Sudan University of Science and Technology

College of Graduate Studies

Antimicrobial Activity of Aqueous Citrus limon Extract against *Streptococcus pyogenes* (Group A) Isolated from Sore Throat Patients in Jeddah City, Saudi Arabia

النشاط المضاد للميكروبات لمستخلص الليمون المائي ضد المكورات السبحية المقيحة (المجموعة أ) المعزولة من مرضى التهاب الحلق بمدينة جدة , المملكة العربية السعودية

A dissertation Submitted in Partial Fulfillment for the Requirement of M.Sc. degree in Medical Laboratory Science (Microbiology)

By:

Maisa Mahdi Salh Salh

B. Sc. (Honors) in Microbiology, Faculty of Medical Laboratories Science, Alzaiem Alazhari University, (2011)

Supervisor:

Dr. Hind Haidar Ahmed

December 2019

الأية الكريمة

﴿ الحَمدُ لِلَّـهِ الَّذي أَنزَلَ عَلى عَبدِهِ الكِتابَ وَلَم يَجعَل لَهُ عِوَجًا ﴿١﴾ قَيِّمًا لِيُنذِرَ بَأسًا شَديدًا مِن لَدُنهُ وَيُبَشِّرَ المُؤمِنينَ الَّذينَ يَعمَلونَ الصّالِحاتِ أَنَّ لَهُم أَجرًا حَسَنًا ﴿٢﴾ ﴾

صدق الله العظيم

سوره الكهف

الايه (١،٢)

DEDICATION

This research is lovingly dedicated to my mother and my father who has been my constant source of inspiration, without their love and support this project would not have been made possible.

To my brothers,
who helped me with their
love.

To my teachers and friends who supported me with their confidence.

To those who help me even with the award.

ACKNOWLEDGMENTS

Almighty **ALLAH** for giving me strength and good health to accomplish this research work.

I am strongly indebted to my director and supervisor Dr. Hind Haidar for her great efforts and valuable pieces of advice correcting and guiding me.

All thanks to Dr. Ghazi Jamjoom for giving me a chance and allowed me to run this research in his laboratory. My gratitude and appreciation to my work colleague Mohammed Sagir and Fatimah Atta, for their assistance and cooperation.

I gratefully appreciate my little brother Mazin Mahdi for his kind help

Last but not least I would like to express my gratitude to everyone encouraged, supported, or assisted me throughout this study.

ABSTRACT

Plant-based therapeutics known to be easily biodegradable, natural and available, with minimal adverse side effects and is easily accessible at low prices.

This study was aimed to determine the antimicrobial activity of aqueous *Citrus limon* on clinically isolated *Streptococcus pyogenes* from sore throat patients in Jeddah, Saudi Arabia, during the period from October 2018 to December 2019.

A total of 135 throat swab samples were collected from patients suffering from sore throat patients, *Streptococcus pyogenes* were isolated by rapid strep test and standerd cultural techniques and identification by using Gram's stain and biochemical tests. Also, the antimicrobial activity of aqueous *Citrus limon* extract was done using a cup plate method.

A total of 135 infected patients with sore throat were enrolled in this study, their age ranged from 3-54 years, with a mean age of 8.84 ± in and 7.54 SD. Patients age were classified into four groups as follow: age ranged (<15) years was 123(91.1%) patients, (15-30) years was 7(5.2%)patients, (31-45) years was 4(3%) patients and (>45) years was 1(0.7%) patient. Females were 74 (54.8%), while males were 61(45.2%).

Out of 135, 72(53.3%) were showed positive growth of *S. pyogenes* by rapid strep test showing (100%) specificity and sensitivity. All 72/135 (53.3%) were showed fever and difficult to swallow, while 64/72 (88.9%) were showed exudate production, only 1/72 (0.7%) patient was on antibiotic treatment and history of recurrent infection.

Regarding age group 68/72 (٨٨.6%) of patients were showed positive growth of *S. pyogenes* in age group (1-15) years, 2/72(2.8%) in (16-30) years, and 2/72 (2.8%) in (31-45) years which showed statistically insignificant difference between *S. pyogenes* growth and age (*P*. value = 0.67).

S. pyogenes were 40/72 (55.6%) isolated from females, and 32/72 (44.4%) from males, there was an insignificant association between the *S. pyogenes* and the gender (*P*. value =0.85).

Regarding exudate, *S. pyogenes* were isolated from 64/72(88.9%) patients with exudate production which showed a highly statistically significant difference between S. *pyogenes* throat infection and exudate production (*P*. value = 0.00).

Considering penicillin treatment and history of recurrent infection, only 1/72 (1.4%) were re-infected with *Streptococcus pyogenes* and was under treatment which showed the

statistically insignificant difference between *S. pyogenes* growth, treatment, and history of recurrent infection (*P*. value = 0.34)

The different concentration (12.5%, 25% 50%, and 100%) (v/v) of *Citrus limon* juice extract was evaluated, *Citrus limon* aqueous extract was showed antimicrobial activity against tested bacteria; the highest mean of zone of inhibition observed in concentration 100% with mean of 21.5± 1.35 STD, 50% the mean was of 17.8 ± 1.59 STD, 25% was 14.2 ± 2.86 STD, while 12.5% was showed no zone of inhibition.

In conclusion *Streptococcus pyogenes* was the most frequent bacterial causes sore throat with no association of *Streptococcus pyogenes* pharyngitis with age and sex as a risk factors, treatment, and history of recurrent infection. Highly associations were found between *Streptococcus pyogenes* and exudate production. *Citrus limon* juice extract was found to be effective against *Streptococcus pyogenes* the effect was found to be increased when increasing the extract concentration.

الخلاصة

من المعروف أن العلاجات النباتية قابلة للتحلل بسهولة ، طبيعية ، متوفرة ، مع الحد الأدنى من الآثار الجانبية الضارة ويمكن الوصول إليها بسهولة بأسعار منخفضة.

هدفت هذه الدراسة إلى تحديد النشاط المضاد للميكروبات من مستخلص الليمون المائي على المكورات السبحية المقيحه المعزولة سريريا من مرضى التهاب الحلق في جدة ، المملكة العربية السعودية ، خلال الفترة من أكتوبر ٢٠١٨ إلى ديسمبر ٢٠١٩.

تم جمع ١٣٥ عينة من مسحة الحلق من مرضى يعانون من التهاب الحلق ، وعُزلت المكورات السبحية المقيحه باستخدام تقنية اختبار مولد المضاد السريع والزراعة ، و التعرف عليها باستخدام صبغة غرام والاختبارات الكيموحيوية. كما تم اجراء و تقييم النشاط المضاد للميكروبات من مستخلص الليمون المائي باستخدام الحفر علي سطح الاجار.

تمت دراسة ١٣٥ مريضا مصابا بالتهاب الحلق ، وتراوحت أعمارهم بين ٣-٥٤ سنوات ، مع متوسط عمر ٨,٨٤±٧,٥٤انحراف معياري. تم تصنيف عمر المرضى إلى أربع مجموعات على النحو التالي: الذين كانت أعمارهم (أقل من ١٥) سنة كانوا ١٢٣ (٩١,١ ٪) مريضا ، (١٥-٣٠) سنة كانوا ٧ (٥,٢ ٪) مريضا ، (٣١-٤٥) سنة كانوا ٤ (٣ ٪) مرضى و (>٤٥) سنة كان ١ (٠,٧ ٪) مريض. كانت الإناث ١٣٥\٧٤ (٥٤,٨ ٪) ، في حين كان الذكور ١٣٥\٦١ (٤٥,٢ ٪).

من أصل ١٣٥عينة ، أظهرت ٧٢ (٥٣,٣ ٪) منها نموا إيجابيا للبكتريا المكورات العقديه المقيحة عن طريق اختبار مولد المضاد السريع الذي أظهر (١٠٠ ٪) خصوصية وحساسية. أظهرت النتائج أن جميع المرضى الذين أصيبوا بحمى وصعوبة في البلع بنسبة ١٣٥/٧٢ (٥٣,٣٪) ، في حين أظهرت نتائج عملية إنتاج الإفرازات ٧٢/٦٤ (٨٨,٩٪) ، كان فقط ٧٢/١ (٠,٧٪) من المرضى يعالجون بالمضادات الحيوية ولديهم تاريخ من الإصابة المتكررة.

فيما يتعلق بالفئات العمرية تم عزل المكورات العقديه المقيحه من ٧٢/٦٨ (٨٨,٦ ٪) من المرضى في الفئة العمرية (<١٥) سنة ، ٧٢/٢ (٢,٨ ٪) في (١٦-٣٠) سنة ، و ٧٢/٢ (٢,٨ ٪) في (٣١-٤٥) سنة والتي لم تظهر فرق ذو دلالة إحصائية بين نمو بكتيريا المكورات السبحية المقيحة والعمر (قيمة $P = 0.67$).

كانت المكورات السبحية المقيحة ٧٢/٤٠ (٥٥,٦ ٪) معزولة عن الإناث ، و ٧٢/٣٢ (٤٤,٤ ٪) من الذكور ، لم يكن هناك ارتباط بين الجينات المقيحة والجنس (القيمة $P = 0.85$).

فيما يتعلق بالإفرازات المقيحه ، تم عزل المكورات السبحية المقيحة من ٧٢/٦٤ (٨٨,٩ ٪) من المرضى والذين كانوا يعانون من إنتاج الإفرازات والتي أظهرت فرقًا ذو دلالة إحصائية عالية بين عدوى المكورات السبحية المقيحة للحلق وإنتاج الإفرازات (القيمة $P = 0.00$).

وبالنظر إلى علاج البنسلين وتاريخ الإصابة المتكررة ، ٧٢/١ (١,٣ ٪) فقط كان لديه اصابه بالمكورات السبحية المقيحة وكان تحت العلاج مما أظهر الفرق غير المهم إحصائياً بين إصابة بكتريا المكورات السبحية المقيحة والعلاج والتاريخ بالإصابة المتكررة (القيمة $P =$٠,٣٤)

تم تقييم تراكيز مختلفة من مستخلص الليمون المائي (١٢,٥ ٪ ، ٢٥ ٪ ٥٠ ٪ ، و ١٠٠ ٪) (ت / ت) ، واظهر مستخلص الليمون المائي نشاط مضاد للميكروبات على البكتيريا المختبرة ؛ أعلى متوسط لمنطقة تثبيط لوحظ في

التركيز ١٠٠ (٪) مع متوسط٢١,٥ ± ١,٣٥ انحراف معياري ، 50 (٪) كان ١٧,٨ ± ١,٥٩ انحراف معياري ، 25 (٪) كان ١٤,٢ ± ٢,٨٦ انحراف معياري ، بينما ١٢,٥ ٪ لم تظهر أي تثبيط.

خلصت الدراسة الي ان البكتريا العقدية السبحية ذات تردد عالي كاكثر بكتريا مسببة للتهاب الحلق مع عدم وجود ارتباط بالتهاب الحلق والبكتريا السبحية المقيحة مع العمر والجنس كعوامل خطر والعلاج وتاريخ الإصابة بالعدوى المتكررة. تم العثور على علاقة عالية بين المكورات السبحية وإنتاج الإفرازات.

وجد ان مستخلص الليمون المائي فعالا ضد المكورات السبحية المقيحة حيث تزيد الكفاءة عند زيادة تركيز المستخلص.

