University of Kerbala

College of Science

Department of Biology

Immunological and Diagnostic Study of the Causes of Otitis Media Infection and the Inhibitory Effect of Some Plant extracts and antibiotic

A Thesis

Submitted to the Council of the College of Science at the University of Kerbala in partial fulfillment of the requirement for the Master degree in Biology

BY

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

اقْرَأْ بِاسْمِ رَبِّكَ الَّذِي خَلَقَ (1) خَلَقَ الْإِنْسَانَ مِنْ عَلَقٍ (2) اقْرَأْ وَرَبُّكَ الْأَكْرَمُ (3) الَّذِي عَلَّمَ بِالْقَلَمِ (4) عَلَّمَ الْإِنْسَانَ مَا لَمْ يَعْلَمْ (5)

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سورة العلق

الآيات (1-5)

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Dedication

"To my parents, who planted the seed of knowledge in my mind and nurtured it."

"To my husband, who has walked every step of this journey with me."

"For my kids, whose laughter is my favorite sound."

"To my professor, who taught me the value of critical thinking and the power of the pen."

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Summary

Otitis media is a middle ear infection that can damage the structures around the ear as well as the auditory system. Due to the anatomical structure and growing immune system, it is one of the most prevalent diseases afflicting young children every year.

During the period three months which extending between March 2022 to May 2022 ,ear swabs and blood samples were collected from 101 otitis media patients and 49 healthy control, their ages was around 1 year to 70 years. Collection of samples carried out in two places (Imam Al-Hussein medical city and private clinic). The bacterial growth was identified by using Vitek -2 system. The human Interleukin-12, Intrleukin-13 and Macrophage Inflammatory Protein -1α were measured by using the ELISA technique .The results showed that infection of males(50.5%) was higher than females(49.5%).On the other hand, the highest rate of infection was 21-30 age group while the lowest age was 61-70 years. The results showed appositive association between respiratory tract infection, recurrent otitis media with otitis media infection while the relation was negative with smoking.

Out of 101 ear swabs75 samples (74.25%) had positive bacterial growth, while 26(25.75%) showed negative growth. The results of bacterial isolates were *Proteus Mirabilis* (17/75) 22.66%, *Staphylococcus aureus* (16/75) 21.31% , *Pseduomonas aeruginosa* (8)10.66%, *Staphylococcus warneri* (6)8%, *Aeromonas sobria* (5)6.66%.

There were significant differences at ($P \leq 0.05$) in serum levels of WBC count between patients ($9.31 mm^3$) and control ($7.52 mm^3$).Furthermore ,Lymphocyte count was significant at ($P \leq 0.05$)and Granulocyte was significant too at($P \leq 0.025$).The interleukins levels were significant also between patients and controls.

IL-12 for levels for patients (33.69pg/mL) and in controls (20.55pg/mL).Also, there were significant differences at (P≤ 0.05) in the serum levels of IL-13 in patients (48.11pg/mL) while in controls (30.23pg/mL).On the other hand, there were significant differences in MIP-1α at (P≤0.05) the level in patients (168.48pg/mL) while in controls (106.26pg/mL).

Also there were significant differences between Interleukins level and bacterial isolates at (P≤0.05) where the higher level of IL-12 was associated with *Staph. aureus* infection (26.6±3.8pg/mL), the higher levels of IL-13 was associated with the bacteria *Aeromonas sobri* (37.9±5 pg/mL) while MIP-1α higher concentration was record also with *Staph. aureus* bacteria (138.6±11pg/mL).

The antibiotic susceptibility for *Staph.aureus* isolates were highly sensitive for (amoxillin/clavonic acid 30mg,ceftazimide 30mg,meropenem 10mg, ciprofloxacin 15mg, amikacin 10mg).

While the bacteria *P. mirabilis* was highly sensitive for (ciprofloxacin 10 mg, gentamycin 10 mg).

The gas chromatography mass spectrophotometry for olive and bitter almond oil showed the presence of different biologically active compounds such as (DPPH% ,Total peroxide ,Vitamin C ,Vitamin E, FRAP ,CUPRAC, thiol ,Phenols ,Flavonoids) the levels of these compound in olive oil was (30.8796 ,13.66667, 1.88 , 2.686, 6.146789, 18.26605, 31.10294, 49, 9.80) respectively.

While the almond oil showed (6.354009, 8.066667, 1.75, 1.75, 16.6055 ,6.761468 ,49.26471, 49.71, 9.9428) respectively.

The stock oil of both (olive oil and bitter Almond oil) showed significant differences at P≤ 0.05 against the growth of *S. aureus* bacteria, however *P. mirabilis* had been resistance for the both oils.

