a time which further delays the time period of the whole experiment. Then, for efficient extraction to occur, soxhlet requires the plant material to be in the finely grounded powdery form, which may not be possible for the fruits. Because, even after an extensive period of drying the fruits, they still remain a little sticky with a little less surface area.

However, soxhlet extraction method is easy to carry out for small scale research regarding plant extraction. For more efficient extraction of the fruits bioactive compounds, other modified extraction methods could also be used. Furthermore, HPLC could be applied to identify and isolate the precise bioactive components of the fruits responsible for antimicrobial activity, so that those components can be further studied to be amplified and be used in treating UTI and many other diseases.

4.6 Conclusion

Use of antibiotics for treating any kind of infection or disease is very common these days. Their use often goes unmonitored or uncontrolled. Such a situation is giving rise to different multi-drug resistant bacteria as well, even in case of UTI. In this scenario, turning to nature is only inevitable, which, considering the advancement in the scientific research field, does not disappoint us. As lemon and strawberry showed antimicrobial potency against tested bacteria according to this study, hence looking for the alternatives using fruits is a dependable choice. All we have to do is to choose the right technique for identifying the right component to be used for treating the appropriate infection or disease.

Chapter Five

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