Table of content

LIST OF TABLES

LIST OF FIGURES

List of Abbreviations

GAS Group A streptococcus

ARF Acute rheumatic fever

APSGN Acute post-streptococcal glomerulonephritis

RHD Rheumatic heart disease

HIV Human immune virus

AID Acquired immune deficiency

BC Before Christ

TCM Traditional Chinese medicine

WHO World Health Organization

USA United States of America

SPEs Streptococcal pyrogenic exotoxins

IL Interleukin

TNF Tumor necrosis factor

Fc Fragment crystallizable region

HLA Human leukocyte antigen

SLS Streptolysin S

SLO Streptolysin O

GN Glomerulonephritis

STSS Streptococcal toxic shock syndrome

SXT Trimethoprim-sulfamethoxazole

NET Nextracellular trap

β Beta

PYR Pyrrolidonyl arylamidase

RADT Rapid antigen-detection

DNA Deoxyribonucleic acid

ETEC Enterotoxigenic *Escherichia coli*

SPP Species

EndoS Endoglycosidase *S. Pyogenes*

CHAPTER ONE

INTRODUCTION

CHAPTER ONE

INTRODUCTION

1.1. Introduction

Streptococci considered one of the predominant flora colonizing the respiratory tract of humans (Todar, 2006). *Streptococcus pyogenes* is an obligate human pathogen causes major human morbidity and mortality worldwide. School-age children (5-15 years) are considered as the major reservoir of group A beta-hemolytic Streptococci (Al Kareem *et al*., 2014).

The group A Streptococci (GAS) causes the broadest range of disease can lead to the asymptomatic carriage, superficial infection of the upper respiratory tract (pharyngitis) and skin (impetigo or pyoderma), or invasive disease (bacteremia or focal infection such as osteomyelitis, pneumonia, and meningitis). GAS also has the potential to release exotoxins, resulting in scarlet fever or streptococcal toxic shock syndrome and to top it all off this is one of the few organisms that unequivocally causes autoimmune disease acute rheumatic fever (ARF) and acute post-streptococcal glomerulonephritis (APSGN). These last two manifestations can, in turn, lead to chronic sequelae, rheumatic heart disease (RHD), which may follow ARF (Carapetis *et al.*, 2005 a).

In a recent analysis of the prevalence of group A streptococcal disease, GAS responsible of causing 18 million cases of severe diseases resulting in 517, 000 deaths each year, although of the sensitivity to penicillin still remain as major public health in both developed and developing countries (Carapetis *et al.*, 2005 b).

Plants extracts are active against human microbial pathogens thus emerging as potential sources of new antimicrobial compounds (Kumar, 2012).

A large number of plants are constantly being screened for their antimicrobial effect as a rich source of compounds with possible antimicrobial activities. Lemon juice is believed to have antimicrobial properties in many cultures of the world (Pandey *et al*., 2011). In South Africa, lemon juice has been used in the treatment of HIV/AID patient oral thrush (Wright *et al*., 2009). In many parts of the world, lemon juice is also used as sanitizers to remove foodborne pathogens from fresh fruits, vegetables and fish (Sengun and Karapinar, 2004; Tomotake *et al*., 2006).

Freshly squeezed lemon juice has antibacterial activity against *Salmonella typhimurium* (Sengun and Karapinar, 2004), Vibrio species (Tomotake *et al*., 2006). *Pseudomonas aeruginosa* and *Escherichia coli* (Kumar *et al*., 2012).

1.2. Rationale

Plant-based therapeutics are known to be easily biodegradable, with minimal adverse side effects and being easily accessible at low prices (Fullerton *et al*., 2011).

Citrus limon fruits are natural, cheap, safe, and its aqueous extracts have an important role as antimicrobial agents against microorganisms (Hindi and Chabuck, 2013). *Citrus limon* juice possesses antibacterial activity against bacterial isolates; *Streptococcus pyogenes* were completely susceptible to lemon juice showed a more inhibitory effect than the antibiotics especially Azithromycin and Amoxicillin-Clavulanic acid (Mshelia, 2018). *Citrus limon* is juice antibacterial agents that can act as alternative medicine treatment, showed significant susceptibility of crude concentrations of *Citrus limon* juice against the clinical bacteria isolates of *Streptococcus pyogenes* from the respiratory tract infection (Eseoghene *et al*., 2017). Antimicrobial activity of *Citrus limon* aqueous extract could be used for the prevention of various diseases caused by *Streptococcus pyogenes* (Abdulhusin *et al.*, 2018).

Lemon is one of home care treatment, *Citrus limon* juice can be used as an antibacterial gargle (Dev and Nidhi, 2016). Improves the symptoms of strep throat, and smooth throat providing pain relief effect. Therefore, it is necessary to investigate those *Citrus limon* juice scientifically to find out a new antimicrobial herbal agent believed to be beneficial, free of side effects, and cheap. This study was designed to answer and verify the claimed activity of *Citrus limon* juice as antimicrobial substances and its effect on *Streptococcus pyogenes* pharyngitis.

1.3. Objectives

1.3.1. General objective

To study the antimicrobial activity of aqueous *Citrus limon* extract against *Streptococcus pyogenes* (Group A) isolated from sore throat patients

1.3.2. Specific objectives

1. To isolate and identify *Streptococcus pyogenes* from throat swabs by the conventional cultural method and rapid strep test.
2. To determine the frequency of *S. pyogenes* among sore throat patients.
3. To determine the antimicrobial activity of aqueous *Citrus limon* extract with different concentrations against *Streptococcus pyogenes* by cup plate method.
4. To compare between cultural methods and rapid strip test in isolation of *Streptococcus pyogenes*.
4. To correlate between *Streptococcus pyogenes* and possible risk factors (sex, age, treatment, symptoms, and recurrent infection).

CHAPTER TWO

LETRITURER REVIEW

CHAPTER TWO

LETRITURER REVIEW

2. Literature review

2.1 Herbal medicine

The plant kingdom is an important source of drug and many plants have been tested for the presence of compounds with therapeutic activity for their biological activities against living organisms causing various diseases (Ali, 2017). Herbal plant drugs are non-conventional therapy that globally expanded during the last decade that has been used to treatment of numerous health problems; the resistance to current antibiotics has become necessary problems to search for an alternative non-conventional effective traditional medicine (Tadeg *et al*., 2005).

Plants had been used for medicinal purposes a long time before the history has been recorded. Earlier in 3,000 BC, the Ancient Chinese and the Egyptian papyrus wrote a plant description for medicinal uses. In indigenous cultures (such as African and Native American), they were using herbs in their healing rituals, while others developed traditional medical systems such as Siddha, Ayurveda, Unani and traditional Chinese medicine (TCM), herbal therapies for medicinal purposes were already used (Ampofo, 2012).

According to the World Health Organization (WHO), the use of herbal remedies throughout the world exceeds the use of conventional drugs by two to three times. The use of plants for healing purposes predates human history and forms the origin of much modern medicine (Gunjan, 2015).

Plant-based medicines and other botanicals in the west has increased manifold in recent years, about two centuries ago, although medicinal practices were largely dominated by plant-based medicines, the medicinal use of herbs rapidly declined in the west when more predictable synthetic drugs were made commonly available (Mosihuzzaman, 2008).

The rising uses of nontraditional herbal medicines remedies led to the establishment of the office of Alternative Medicine by the National Institute of Health USA, in 1992. Worldwide, herbal medicine received a boost when the WHO encouraged developing countries to use traditional plant medicine to fulfill needs unmet by modern systems (Ekwenye and Edeha, 2010).

In the early 19th century, chemical analysis first became available, scientists began to extract and modify the active ingredients from plants. Lately, chemists began making their own version of plant compounds and over time, the uses of herbal medicines declined in favor of drugs (Steven and Ehrlich, 2011).

Almost one-fourth of pharmaceutical drugs are derived from the plant the herbal medicine is used to treat many conditions, such as asthma, eczema, premenstrual syndrome, rheumatoid arthritis, migraine, menopausal symptoms, chronic fatigue, irritable bowel syndrome and cancer (Steven and Ehrlich, 2011).

Herbal medicine is still the mainstay of about 75 - 80% of the world population, for primary health care in the developing countries, because generally, they believed that herbal drugs are cheap, locally available and without any side effects (Gunjan, 2015). In contrast, many developing nations carry on benefit from the rich knowledge of medical herbalism, for example, Siddha and Ayurveda medicines in India, TCM, Kampo medicine in Japan, and Unani medicine in the Middle East and South Asia are still used by a large majority of people (Mosihuzzaman, 2008).

2.1.1. Herbal medicine in Sudan

Local people in Sudan based on acquired folklore and local traditions that still rely on traditional medicines to treat several diseases and microbial infections. The traditional medicinal applications of Sudanese plants have encouraged many pharmacological investigations that depends on the phytochemical and pharmacological investigation of these extracts and purified compounds, that have been assessed for their biological activities, especially antibacterial, antioxidant, antimalarial, antifungal, anti-inflammatory, anticancer, and antidiabetic activities (Karar and Kuhnert, 2017).

2.2. *Citrus limon*

Citrus limon is an important medicinal plant of the family Rutaceae. It is cultivated mainly for its alkaloids, which have antibacterial potential and anticancer activities in crude extracts of different parts against clinically significant bacterial strains have been reported (Kawaii *et al*., 2000).

Flavonoids have a wide pattern of biological activity including antibacterial, antiviral, antifungal, antidiabetic, anticancer activities (Burt, 2004 and Ortuno *et al*., 2006).

Flavonoids act as direct antioxidants free radical scavengers, having a capacity of modulating enzymatic activities and inhibit cell proliferation (Duthie and Crozier, 2000).

2.2.1. Scientific Classification of *Citrus limon*

Kingdom -Plantae, Angiosperms, Eudicots, Rosids, Order -Sapindales, Family -Rutaceae, Genus - Citrus, Species - C. × Limon, Binomial name - Citrus × limon (Mohanapriya, 2013)

2.2.2. Nutritional value

Nutritional value of 100 grams of raw *Citrus limon* without peel is Energy – 121 kJ (29 kcal), Carbohydrates – 9.32 g, Sugars -2.50 g, Dietary fibre – 2.8 g, Fat – 0.30 g, Protein – 1.10 g, Thiamine (Vit.B1) – 0.040 mg (3%), Riboflavin (Vit. B2) – 0.020 mg (1%), Niacin (Vit. B3) – 0.100 mg (1%), Pantothenic acid (B5) – 0.190 mg (4%), Vitamin B6 – 0.080 mg (6%), Folate (Vit. B9) – 11 μg (3%), Vitamin C –53.0 mg (88%), Calcium – 26 mg (3%), Iron – 0.60 mg (5%), Magnesium – 8 mg -(2%), Phosphorus – 16 mg (2%), Potassium – 138 mg (3%), Zinc – 0.06 mg (1%) (Mohanapriya, 2013).

2.2.3. Pontifical distribution

Citrus limon grows on small, thorny trees that reach in height of 10 to 20 feet. The *Citrus limon* has a white, fragrant flower with five petals; this particular flower comes from cultivar called “Pink Lemonade”. The leaves of the *Citrus limon* are dark green in color and they are arranged alternately on the stem. *Citrus limon* fruits are oval with smooth porous skin; some fruits have a pointed tip on the bottom of the fruit while others are rounded at the base. Some kinds of *Citrus limon* fruits are quite larger than other, varieties and resemble elongated grapefruits. The color of fruits ranges from greenish-yellow to bright yellow. *Citrus limon* looks very similar to limes, limes are green, where *Citrus limon* tend to be a little larger and are yellow (Mohanapriya, 2013).

2.2.4. Pharmacological uses

Lemon juice helps in sugar balance, blood purifier, osteoporosis, asthma, control nausea and vomiting, arthritis and bone-related diseases, affect acne, spots, and pimples, and help in the smooth throat. Also use to prevent kidney stones, supporting weight loss, anti-cancer properties, act as potassium power that supports in normalizing blood pressure, and prevent scurvy because of a high amount of Vitamin *Citrus limon* juice shows anti-inflammatory and anesthetic effects and bring your fever down faster (Dev and Nidhi, 2016).

2.3. *Streptococcus pyogenes* (Group A streptococcus)

2.3.1. History

Streptococci were discovered by Billroth in 1874 from many cases of erysipelas and wound infections. In 1884 Pasteur was the first one who reported the isolation of these bacteria from the blood of a woman with puerperal sepsis. The organism was designated as *Streptococcus pyogenes* by Rosenbachin in the late 19th century. In the early 1900s, Donchez, George, and Dick identified that scarlet fever was caused by hemolytic streptococci (Parija, 2014).

2.3.2. Habitat

Streptococcus pyogenes is the main human pathogen that leads to local or systemic invasion and post-streptococcal immunologic disorders. GAS typically colonizes the throat and skin. However, with a 20–40% rate of asymptomatic carriers. The infection transmitted through person to person via respiratory droplets and through the skin by direct contact with an infected patient, fomites, or arthropod vectors (Jawetz*et et al*., 2010).

2.3.3. Morphology

GAS is aerobic, facultative anaerobic Gram-positive coccus that grows in chains, producing small white to gray colonies with a clear zone of β-hemolysis on blood agar. It is distinguished from other groups of β-hemolytic streptococci by a group-specific polysaccharide (C antigen) in the cell wall (van Sorge *et al.,* 2014).

2.3.4. Classification of Streptococci

2.3.4.1. Specific carbohydrates antigen

Lancefield classification is a serological classification of streptococci based on the presence of group-specific carbohydrates antigen (C antigen) on the cell wall, streptococci classified into 21 serological groups from A to V (with exception of I and J). *S. pyogenes* is belonged to group A according to Lancefield's method (Parija, 2014).

2.3.4.2. Type-specific protein

Griffith typing based on the M, T and R protein present on the cell wall surface, classified *Streptococcus pyogenes* into 80 serotypes M protein (Parija, 2014).

2.3.5. Virulence factors

2.3.5.1. Streptococcal pyrogenic exotoxins

Streptococcal pyrogenic exotoxins (SPEs) are secreted factors that trigger T-lymphocyte proliferation and cytokine release (Spaulding *et al*., 2013). They are heat-labile act as superantigens activating macrophages and T-helper cells inducing the release of powerful immune mediators including IL-1, IL-2, IL-6, TNF-alpha, TNF-beta, interferons, and cytokines, which induce scarlet fever that occurs in association with streptococcal pharyngitis and is manifested by a rash of the face and upper trunk, shock and organ failure (Tille, 2014). The SPEs are a family of more than 15 bacterial superantigens, including the bacteriophage encoded SPE A and SPEC (Alouf and Muller, 2003).

2.3.5.2. M protein

M protein is the chief virulence factor, heat stable and trypsin sensitive proteins (Parija, 2014). During invasive infection, significant quantities of M protein are released from the cell surface by proteolysis forming pro-inflammatory, clot like complex with human fibrinogen, leading to uncontrolled neutrophil activation, vascular leakage, and toxic shock symptomatology (Herwald *et al*., 2004; Macheboeuf *et al*., 2011).

M protein and fibronectin-binding proteins are important for subsequent endocytosis uptake of GAS into respiratory epithelial cells (Bisno *et al*., 2003). This process of intracellular invasion allows GAS access to a privileged intracellular niche, and it represents a proximal step in the pathogenesis of systemic infection (Courtney *et al*., 2002).

M protein a multifaceted immune resistance factor that promotes GAS resistance to phagocytosis through multiple mechanisms, including the binding of fibrinogen, complement inhibitory factor H, host antimicrobial peptides, and the Fc region of immunoglobulins (Ghosh, 2011; LaRock *et al.*, 2015). M protein also collaborates with the GAS virulence factor streptokinase to bind host plasminogen to the GAS surface; this generates plasmin activity, effectively coating the bacterial surface with a powerful protease to facilitate tissue spread (D McArthur, 2012).

Susceptibility to Streptococcal Toxic Shock Syndrome STSS appears to be related to the absence of antibodies to both M protein and superantigens, in addition to the presence of specific human leukocyte antigen (HLA) haplotypes (Kotb *et al.*, 2002).

2.3.5.3. Streptolysin S and streptolysin O

Streptolysin S is non-immunogenic oxygen stable, which causes erythrocytes, leukocytes, and platelets lysing in the presence of air. While Streptolysin O is immunogenic, oxygen labile produces hemolysis only in the absence of air, causing β-hemolytic pattern on blood agar (Tille, 2014).

The pore-forming toxins SLS and SLO are toxic to multiple host cell types, including macrophages and neutrophils, and therefore promote GAS tissue damage and resistance to phagocytic clearance. SLO, in particular, can induce accelerated apoptosis of immune cells (Timmer *et al.,* 2009) and inhibit neutrophil oxidative burst and neutrophil extracellular trap (NET) production (Uchiyama *et al.,* 2016).

2.3.5.4. Streptokinase

Streptokinase also known as fibrinolysin, which is an active proteolytic enzyme that digests fibrin and other proteins, transforms the plasminogen of into plasmin, allowing the bacteria to escape from blood clots (Jawetz*et et al*., 2010)

2.3.5.5. Hyaluronic acid

Hyaluronic acid is a capsule inhibits phagocytosis, mimicking a common human matrix component, and cloaks opsonic targets on the bacterial surface from phagocyte recognition (Stollerman and Dale, 2008).

2.3.5.6. Deoxyribonucleases

Deoxyribonucleases A, B, C, and D degrade DNA (DNases), facilitate the streptococci spread in tissue by liquefying pus (Jawetz*et et al*., 2010)

2.3.5.7. Hyaluronidase

Hyaluronidase splits hyaluronic acid, acts as a spreading factor that helps in spreading infecting microorganisms (Jawetz*et et al*., 2010).

Protein F mediates epithelial cell attachment (fibronectin-binding). A large number of adherence factors for epithelial cells and extracellular GAS, including lipoteichoic acid, M protein, pili, and fibronectin or laminin-binding proteins, including Sfb1, SOF and Lbp (Bisno, 2003).

2.3.6. Pathogenicity

GAS is a powerful modulator of the host immune system (Tille, 2014). That produces serious infection in healthy children and adults, defines the pathogen's ability to resist innate immune clearance mechanisms that normally prevent microbial dissemination (Walker *et al*., 2014). The first step in the pathogenesis of GAS disease in humans is adherence and colonization of the upper respiratory mucosa or skin (Bisno, 2003). The formation of a GAS biofilm facilitates persistence in humans (Doern *et al*., 2009). Secondly, *S. pyogenes* invades into epithelial cells mediated by M protein, F protein, and other antigens of the cocci. Lastly produces a wide variety of toxins and enzymes that contribute to the pathogenesis of many streptococcal diseases (Jawetz*et et al*., 2010).

The infections caused by *S. pyogenes* may be localized or systemic, localized infections include acute pharyngitis (sore throat), and skin soft tissue infections, such as erysipelas and impetigo, GAS infections may involve of deeper tissues and organs, designated in general publications as the flesh-eating bacteria (Tille, 2014).

2.3.6.1. Pharyngitis

Pharyngitis is inflammation of posterior pharynx and tonsils, GAS is the most common bacterial cause of pharyngitis, with over 600 million cases per year (Carapetis *et al*., 2005) (b). Pharyngitis is spread by person to person contact, presumably via nasal secretion or saliva droplets from carriers or infected individuals (Wessels, 2011). Pharyngitis incidence is higher in places of crowded, such as schools and military training facilities, approximately 4 to 10% of adults and15% of schoolchildren may suffer an episode of GAS pharyngitis each year in developed countries, whereas incidence rates are 5 to 10 times higher in developing countries (Carapetis *et al*., 2005) (b).

The clinical symptoms of GAS pharyngitis include fever accompanying a sore throat, often with patchy exudates and cervical lymph node adenopathy. Other uncommon symptoms include malaise, headache, nausea, abdominal pain, and vomiting (Choby, 2009; Wessels, 2011).

GAS pharyngitis is typically a self-limiting disease, antibiotic treatment is recommended for symptomatic individuals, confirmed by throat culture or a rapid antigen detection test, to prevent progression to acute rheumatoid fever ARF (Shulman *et al*., 2012). Additional benefits of treatment of GAS pharyngitis include amelioration of clinical symptoms, a rapid

decrease in contagiousness for close contacts, and prevention of suppurating complications, including peritonsillar abscess, cervical lymphadenitis, and, possibly, invasive infections (Shulman *et al*., 2012)

2.3.6.2. Erysipelas

Erysipelas massive brawny edema with a rapidly advancing margin of infection (Jawetz*et et al*., 2010).

2.3.6.3. Cellulitis

Cellulitis is an acute, rapidly spreading infection of the skin and subcutaneous tissues. It follows infection associated with mild trauma, burns, wounds, or surgical incisions. Pain, tenderness, swelling, and erythema occur. Cellulitis is differentiated from erysipelas by two clinical findings: In cellulitis, the lesion is not raised, and the line between the involved and uninvolved tissue is indistinct (Jawetz*et et al*., 2010).

2.3.6.4. Streptococcal pyoderma

Local infection of superficial layers of the skin occurs especially in children, called impetigo. Superficial vesicles break down and eroded areas whose denuded surface is covered with pus and later is encrusted, highly communicable, spread especially in hot, humid climates. More widespread infection occurs in eczematous or wounded skin or in burns and may progress to cellulitis (Tille, 2014).

Group A streptococcal skin infections are often attributable to M types 49, 57, and 59–61 and may precede glomerulonephritis but do not lead to rheumatic fever (Tille, 2014).

2.3.6.5. Necrotizing fasciitis (streptococcal gangrene)

Extensive rapidly spreading necrosis of the skin, tissues, and fascia (Jawetz*et et al*., 2010).

2.3.6.6. Puerperal fever

GAS when entering the uterus after delivery, puerperal fever develops, which essentially septicemia is originating in the infected wound (Jawetz*et et al*., 2010).

2.3.6.7. Bacteremia

Traumatic or surgical wounds infection with GAS results in bacteremia (Jawetz*et et al*., 2010).

2.3.6.8. Streptococcal Toxic Shock Syndrome and Scarlet Fever

SPEs superantigens induce antigen nonspecific T lymphocyte activation, suppress antibody synthesis, potentiate endotoxic shock, induce fever, promote the release of pro-inflammatory cytokines, produce reticuloendothelial blockade, and may contribute to the multi-organ failure characteristic of STSS (Tille, 2014).

2.3.7. Host immunity

S. pyogenes is a highly communicable bacterium, causes disease in people of all ages who do not have type-specific immunity against the specific serotype. Acquired immunity to streptococcal infection is based on the development of specific antibodies against the antiphagocytic epitopes of M protein. The acquired immunity against a particular M type of streptococci lasts longer in untreated persons than in treated persons. Although such antibodies protect from infection against a homologous M protein type, they confer no immunity against other M serotypes (Parija, 2014).

Protective immunity toward GAS is generally poor, and recurrent infections are not uncommon, especially in children (Sauver *et al*., 2006). This is despite the fact that most people do in fact raise an adaptive immune response and exhibit high titers of IgG antibodies toward different GAS antigens (Akesson *et al*., 2004). The reason for the lack of protection is not entirely understood but can in part be attributed to a large number of different GAS serotypes and the surface antigen variability this entails (McMillan *et al*., 2013).

GAS is also able to counteract adaptive immunity by specifically impairing IgG function, this can be mediated by non-immune IgG binding to Fc (fragment crystallizable) binding proteins on the streptococcal surface such as the M and M related proteins (Akesson *et al*., 1990; Akesson, 1994) or through specific degradation of the IgGs themselves. GAS secretes, for example, the IgG degrading enzyme of *S. pyogenes* (IdeS), and IgG specific protease that is able to cleave the antibody in the hinge region, separating the antigen-binding (Fab) from the effector function promoting Fc region (von Pawel Rammingen *et al*., 2002). AS is further able to degrade IgGs by secretion of the endoglycosidase (EndoS) of *S. Pyogenes* This enzyme cleaves the conserved FcN glycan from IgGs with great specificity (Collin and Olsén, 2001). This glycan is situated at the interaction surface between the IgG and Fc receptors (Subedi and Barb, 2015; subedi, 2016) as well as the

complement system (Burton, 1985) and is therefore ideally located to influence IgG effector function. While an antibody's specificity is determined by the Fab regions, the Fc region determines which effector functions are elicited, and the structure of the Fc glycan has been shown to be crucial in the regulation of this process (Burton and Dwek, 2006). For example, IgGs lacking core fucosylation exhibit increased affinity for Fcγ RIIIA and are therefore significantly more potent in eliciting antibody-dependent cellular cytotoxicity (Shinkawa *et al*., 2003; Okazaki *et al*., 2004). Furthermore, the degree of galactosylation of the Fc glycan influences IgG stability to activate the complement system (Peschke *et al*., 2017).

2.3.8. The complication of *Streptococcus pyogenes* infections

Post *streptococcus pyogenes* infection occurs in 1-3% of untreated infections, such as acute rheumatic fever and acute glomerulonephritis (Todar, 2006). The post-streptococcal diseases are mediated by the presence of the M protein, which is anti-phagocytic consisting of greater than 100 serotypes, class 1 M protein is associated with rheumatic fever, and class I or II is typically associated with glomerulonephritis. Rheumatic fever, which is manifested by fever, endocarditis (inflammation of heart muscle), subcutaneous nodules, and heart tissue, usually follows respiratory tract infections mediated by antibodies produced against *S. pyogenes* M protein that cross-react with human heart tissue. Acute glomerulonephritis, characterized by hypertension, edema, proteinuria, and hematuria, mediated by antigen-antibody complexes that deposit in glomeruli (Tille, 2014).

2.3.9. Laboratory diagnosis of *Streptococcus pyogenes*

2.3.9.1. Specimen collection

A throat swab is a specimen, swabbing the posterior pharynx and tonsils avoid touching tongue, lips, and buccal mucosa (Fox *et al*., 2006).

2.3.9.2. Cultural technique

Throat swabs are the gold standard for diagnosing GAS pharyngitis, inoculated throat swab on a 5% sheep blood agar and incubated at 37°C aerobically in the presence of 5–10% CO5%, selective media as crystal violet blood agar (1 in 50 000) also can be used (Chessbrough, 2006). 5% sheep blood agar supplemented with trimethoprim-sulfamethoxazole (SXT) can be also used as selective media suppressing the growth of

normal flora and inhibit the growth of groups C, F, and G β-hemolytic streptococci (Tille, 2014).

2.3.9.2.1. Colonial morphology

S. *pyogenes* characterize on blood agar, colonies are small, colorless, dry, shiny β-hemolytic colonies (Chessbrough, 2006).

2.3.9.2.2. Biochemical test

Catalase test negative, sensitive to bacitracin disk, positive pyrrolidonyl arylamidase (PYR) test , Lancefield grouping positive agglutination with antigen A (Chessbrough, 2006).

2.3.9.3. Advanced technique Vitek 2 system

The Vitek 2 rapid System was used to confirm the biochemical test of *Streptococcus pyogenes* the assay had been performed according to the manufacturer's instructions. (ALTaei *et al*., 2016)

2.3.9.4. Rapid antigen detection

Rapid antigen detection tests (RADT) have been developed to detect *S. pyogenes* directly from throat swabs, generally within minutes. These tests are based on acid extraction of cell wall carbohydrate antigen and detection of the antigen with the use of a specific antibody (Gerber and Shulman, 2004).

Good sampling increases the sensitivity of both the culture and the rapid antigen-detection test (Fox *et al*., 2006).

2.3.9.4. Serological methods

Serological tests detect a high level of antibodies produced against many streptococcal antigens. The tests detecting antibodies against SLO antistreptolysin O (ASO), anti-DNase B, anti-streptokinase, and anti hyaluronidase (Parija, 2014). Neutralizing antibody titers peak at 3 to 6 weeks for SLO and at 6 to 8 weeks for DNase B. Antibody titers against GAS extracellular antigens reported by clinical immunology laboratories may vary. The upper limits of normal are higher for children than for adults, and these values, even for the same age group, are higher in some populations than in others (Shet and Kaplan, 2002).

2.3.9.5. Molecular methods

An alternative rapid identification of *S. pyogenes* specific DNA sequences by means of hybridization with a DNA probe or by means of a real-time polymerase chain reaction (PCR) assay (Edmonson and Farwell, 2005; Tanz *et al*., 2009).

2.3.10. Treatment

GAS remains exquisitely and universally sensitive to penicillin, while antibiotics such as cephalosporins, macrolides, and clindamycin are also used clinically (Hasenbein *et al*., 2004). Despite universal sensitivity to penicillin, GAS infections may fail to respond to penicillin therapy, leading to persistent throat carriage and recurrent infections (Kaplan and Johanson, 2001). It has been suggested that GAS may escape penicillin treatment by entering epithelial cells, which are poorly penetrated by penicillin (Kaplan, 2006), or by forming a biofilm (Baldassarri *et al*., 2006; Ogawa *et al*., 2011), or alternatively, failure of penicillin treatment may possibly be due to the protection of GAS by other β-lactamase-producing bacterial species; however, this has not been proven (Brook and Gober, 2008).
GAS resistance to antibiotics such as macrolides, clindamycin, and lincosamide has become an increasing concern (Chen *et al*., 2012).

2.3.11. Prevention

Penicillin indicates control outbreaks of *S. pyogenes* in individuals in close physical contact, such as in households, military populations, or newborn nurseries. Lifetime chemoprophylaxis with penicillin, given monthly (intramuscular administration) or daily (oral administration), is recommended for patients with rheumatic heart disease to prevent the development of bacterial endocarditis on a damaged heart valve (Tille, 2014). Immunization with M protein provides strong protection against infection with a type-specific GAS strain (Herwald *et al.*, 2004; Macheboeuf *et al.*, 2011).

2.4. Antibacterial activities of *Citrus limon*

Lemon is very rich in important natural compounds, including citric acid, ascorbic acid, flavonoids, minerals, and essential oils. Citric acid is naturally concentrated in citrus fruits that are weak tricarboxylic acid increases the acidity of the bacterial environment making it difficult for it and microbes to survive (Khusro *et al*., 2013).
In 2015 a study was done in Nigeria, the Nigerian herbalists evaluated the antibacterial activity of the *Citrus limonum* extract, was done on ten strains of bacteria was determined

by both agar well diffusion and macro broth dilution methods against *Bacillus subtilis* ATCC 6051, *Staphylococcus aureus* ATCC 12600, *Escherichia coli* ATCC 11775, *Pseudomonas aeruginosa* ATCC 10145 (also clinical isolated strains), *Salmonella kintambo, Salmonella typhi,* and *Proteus* spp, showed broad-spectrum activity similar to gentamycin with the exception of *B. subtilis* ATCC 6051, *P.aeruginosa* ATCC 10145 and *Proteus* spp (clinical), the *Citrus limon* fruit juice extract has low MBC/MIC ratio, provide scientific justification for the medicinal use of extract in treatment of disease caused by tested organism (Okeke *et al*., 2015).

Clinical isolates of multidrug-resistant *Staphylococcus aureus, P. aeruginosa* were tested against aqueous *Citrus limon* that showed great potential as an antibacterial compound against those resistant bacteria (Verlekar and Chandak, 2018).

In study 2019 *Citrus limon* extract showed antidiarrheal potential activity inhibiting the growth of diarrhea-causing Enterotoxin *Escherichia coli* (ETEC) with optimum dose was 900mg/ml (Ekawati and Darmanto, 2019). Antimicrobial activities different concentrations of *Citrus limon* extracts (juice) were showed effectivity against *Staphylococcus aureus*, *Proteus Vulgaris* and *Pseudomonas aeruginosa* in the study done in 2010 (Tawfik *et al*., 2010).

2.5. *Streptococcus pyogenes* and *Citrus limon*

Antibacterial activity of *Citrus limon* aqueous extracts was proved against *Streptococcus pyogenes*, which extracts showed antimicrobial activity and can be used to prevent various diseases caused by *Streptococcus pyogenes* (Hindi and Chandak, 2013). In another study was done in 2018, the antimicrobial activity of *Citrus limon* aqueous extracts was judged against bacterial causing diseases was *Streptococcus pyogenes* (Abdulhusin *et al*., 2018).

Antibacterial activity of *Citrus limon* aqueous extracts was investigated against *Streptococcus pyogenes* causes respiratory tract infections caused (Eseoghene *et al*., 2017).

CHAPTER THREE

MATERIALS AND METHODS

CHAPTER THREE
MATERIALS AND METHODS

3.1. Study design

This study was a descriptive, cross-sectional laboratory-based study.

3.2. Study area and duration

This study was conducted at Advanced Med Lab - Jeddah - Saudi Arabia from October 2018 to December 2019.

3.3. Study population

All clinical cases of a sore throat admitted to Hashim Clinic or Advanced Med Lab were included in this study.

3.4. Inclusion criteria

Patients admitted with sore throat manifestations.

3.5. Exclusion criteria

Patients with no sore throat manifestation.

3.6. Sample size

A total of a hundred thirty-five throat swab samples (n=135) were collected.

3.7. Data collection

Data were collected using direct review (questionnaire) (Appendix 1), which including demographic and clinical data.

3.8. Sampling technique

This study based on a non-probability convenience sampling technique. Samples were taken from attended agreed patients.

3.9. Ethical consideration

This research was authorized and approved by the Research Committee of the College of Medical Laboratory Science - Sudan University of Science and Technology, and informed consent was taken from patients before enrollment on the study.

3.10. Methodology

3.10.1. Collection of specimens

Under aseptic conditions and good light, the patient was asked to tilt the head back and open mouth widely, 2 swabs were collected from each patient by depressing the tongue with depressor, swabbing irritated inflamed swelled area, posterior pharynx, and tonsils by

sterile cotton swabs, avoid touching cheeks and tongue, the swabs were placed on Amie's transport medium.

3.10.2. Rapid antigen detection from throat swab

Bioline SD Strep A strip test (Standard diagnostic INC company from Germany) appendix No 2, is one step lateral flow chromatographic immunoassay for the qualitative detection of group A antigen directly from throat swabs.

First, the test reagent and strip were brought to room temperature before use, in the test tube 3 drops of solution A and 3 drops of solution B were added after mix throat swab was put into the test tube containing solution. The swab was swirled 10 times into the test tube and was left for 1 minute, then the swab was removed, the test strip was dipped into the test tube, and the result was read after 10 minutes. 2 lines were developed, the test line and control line were indicated the presence of group A streptococcal antigen.

3.10.3. Swabs culture

Throat swabs were inoculated on 5% sheep blood agar plates, after streaking, the plates were stabbed vertically by inoculating loop allowing better visualizing of the hemolysis caused by streptolysin O, using sterile forceps, bacitracin dick (0.04 IU) were placed in the first heaviest growth area (Forbes *et al*., 2007). The plates were incubated at 37º C for 18 to 24 hours under 10 % Co_2 which often speeds hemolysis (Jawetz*et et al*., 2010).

3.10.4. Identification of the growth

3.10.4.1. Colonial morphology

S. pyogenes colonies were showed small, colorless, dry and shiny colonies were surrounded by a clear zone of beta-hemolytic colonies on sheep blood agar (Chessbrough, 2006).

3.10.4.2. Gram's stain

On dry clean clearly labeled slide, the smear was made by transfer a loop full of colony emulsified into a drop of normal saline to made a thin smear, the slide was left for drying then fixed with heat.

The slide was flooded with crystal violet stain (basic stain) for 30-60 seconds, rapidly the slide was washed with clean tap water, slide was covered with Lugol's iodine solution (mordant), for another 30-60 seconds, repeat washing step, the slide was decolorized rapidly with acetone alcohol for 10 seconds, repeat the washing process, finally, the slide was covered with saffranin solution for 2 minutes, then was washed with clean tap water,

and left to dry and examined for morphological appearance and arrangement with oil immersion 100x.

3.10.4.3. Biochemical tests

3.10.4.3.1. Catalase test

Catalase enzyme produced by the bacteria to break down hydrogen peroxide (H_2O_2) into H_2O and O_2. 1-2 ml 3% hydrogen peroxide solution (H_2O_2) was placed into on test tube, using a sterile wooden stick, several colonies bacterial growth was taking and immersed in the hydrogen peroxide solution, the result was observed for immediate bubbling (Forbes *et al*., 2007).

3.10.4.3.2. Bacitracin sensitivity test

This test was used for presumptive identification and distinguish of *S. pyogenes* from other beta-hemolytic Streptococcus, a zone of inhibition greater than10 mm around bacitracin disk (0.04IU) considered susceptible and confirms the presence of *S. pyogenes* (Tille, 2014).

3.10.4.3.3. Lancefield antigen determination

Streptococcus grouping kit (Mircogen bioproduct from the United Kingdom) appendix No 2 was used and the manufacturer's instructions were followed; the first latex reagent and positive control were to bring to room temperature.

About 0.4 ml of the extract solution was dispensed into the test tube, around 4-5 averaged size colonies were picked up using a bacteriological loop, were emulsified through enzymatic extraction solution. The test tube was incubated 15 minutes in the incubator, after the incubation period the test tube and latex reagent was shaken vigorously for a few seconds. On the reaction card, a drop of well-mixed reagent of antigen A and one drop of well-mixed extract solution were placed into circle one, in circle two (for control) one drop of antigen A reagent and positive control were added, using mixing sticks, each circle was mixed separately, and the card was gently and slowly rotated for 1 minute; the result was appeared to inform of agglutination by naked eye, positive control result appeared as agglutination, *S.pyogenes* possess antigen A, the agglutination appeared with latex antigen A reagent.

3.11. Plant material

3.11.1. Collection of lemon fruits

Fresh 5 fruits of *Citrus Limon* were obtained from the local market in Jeddah City, Saudi Arabia, 2019; they were detected, identified, and authenticated by researcher Montasir A.O. Saad from agriculture research cooperation research center, King Saud University.

3.11.2 Preparation of the aqueous extract

Fresh 5 *Citrus limon* fruits were washed in running tap water in the laboratory, and the surface was sterilized with 70% alcohol, then rinsed in sterile distilled water. By sterile knife, the fruits were cut and the juice was pressed out into a sterile universal container, then filtered using Millipore 0.45 filter paper into another sterile container to remove the seeds and other tissues, the freshly extract was used without refrigeration (Hindi and Chabuck 2013; Abdulhusin *et al*., 2018). The crude extracts of *Citrus limon* were diluted by distilled water into different concentrations as follows: 100%, 50%, 25% and 12.5% (v/v) to be used against the selected organisms.

3.12. Preparation of bacterial suspension:

S. pyogenes isolate were inoculated into 3.0 ml of sterile normal saline. The inoculum density was compared with the McFarland standard solution.

3.13. Antimicrobial activity by cup plate method

Cup plate method was used in the investigation of antimicrobial activities of *Citrus limon* aqueous extract on the clinically isolated *S. pyogenes*, in which Mueller Hinton's blood agar was used to cultured bacterial isolates, each microbial isolate was inoculated on plate culture. 4 Wells of 10 mm diameter were made on the agar plate using a sterile borer. Approximately 20µl of extract (100%, 50%, 25% and 12.5%)(v/v) was inoculated into each well were made in the agar plate, and the plates were allowed to diffuse at room temperature for 30 min, then were incubated at 37°C for overnight for 24 h of incubation (Prescott *et al.,* 2002).

Distilled water as a negative control. Confluent bacterial growth was observed; each extract has noted the diameters of inhibition zone that formed around the wells in millimeter for all isolates. The inhibition zones with a diameter of less than 12mm were considered having no antibacterial activity (Srinivasan *et al*., 2001).

3.14. Data analysis

Data were entered, check, and analyzed using Microsoft Excel 2010 and SPSS (Statistical Package Social Science) program version 25. Frequency and mean were included. Data were presented in the form of tables and figures. A chi-square test was used to detect the correlation between the qualitative variable. A *P*. *value* of <0.05 was considered significant for all results.

CHAPTER FOUR

RESULTS

CHAPTER FOUR
RESULTS

4.1. Demographic data of the study population

A total of 135 infected patients with sore throat were enrolled in this study, their age ranged from 3-54 years, with a mean age of 8.84 ± in and 7.54 SD. Patients age were classified to four groups as follow: age ranged (<15) years were 123(91.1%) patients, (15-30) years were 7(5.2%) patients, (31-45) years were 4(3%) patients and (>45) years was 1(0.7%) patient. Females were 74 (54.8%), while males were 61(45.2%) as shown in table (4.1)

Out of 135 sore throat patients, 72(53.3%) were showed positive growth of *S. pyogenes*, while 63 (46.7%) were negative for *S. pyogenes.* Only 1(0.7%) patient was on antibiotic treatment (Penicillin), while 134 (99.3%) were not, and 1 (0.7%) patient was showed recurrent *S. pyogenes* infection as mentioned in table (4.1).

All patients 135 (100%) were showed fever and difficult to swallow. Regarding exudate 81 (60%) patients were showed the presence of exudate, while 54 (40%) patients were not, as pointed in the table (4.1).

4.2. Correlation between *S. pyogenes* and demographic data

Regarding age group and out of positive specimen, 68/72 (94.4%) of patients were showed positive growth of *S. pyogenes* in age group <15 years, 2(2.8%) in (15-30) years, and 2(2.8%) in (31-45) years with statistical insignificant difference between *S. pyogenes* growth and age (*P*. value = 0.67).

Out of total positive specimen, *S. pyogenes* were isolated from 40/72 (55.6%) females, and 32/72 (44.4%) from males, with insignificant association between the isolated bacteria and the gender (*P*. value =0.85).

According to exudate, 64/72 (88.9%) with positive *S. pyogenes* showed exudate production which showed a high statistically significant difference between *S. pyogenes* growth and exudate production (*P*. value = 0.00).

Considering penicillin treatment and history of recurrent infection, only 1/72 (1.4%) were re-infected with *Streptococcus pyogenes* and was under treatment which showed a statistically insignificant difference between *S. pyogenes* growth, and treatment and history of recurrent infection (*P*. value = 0.34) for both of them as shown in table (4.2)

4.3. Compression between cultural technique and rapid strep test

In compression between cultural technique as the gold standard method in detecting *Streptococcus pyogenes* and rapid strep test. All 72(53.3%) positive specimens that showed *Streptococcus pyogenes* by the cultural method were also positive, 72 (53.3%) in rapid strep test with (100%) specificity and sensitivity, as mentioned in table (4.3).

4.4. The activity of aqueous lemon extract on clinical isolates of *S. pyogenes*

The antimicrobial potential of the aqueous extract of the medicinal plant *Citrus limon* extract was evaluated against 72 clinical isolates of *S. pyogenes*; (v/v) 100(%) the mean 21.5± 1.35 STD; with a range of 20-24 mm. In the concentration 50%, the mean was 17.8± 1.59 STD; with a range of 15- 22 mm. In the concentration 25%, the mean was 14.2± 2.86 STD; with a range of 11- 19 mm, and finally the concentration of 12.5% was showed no zone of inhibition, as shown in table (4.4).

Table (4.1): Demographic data of the study population

Character		**Frequency**	**Percentage**
Age group / years	<15	123	91.1 %
	15-30	7	5.2 %
	31-45	4	3 %
	>45	1	0.7 %
	Total	135	100 %
Gender	Males	61	45.2 %
	Females	74	54.8 %
	Total	135	100 %
***S.pyogenes* growth**	Yes	72	53.3%
	No	63	46.7%
	Total	135	100 %
Penicillin treatment	Yes	1	0.7%
	No	134	99.3%
	Total	135	100 %
History of recurrent infection	Yes	1	0.7%
	No	134	99.3%
	Total	135	100 %
Fever	Yes	135	100 %
	No	0	0%
	Total	135	100 %
Symptoms Sore throat / difficult to swallow	Yes	135	100%
	No	0	0%
	Total	135	100 %
Exudate	Yes	81	60%
	No	54	40%
	Total	135	100 %

Table (4.2): Association between *S. pyogenes* and demographic data

Character		*S.pyogenes*		Total	*P.value*
		Positive No (%)	**Negative No (%)**		
Age group / years	1-15	68(50.3%)	55 (40.7%)	123 (91.1%)	0.678
	16-30	2 (1.5%)	5 (3.7%)	7 (5.2%)	
	31-45	2 (1.5%)	2 (1.5%)	4 (3%)	
	< 45	0	1 (0.7%)	1 (0.7)	
	Total	72 (53.3%)	63 (46.7%)	135 (100%)	
Gender	Male	32 (23.7%)	29 (21.5%)	61 (45.2%)	0.853
	Female	40 (29.6%)	34 (25.2%)	74 (54.8%)	
	Total	72 (53.3%)	63 (46.7%)	135 (100%)	
Penicillin treatment	Yes	1 (0.7%)	0	1(0.7%)	0.348
	No	71(52.6%)	63 (46.7%)	134(99.3%)	
	Total	72 (53.3%)	63 (46.7%)	135 (100%)	
History of recurrent infection	Yes	1 (0.7%)	0	1 (0.7%)	0.348
	No	71	63 (46.7%)	0	
	Total	72 (53.3%)	63 (46.7 %)	135 (100%)	
Exudate	Yes	64 (47.4%)	17(12.6%)	81 (60%)	0.0
	No	8 (5.9%)	46 (34.1%)	54 (40%)	
	Total	72 (53.3%)	63 (46.7%)	135 (100%)	
Fever	Yes	72(53.3%)	63 (45.7%)	135 (100%)	-
	No	0	0	0	
	Total	72 (53.3%)	63 (46.7%)	135 (100%)	
Symptoms Sore throat / difficult to swallow	Yes	72(53.3%)	63(46.7%)	135(100%)	-
	No	0	0	0	
	Total	72 (53.3%)	63 (46.7%)	135 (100%)	

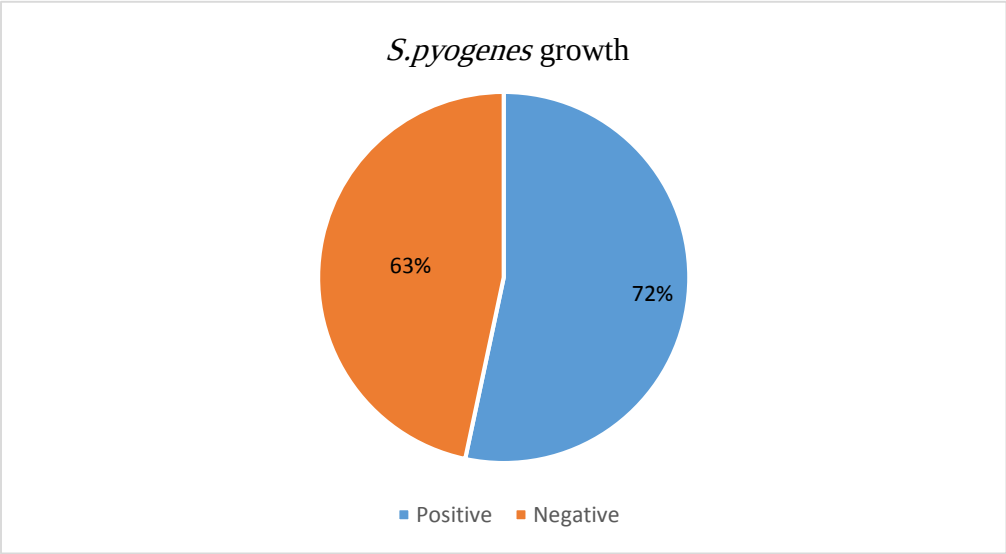


Figure (4.1) Frequency of positive *S. pyogenes* among the study population

Table (4.3): Comparison between cultural method and rapid strep test

Test	*Streptococcal pyogenes* results		Total
	Positive %	Negative%	
Cultural method	72 (53.3%)	63 (46.7%)	135 (100%)
Rapid strep test	72 (53.3%)	63 (46.7%)	135 (100%)

Sensitivity: 100%

Specificity: 100%

Table (4.4): The activity of aqueous lemon extract on clinical isolates of *S. pyogenes*

The concentration of lemon juice extract (v/v)	Inhibition zone (mm) Minimum-maximum	Mean of inhibition zone ±STD
12.5 %	0	-
25 %	11-19	14.22± 2.86
50 %	15-22	17.85±1.59
100 %	20-24	21.51±1.35

CHAPTER FIVE
DISCUSSION, CONCLUSION, AND RECOMMENDATIONS

CHAPTER FIVE

DISCUSSION, CONCLUSION, AND RECOMMENDATIONS

5.1. Discussion

GAS is the most common bacterial cause of pharyngitis, with is a higher incidence in places crowded places, such as schools and military training facilities, approximately 4 to 10% of adults and 15% of schoolchildren may suffer an episode of GAS (Carapetis, 2005b). Plants and plant products have been used extensively throughout history to treat medical problems. Numerous studies have been carried out to extract various natural products for screening antimicrobial activity (Nita *et al*., 2002).

In this study 135 (100%) throat swabs were collected from patients of sore throat, 72(53.3%) *Streptococcus pyogenes* were positive by both cultural method and rapid strep test (SD bioline one-step lateral flow chromatographic immune assay), this result was in agreement with Carapetis and his collogues (2005 a) in Australia who observed that GAS is the most bacterial cause of pharyngitis.

According to Leung and his collogues (2006) in united kigdom the most rapid detection test excellent specificity of greater than 95 % and sensitivity greater or equal 90%, similar to this result, which the specificity and sensitivity of rapid strip (SD bioline) were 100% for both.

In the current study, the age of participants was divided into four categories (<15), (15-30), (31-45), and (>45) years. Although the highest frequency of infection was observed in the first group (94.4%), there was no association between *Streptococcus pyogenes* infection and age (P=0.67), these results disagreed with AL Kareem *et al*., (2014) in Iraq (P=0.021)

The results of this study were pointed to slightly increased frequency of *S. pyogenes* pharyngitis in females 40 (55.6%) versus in male 32 (44.4%), with no association was found between gender and *Streptococcus pyogenes* infection (P= 0.853). This result of this study was compatible with Nandi and his collogues (2001) in India, Sauver with his collogues (2006) in rochester of Minnesota who reported the gender wasn't a risk factor in increasing or decreasing the prevalence rate of infection. Differ finding was reported by ALtaei and his collogues (2016) in Iraq who reported the females were more susceptible and responsible for infection. Chung (2003)in Korea who reported the males were more susceptible to infection compared with females (P=0.51)

Considering penicillin treatment and history of recurrent infection, only 1/72 (1.4%) was re-infected with *Streptococcus pyogenes* and was under treatment which showed a statistically insignificant difference between *S. pyogenes* growth, treatment, and history of recurrent infection (*P*. value = 0.34) for both of them, these findings were in agreement with Kaplan (2001) who was reported failure to respond to penicillin therapy recurrent infections, may due to GAS may escape penicillin treatment by entering epithelial cells, which are poorly penetrated by penicillin Kaplan and his collogues (2006), Brook and Gober (2008) showed that failure of penicillin treatment may be due to the protection of GAS by other β-lactamase producing bacterial species.

In this study, *S. pyogenes* were isolated from 64/72 (88.9%) patients with exudate production which showed a highly statistically significant association between *S. pyogenes* growth and exudate production (*P*. value = 0.00) agreeing with Choby (2009) and Wessels (2011) in USA who found that the frequeny of *S.pyogenes* from the patients was 85% .

The determination of the antimicrobial activity of *Citrus limon* aqueous extract against 72 clinically isolated *S. pyogenes* from sore throat patients, this finding was in agreement with Suleiman (2013) in England the means diameter of inhibition zone increases with the increase of extract concentration.

This study determine of the antimicrobial activity of *Citrus limon* aqueous extract against 72 clinically isolated *S. pyogenes* from sore throat patients. The mean inhibition zone of 100% *Citrus limon* concentration was 21.5 mm. This finding is higher than the results observed by Hindi and Chabuck (2013) in Hilla City, Iraq also to that observed by Abdulhusin and his collogues (2018) in Iraq was 20mm. In this present study the means diameter of inhibition zone were increases with the increase of extract concentration, other concentration results were 50% mean 17.8mm, 25% mean 14.2 mm, and 12.5% was showed no zone of inhibition.

C*itrus limon* was effective against *S. pyogenes*, these results were agreed with Abdullah results (2009) in Malaysia that the activity due to the acidic pH of this juice that will affect the charges of the amino acids that constitute the peptidoglycan and it may affect the active sites of enzymes leading to defect in their activity.

5.2. Conclusion

Citrus limon aqueous extract was showed remarkable antimicrobial activity against *Streptococcus pyogenes*, The antimicrobial efficiency of *Citrus Limon* juice was found to be increased when increasing the extract concentration.

Streptococcus pyogenes was the most frequent bacterial causes sore throat with no association of *Streptococcus pyogenes* pharyngitis with age, sex, treatment and history of recurrent infection, while the high association was found between *Streptococcus pyogenes* and exudate production.

Bioline SD (one-step lateral flow chromatographic immune assay) antigen detection test is easy to perform with a short time showed a higher advantage for diagnosis, was highly specific and sensitive results comparing with the standard cultural techniques.

5.3. Recommendations

Further research with large sample size is recommended to verify these results.

Antigen detection test (one-step lateral flow chromatographic immune assay) can be used routinely as diagnostic testing for acute GAS pharyngitis because of it easy to perform and has a short turnaround time

Determination of minimum inhibitory concentration and minimum bactericidal concentration of *Citrus Limon* juice active ingredients against *Streptococcus pyogenes* is recommended.

Further investigations with reference strains are also essential.

References

Abdulhusin, I.F., Al musawi, S., Hindi, A.N.K.K. and Abdulmahdi, S. (2018). Aqueous lemon extracts as an antimicrobial agent against some pathogenic bacteria. *Plant Archives*, **18**(1):431-434.

Abdullah NY. (2009). Effect of some plant extracts against *Staphylococcus aureus* and *Klebsiella pneumonia*e. *Iraqi academy of science journal*, **1**(2): 32-36

Åkesson, P., Cooney, J., Kishimoto, F. and Björck, L. (1990). Protein H— a novel IgG-binding bacterial protein. *Molecular immunology*, **27**(6):523-531.

Åkesson, P., Rasmussen, M., Mascini, E., Ulrich, V.P.R., Janulczyk, R., Collin, M., *et al*. (2004). Low antibody levels against cell wall attached proteins of Streptococcus pyogenes predispose for severe invasive disease. *Journal of Infectious Diseases*, **189**(5):797-804.

Åkesson, P., Schmidt, K.H., Cooney, J. and Björck, L. (1994). M1 protein and protein H: IgGFc-and albumin-binding streptococcal surface proteins encoded by adjacent genes. *Biochemical Journal*, **300**(3):877-886.

Al Kareem, F.E.A., Abbas, A.K. and Hussein, M.A. (2014). Comparative study of the Antibody Responses to Streptococcus pyogenes between school Children carriers and patients with Tonsillitis. *Iraqi Journal of Science*, **55** (2): 403-410.

Ali, J., Das, B. and Saikia, T. (2017). Antimicrobial activity of lemon peels (Citrus limon) extract. *Clinical microbiology reviews*, **26**(3):422-447.

Alouf, J.E. and Müller-Alouf, H. (2003). Staphylococcal and streptococcal superantigens: molecular, biological and clinical aspects. *International journal of medical microbiology*, **292**(7-8):429-440.

ALTaei, F.A., AlKhafaji, J.K. and AlGazally, M.E. (2016).Characterization of Streptococcus pyogenes Isolated from Throat Swabs in Baghdad Children Patients. *Journal of the University of Babylon*, **24**(5):1227-1233.

Ampofo, J.A., Andoh, A., Tetteh, W. and Bello, M. (2012). Microbiological Profile of Some Ghanaian Herbal Preparations Safety Issues and Implications for the Health Professions. *Open Journal of Medical Microbiology*, **2**(03)121.

Baldassarri, L., Creti, R., Recchia, S., Imperi, M., Facinelli, B., Giovanetti, E., *et al.* (2006). Therapeutic failures of antibiotics used to treat macrolide-susceptible

Streptococcus pyogenes infections may be due to biofilm formation. *Journal of Clinical Microbiol*ogy, **44**:2721– 2727.

Bisno, A.L., Brito, M.O. and Collins, C.M. (2003). Molecular basis of group A streptococcal virulence. *The Lancet infectious diseases*, **3**(4):191-200.

Brook I, and Gober AE. (2008). Failure to eradicate streptococci and beta-lactamase-producing bacteria. *Acta Paediatr*, **97**:193–195.

Burt, S., (2004). Essential oils: their antibacterial properties and potential applications in foods a review. *International journal of food microbiology*, **94**(3):223-253.

Burton, D.R., and Dwek, R.A. (2006). Sugar determines antibody activity. *Science*, **313**(5787):627-628.

Burton, D.R., (1985). Immunoglobulin G: functional sites. *Molecular immunology*, **22**(3):161-206.

Carapetis, J.R., McDonald, M. and Wilson, N.J. (2005a). Acute rheumatic fever. *The Lancet*, **366**(9480):155-168.

Carapetis, J.R., Steer, A.C., Mulholland, E.K. and Weber, M. (2005b). The global burden of group A streptococcal disease. *The Lancet infectious diseases*, **5**(11):685-694.

Chen M., Yao, W., Wang, X., Li, Y., Chen, M., Wang, G., *et al*. (2012). The outbreak of scarlet fever associated with *emm12* type group A *Streptococcus* in 2011 in Shanghai, China. *Pediatric Infectious disease Journal*, **31**: 158-162.

Chessbrough, M. (2006). District Laboratory practice for tropical countries, 2nd ed. pp: 38,162

Choby BA. (2009). Diagnosis and treatment of streptococcal pharyngitis. American Family *Physician*, **79**:383-390.

Chung, J.Y., Sin, S.H., Ahn, Y.M., Ahn, B.M., Sin, Y.G., Bae, Y.M., *et al*. (2003). Clinical and bacteriologic efficacy of cefdinir on pharyngitis and pharyngotonsillitis caused by group A beta-hemolytic streptococci in Children. *Korean Journal of Pediatric Infectious Disease*, **10** (1):95-101.

Collin, M. and Olsén, A. (2001). EndoS, a novel secreted protein from Streptococcus pyogenes with endoglycosidase activity on human IgG. *The EMBO journal*, **20**(12):3046-3055.

Courtney, H.S., Hasty, D.L. and Dale, J.B. (2002). Molecular mechanisms of adhesion, colonization, and invasion of group A streptococcus. *Annals of medicine*, **34**(2):77-87.

Dev, C. and Nidhi, S.R.R.S. (2016). Basketful benefit of *Citrus limon*. *International Research Journal of Pharmacy*, **7**(6):1-3.

D McArthur, J., M Cook, S., Venturini, C. and J Walker, M. (2012). The role of streptokinase as a virulence determinant of Streptococcus pyogenes potential for therapeutic targeting. *Current drug targets*, **13**(3): 297-307.

Doern, C.D., Roberts, A.L., Hong, W., Nelson, J., Lukomski, S., Swords, W.E. and Reid, S.D., (2009). Biofilm formation by group A Streptococcus: a role for the streptococcal regulator of virulence (Srv) and streptococcal cysteine protease (SpeB). *Microbiology (Reading, England)*, **155** (1):46.

Duthie, G. and A. Crozier. (2000). Plant-derived phenolic antioxidants, *Current Opinion in Lipidology*, **11**: 43-47.

Edmonson, M.B. and Farwell, K.R. (2005). Relationship between the clinical likelihood of group A streptococcal pharyngitis and the sensitivity of a rapid antigen detection test in pediatric practice. *Pediatrics*, **115**(2):280-285.

Ehrlich, S.D. and NMD, S.A. (2011). a private practice specializing in complementary and alternative medicine, Phoenix, AZ. *Review provided by Veri Med Healthcare Network*.

Ekawati, E.R. and Darmanto, W. (2019). Lemon (*Citrus limon*) Juice Has Antibacterial Potential against Diarrhea-Causing Pathogen. IOP Conference Series: *Earth and Environmental Science* **217**(1):012023

Ekwenye, U.N., and Edeha, O.V. (2010). The antibacterial activity of crude leaf extract of Citrus sinensis (sweet orange). *International Journal of Pharmacy and Biological Science*, **1**(4):742-750.

Eseoghene, O., Banu, A. and Nisha, M. (2017). Assessing the Antibacterial Activity of Honey and Lemon Juice against Bacterial Isolated from Upper Respiratory tract infection, International Conference, Kuala Lumpur, Malaysia

Forbes, B.A., Sahm, D.F. and Weissfeld, A.S. (2007). Bailey and Scott's diagnostic microbiology, 12th ed. pp: 266-268.

Fox, J.W., Marcon, M.J. and Bonsu, B.K. (2006). Diagnosis of streptococcal pharyngitis by detection of Streptococcus pyogenes in posterior pharyngeal versus oral cavity specimens. *Journal of clinical microbiology*, **44**(7):2593-2594.

Fullerton, M., Khatiwada, J., Johnson, J.U., Davis, S. and Williams, L.L. (2011). Determination of the antimicrobial activity of sorrel (Hibiscus sabdariffa) on Escherichia coli O157: H7 isolated from food, veterinary, and clinical samples. *Journal of medicinal food*, **14**(9): 950-956.

Gera, K. and McIver, K.S. (2013). Laboratory growth and maintenance of Streptococcus pyogenes (the Group A Streptococcus, GAS). *Current protocols in microbiology*, **30**(1): 9D-2.

Gerber MA, and Shulman ST. (2004). Rapid diagnosis of pharyngitis caused by group A streptococcus. *Clinical Microbiological Review*, **17**:571–80.

Ghosh, P. (2011). The non-ideal coiled-coil of M protein and its multifarious functions in pathogenesis. *In Bacterial Adhesion*, 197-211.

Gunjan, M., Naing, T.W., Saini, R.S., Ahmad, A., Naidu, J.R. and Kumar, I. (2015). Marketing trends & future prospects of herbal medicine in the treatment of various diseases. *World Journal of Pharmaceutical Research*, **4**(9):132-155.

Hasenbein ME, Warner JE.,Lambert, KG, Cole SE, Onderdonk AB, and McAdam AJ. (2004). Detection of multiple macrolides- and lincosamide-resistant strains of *Streptococcus pyogenes* from patients in the Boston area. *Journal of Clinical Microbiology*, **42**:1559–1563

Herwald, H., Cramer, H., Mörgelin, M., Russell, W., Sollenberg, U., Norrby-Teglund, A., Flodgaard., *et al.* (2004). M protein, a classical bacterial virulence determinant, forms complexes with fibrinogen that induce vascular leakage. *Cell*, **116**(3):367-379.

Hindi, N. K. K., and Chabuck, Z. A. G. (2013). Antimicrobial Activity of Different Aqueous Lemon Extracts, *Journal of Applied Pharmaceutical Science*, **3**(6):74–78.

Jawetz F, Melnick C and Adelberg S. (2010). Medical microbiology, 24th ed. McGraw-Hill Companies, USA. pp: 237-238.

Kaplan EL, Chhatwal GS, and Rohde M. (2006). Reduced ability of penicillin to eradicate ingested group A streptococcus from epithelial cells: clinical and pathogenetic implications. *Clinical Infectious* Disease, **43**:1398 –1406.

Kaplan EL, and Johnson DR. (2001). Unexplained reduced microbiological efficacy of intramuscular benzathine penicillin G and of oral penicillin V in the eradication of group A streptococcus from children with acute pharyngitis. *Pediatrics,* 108:1180 –1186.

Karar, M.G.E., and Kuhnert, N. (2017). Herbal drugs from Sudan: Traditional uses and phytoconstituents. *Pharmacognosy Reviews*, **11**(22): 83

Kawaii, S., Tomono, Y., Katase, E., Ogawa, K., Yano, M., Koizumi, M., *et al*. (2000). A quantitative study of flavonoids in leaves of *Citrus* plants, *J. Agric. Food Chemistry*, **48**: 3865-3871.

Khusro A., Preetamraj, J. and Panicker S. (2013). A comparative analysis of the antibacterial activity of *Citrus limonium* juice extracts antibiotics and commercially available citric acid against new strains of bacteria for the prevention of eye infections. *International Journal of Advanced Research,* Article **1**(9): 104-111.

Kotb, M., Norrby-Teglund, A., McGeer, A., El-Sherbini, H., Dorak, M.T., Khurshid, A., *et al*. (2002). An immunogenetic and molecular basis for differences in outcomes of invasive group A streptococcal infection. *Nature medicine*, **8**(12):1398.

Kumar, S., Marwaha, N., Singh, D. and Kumar, V. (2012). Evaluating the antibacterial activity of plant extracts against bacterial pathogens. *Journal of Drug Delivery and Therapeutics*, **2**(4).

LaRock, C.N., Döhrmann, S., Todd, J., Corriden, R., Olson, J., Johannssen, T., *et al.* (2015). Group A streptococcal M1 protein sequesters cathelicidin to evade innate immune killing. *Cell host & microbe*, **18**(4):471-477.

Leung AK, Newman R, Kumar A, Davies HD. (2006). Rapid antigen detection testing in diagnosing group A beta-hemolytic streptococcal pharyngitis. Expert Review of Molecular Diagnostics, 6:761–716.

Macheboeuf, P., Buffalo, C., Fu, C.Y., Zinkernagel, A.S., Cole, J.N., Johnson, *et al*. (2011). Streptococcal M1 protein constructs a pathological host fibrinogen network. *Nature*, **472**(7341):64.

McMillan, D.J., Drèze, P.A., Vu, T., Bessen, D.E., Guglielmini, J., Steer, A.C., *et al*. (2013). The updated model of group A Streptococcus M proteins based on a comprehensive worldwide study. *Clinical Microbiology and Infection*, **19**(5):222-229.

Mohanapriya, M., Ramaswamy, L. and Rajendran, R. (2013). Health and medicinal properties of lemon (*Citrus limonum*). *International Journal of Ayurvedic and Herbal Medicine*, **3**(1):1095-1100.

Mosihuzzaman, M. and Choudhary, M.I. (2008). Protocols on safety, efficacy, standardization, and documentation of herbal medicine (IUPAC Technical Report). *Pure and Applied Chemistry*, **80**(10):2195-2230.

Mshelia, B.M. (2018). The antibacterial activity of honey and lemon juice against Streptococcus pneumoniae and *Streptococcus pyogenes* isolates from respiratory tract infections. *Acta Scientific. Microbiology*, **1:** 22-7.

Nandi, S., Kumar, R., Ray, P., Vohra, H. and Ganguly, N.K. (2001). Group A streptococcal sore throat in a periurban population of northern India: a one-year prospective study. *Bull World Health Organization*. **79**:528-533.

Nita T, Arai T and Takamatsu H. (2002). Antibacterial activity of extracts prepared from tropical and subtropical plants on methicillin-resistant *Staphylococcus aureus*. *Journal of Health and Sciences*; **48**: 273-276

Ogawa, T., Terao, Y., Okuni, H., Ninomiya, K., Sakata, H., Ikebe, K., *et al*. (2011). Biofilm formation or internalization into epithelial cells enables Streptococcus pyogenes to evade antibiotic eradication in patients with pharyngitis. *Microbial Pathog*enesis, **51**:58 –68.

Okazaki, A., Shoji-Hosaka, E., Nakamura, K., Wakitani, M., Uchida, K., Kakita, S., *et al*. (2004). Fucose depletion from human IgG1 oligosaccharide enhances binding enthalpy and association rate between IgG1 and FcγRIIIa. *Journal of molecular biology*, **336(**5):1239-1249.

Okeke, M.I., Okoli, A.S., Eze, E.N., Ekwume, G.C., Okosa, E.U. and Iroegbu, C.U. (2015). Antibacterial activity of Citrus limonum fruit juice extract. *Pak. Journal of Pharmacy and Science*, **28**(5): 1567-1571.

Ortuno, A.A., P. Baidez, M.C. Gomez, I. Arcas, A.G. Porras, and J.A. Del Rio, (2006), "*Citrus paradise* and *Citrus sinensis* flavonoids: Their influence in the defense mechanism against *Penicillium digitatum*. *Food Chemistry*, **98**(2): 351-358.

Pandey, A., Kaushik, A. and Tiwari, S.K. (2011). Evaluation of antimicrobial activity and phytochemical analysis of *Citrus limon. Journal of Pharmaceutical Biomedical Sciences*, **13**(17): 1-7.

Parija, S.C., (2014). Textbook of Microbiology & Immunology. 2nd ed. Elsevier Health Sciences.pp:183-190

Peschke, B., Keller, C.W., Weber, P., Quast, I. and Lünemann, J.D. (2017). Fc galactosylation of human immunoglobulin gamma isotypes improves C1q binding and enhances complement-dependent cytotoxicity. *Frontiers in immunology*, 8:646.

Prescott LM, Harley J, and Klein DA**.** (2002). Microbiology 5th ed, pp: 809-811.

Sauver, J.L.S., Weaver, A.L., Orvidas, L.J., Jacobson, R.M. and Jacobsen, S.J. (2006), September. Population-based prevalence of repeated group A β-hemolytic streptococcal pharyngitis episode. *In Mayo Clinic Proceedings*, **81**, **9**:1172-1176.

Sengun, I.Y. and Karapinar, M. (2004). Effectiveness of lemon juice, vinegar and their mixture in the elimination of Salmonella typhimurium on carrots (Daucus carota L.). *International journal of food microbiology*, **96** (3): 301-305.

Shet, A. and Kaplan, E.L. (2002). Clinical use and interpretation of group A streptococcal antibody tests: a practical approach for the pediatrician or primary care physician. *The Pediatric infectious disease journal*, **21**(5):420-426.

Shinkawa, T., Nakamura, K., Yamane, N., Shoji-Hosaka, E., Kanda, Y., Sakurada, M., *et al*. (2003). The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. *Journal of Biological Chemistry*, **278**(5):3466-3473.

Shulman, S.T., Bisno, A.L., Clegg, H.W., Gerber, M.A., Kaplan, E.L., Lee, G., *et al*. (2012). Clinical practice guideline for the diagnosis and management of group A streptococcal pharyngitis: 2012 update by the Infectious Diseases Society of America. *Clinical Infectious Disease*, 55: 86 – 102.

Suleiman, S.M. (2013). Antibacterial activity of Guava (Psidium guajava) leaves and Safflower (Carthamus tinctorius) seeds extracts against wound infection bacteria. M.Sc. Thesis, Sudan University of Science and Technology.

Spaulding, A.R., Salgado-Pabón, W., Kohler, P.L., Horswill, A.R., Leung, D.Y. and Schlievert, P.M. (2013). Staphylococcal and streptococcal superantigen exotoxins. *Clinical microbiology reviews*, **26**(3):422-447.

Srinivasan, D., Sangeetha, N., Suresh, T. and lackshmanaperumalsamy, p. (2001). Antimicrobial activity of certain Indian medicinal plants used in folkloric medicine. *Journal of Ethnopharmacology*, 74: 217-220.

Steven D., and Ehrlich, N.M.D. (2011). A private practice specializing in complementary and alternative medicine, Phoenix, AZ. *Review provided by VeriMed Healthcare Network*.

Stollerman, G.H., and Dale, J.B. (2008). The importance of group A Streptococcus capsule in the pathogenesis of human infections: a historical perspective. *Clinical Infectious Diseases*, **46**(7):1038-1045.

Subedi, G.P. and Barb, A.W. (2015). The structural role of antibody N-glycosylation in receptor interactions. *Structure*, **23**(9):1573-1583.

Subedi, G.P. and Barb, A.W. (2016). The immunoglobulin G1 N-glycan composition affects binding to each low-affinity Fc γ receptor. In *MAbs*, **8**(8):1512-1524.

Tadeg, H., Mohammed, E., Asres, K. and Gebre-Mariam, T. (2005). Antimicrobial activities of some selected traditional Ethiopian medicinal plants used in the treatment of skin disorders. *Journal of Ethnopharmacology*, **100**(1-2):168-175.

Tanz, R.R., Gerber, M.A., Kabat, W., Rippe, J., Seshadri, R. and Shulman, S.T. (2009). Performance of a rapid antigen-detection test and throat culture in community pediatric offices: implications for management of pharyngitis. *Pediatrics*, **123(**2):437-444.

Tawfik, N.O., Al Haliem, S.M. and Al-Ani, W.N. (2010). Evaluation of the antibacterial activity of citrus juices: an in vitro study. *Al Rafidain Dental Journal*, (12):376-382.

Tille, P.M. (2014). Bailey & Scott's Diagnostic Microbiology, 13th ed, *South Dakota*, *Elsevier* **pp**: 198,248-257.

Timmer, A.M., Timmer, J.C., Pence, M.A., Hsu, L.C., Ghochani, M., Frey, T.G., *et al.* (2009). Streptolysin O promotes group A Streptococcus immune evasion by accelerated macrophage apoptosis. *Journal of Biological Chemistry*, **284**(2):862-871.

Todar, K. (2006). Todar's online textbook of bacteriology. pp: 344-350.

Tomotake, H., Koga, T., Yamato, M., KASSU, A. and OTA, F. (2006). Antibacterial activity of citrus fruit juices against Vibrio species. *Journal of nutritional science and vitaminology*, **52**(2): 157-160.

Uchiyama, S., Döhrmann, S., Timmer, A.M., Dixit, N., Ghochani, M., Bhandari, T., *et al.* (2016). Streptolysin O rapidly impairs neutrophil oxidative burst and antibacterial responses to group A Streptococcus. *Frontiers in immunology*, **6**:581.

Van Sorge, N.M., Cole, J.N., Kuipers, K., Henningham, A., Aziz, R.K., Kasirer-Friede, A., *et al.* (2014). The classical Lancefield antigen of group A Streptococcus is a virulence determinant with implications for vaccine design. *Cell host & microbe*, **15**(6):729-740.

Verlekar, P. and Chandak, N. (2018). Antibacterial and antibiotic potential activities against drug-resistant pathogens. *International journal of pharmaceutical science and research*, **9**(10): 4373-4381.

Von Pawel Rammingen, U., Johansson, B.P. and Björck, L. (2002). IdeS, a novel streptococcal cysteine proteinase with unique specificity for immunoglobulin G. *The European Molecular Biology Organization Journal*, **21**(7):1607-1615.

Walker, M.J., Barnett, T.C., McArthur, J.D., Cole, J.N., Gillen, C.M., Henningham, A., *et al.* (2014). Disease manifestations and pathogenic mechanisms of group A Streptococcus. *Clinical microbiology reviews*, **27**(2):264-301.

Wessels MR. (2011). Streptococcal pharyngitis. *New England Journal of Medicine*, 364:648-655.

Wright, S.C., Maree, J.E. and Sibanyoni, M. (2009). Treatment of oral thrush in HIV/AIDS patients with lemon juice and lemongrass (Cymbopogon citratus) and gentian violet. *Phytomedicine*, **16**(2-3): 118-124.

APPENDICES

Appendix1

Sudan University of Science and Technology

College of Graduate Studies

Antimicrobial Activity of Aqueous *Citrus limon* Extract against *Streptococcus pyogenes* (Group A) Isolated from Sore Throat Patients In jaddah,Saudi Arabia

Questionnaire

1- Patient ID ……..

2- Sex……..

3- Age…….

4- Histry of treatment? Yes ☐ NO ☐

5- History of recurrent infection? Yes ☐ NO ☐

6- Symptoms:

Sore throat? Yes ☐ NO ☐

Fever? Yes ☐ NO ☐

Exudate production? Yes ☐ NO ☐

7- Results

Culture…….

Rapid test…….

Inhbition zone :

100%..... 50%.....

25% …… 12.5%.....

Appendix 2

Materials

A-Equipment

Autoclave

Bunsen burner

1- Incubator

2- Deep freezer

4- Hot air oven

5- Cork borer

6- Light microscope with an oil immersion lens

7- Refrigerator

8- Automated microtiter pipette

8- Rack

9- Loop

B-Glass wares

1- Sterile 5 ml test tube

2- Measuring cylinder

3- Beakers.

4- Slides

C-Disposable materials

1- Petri dishes (plates)

2- Sterile containers

3- Millipore Filter papers

4- Disposable syringes

5- Sterile pasture pipette

D-Culture media

Different culture media were used for the isolation, and identification of organisms includes:

Blood agar

1. Suspend 28 g of nutrient agar powder in 1 litre of distilled water.

2. Mix to dissolve all components.
3. Autoclave the mixture at 121 °C for 15 minutes.
4. Allow it to cool but not solidify, when the agar has cooled to 45-50 °C, Add 5% (vol/vol) Sheep blood that has been warmed to room temperature and mix gently but well.
5. Pour the media into sterile petri dishes, cool and store

Mueller Hinton 5% blood agar :

1. Suspend 38 gm of the medium in 1L of distilled water.
2. Heat with frequent agitation and boil for one minute to completely dissolve the medium.
3. Autoclave at 121°C for 15 minutes, cool to room temperature then add 5% of sheep blood agar
4. Pour the media into sterile petri dishes on a level, horizontal surface to give uniform depth.
5. Cool , store the plates at 2-8 ºC.

E- Reagent

Mc Farland turbidity standard

A 0.5 McFarland standard is prepared by mixing 0.05 mL of 1% barium chloride ($BaCl_2$), with 9.95 mL of 1% sulfuric acid (H_2SO_4)

G-Gram's stain:

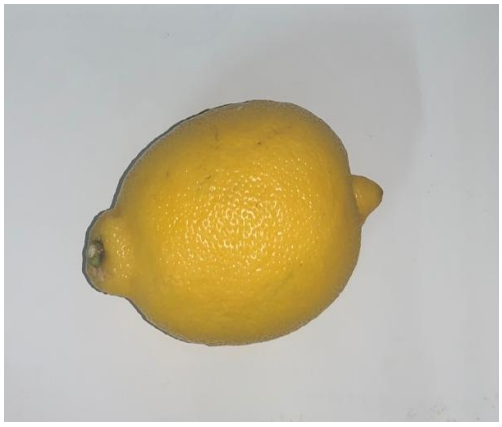
Crystal violet

Iodine

Decolorization with ethanol or acetone

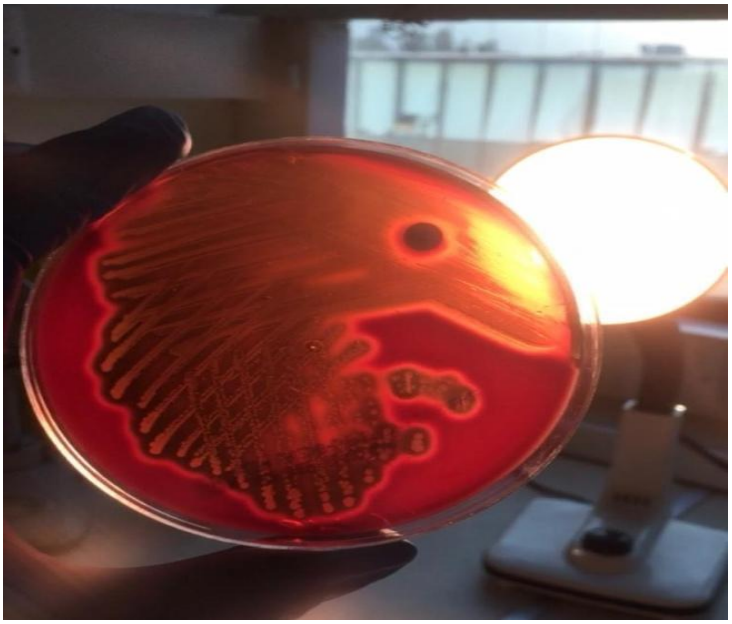
Safranin

Appendix 3

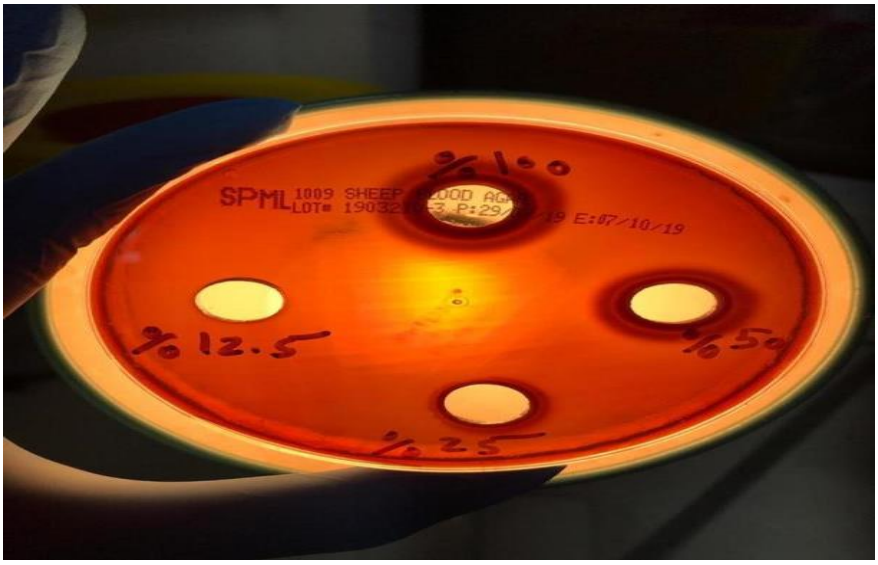


Color plate 1: *Citrus limon* fruit

Color plate 2: Throat swab on 5% sheep blood agar showing β-hemolytic colonies and throat normal flora. Bacitracin dick sensitive.



Color plate 3: Pure *S. pyogenes* culture



Color plate 4: Activity of *Citrus limon* juice against *S. pyogenes*

Color plate5: SD bioline strep A kit

Color plate 6: Microgen strep (Streptococcus grouping Kit)

Color plate 7: Millipore filter (syringe filter)