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List of abbreviations

Word	Abbreviations
Acute otitis media	AOM
Chronic Otitis Media	C O M
Chronic Suppurative Otitis Media	CSOM
C-Type Lectin Receptors	CLRs
Extra Virgin Olive Oils	EVOOS
Enzyme Linked ImmunoSorbent Assay	ELISA
Gas Chromatography –Mass Spectrophotometry	GC-MS
Intensive Care Unit	ICU
Interleukin -13	IL-13
Interleukin-12	IL-12
Macrophage Inflammatory Protein -1α	MIP-1α
Methicillin Resistant *Sthaphylococcus Aureus*	MARSA
Middle ear effusion	MEE
Natural killer cell	NK
Neutrophil Extracellular Traps	NETs

Otitis media	**OM**
Otitis Media with Effusion	**OME**
Pathogen Associated Molecular Patterns	**PAMPs**
Pattern Recognition Receptors	**PRRs**
Polymorphonuclear leukocytes	**PMNs**
Retinoic Acid-Inducible Gene	**RIG-I**
Tympanic Membrane	**TM**
Upper Respiratory Infection	**URI**

Chapter one

Introduction

1.Introduction:

Ear infections can take many different forms depending on whether the infection is in the external, middle, or internal ear and if the pathogen is viral, bacterial, or fungal. Aural discharge is common with acute middle ear infections (Al-Hamamy *et al*.,2021).

Over 330 million individuals worldwide suffer from ear infections, with over 60% losing hearing capacity. Its widespread prevalence has caused difficulties for public health in both developing and industrialized countries (Javed *et al*.,2020).

Otitis media is a group of disorders characterized by inflammation of the middle ear. It is the most prevalent type of illness in children, and it frequently follows an acute upper respiratory tract infection. Otitis media is the most common cause of hearing loss and the fifth leading cause of disease worldwide, with a higher frequency in developing countries such as Sub-Saharan Africa and South Asia (Tesfa *et al*.,2020).

The development of problems linked with the migration of the infection process to the inner ear or the cranial cavity is a serious consequence of otitis media: facial nerve paresis, labyrinthitis , meningitis, sigmoid sinus thrombosis, brain and cerebellar abscesses (Venekamp., 2020).

Otitis media affects around 16% of Nepalese children over the age of five. Nearly two-thirds of these cases involve elementary or middle school-aged children, the majority of whom come from low-income families (Almayali *et al*.,2023).

The global prevalence of OM is considerable, with rates ranging from 9.2% in Nigeria to 10% in Egypt, 6.7% in China, 9.2% in India, 9.1% in Iran, and 5.1-7.8%

in Russia . Furthermore, the prevalence of OM among native Australian children is 90%, the highest in the world (Song *et al*., 2023).

The origin and pathophysiology of OM are both complicated. The most important component is pathogenic infection of the upper respiratory tract. Viral or viral antigen was found in the middle ear of 5% to 25% of AOM patients. Bacteria are identified in around 40% to 70% of patients. Furthermore, there was a significant association between the bacteria recovered from individuals with middle ear OM and the primary organisms detected in the nasopharynx. Bacterial ear infections are common following a throat infection, flu, or other respiratory system infection (Spoială *et al*.,2021; Hateet *et al.*,2022)

Pseudomonas sp., S. aureus, K. pneumoniae, and Proteus sp. have been shown in numerous investigations to be the common microorganisms found in cases of otitis media. Moreover, it has been noted that bacteremia might develop in people who have had tympanomastoidectomy following surgery, which can have serious consequences (Shishegar & Jahangiri .,2021).

The human immune system controls the response to infectious stimuli in health by providing vigorous action against non-self-antigens while tolerating the host's own cells .However, failure of this mechanism generates numerous inflammatory disease processes at multiple anatomical locations. However, the particular immune cell groups found in MEE from individuals with OME are still poorly understood. Early observations showed that, with the exception of purulent MEE, such as AOM, where neutrophils predominate, macrophages are the most prevalent immune cell type in all MEE regardless of appearance. T cells and B lymphocytes have also been seen in MEEs with serous or mucoid characteristics to varying degrees. Many studies have been conducted to examine the content of

inflammatory mediators in serous and mucoid MEEs, with mucoid MEEs including higher inflammatory mediators such interleukin-8 (IL-8) and RANTES (Enoksson *et al*.,2020).

Medicinal plants are employed as a source of medicine in basically all historical periods. Ensure the safety, purity, and efficacy of medicinal plants and herbal medications has only lately emerged as a critical concern in both developed and developing countries. Herbal medications can aid the creation of a new age of the healthcare system to cure human ailments in the future by standardizing and analyzing the health of active plant-derived chemicals. Traditional knowledge and medicinal plants awareness can play an important role in the exploitation and discovery of natural plant resources (Ahad *et al* .,2021).

1.2.Aims of this study:

This study aims to evaluate the association between the types of pathogenic bacteria isolated from patients with Otitis Media infection and levels of some Interleukins in patients serum as well as determine the antimicrobial activity of some medical plants against the most common isolate pathogenic bacteria through the following objects:

1. Culture ear swab samples collect from patients with Otitis Media infections and healthy people ,in addition take some information about each subject such as age, sex ,respiratory infection , smoking and recurrent Otitis media.

2. Isolation and identification bacterial isolates using the general and selective medium and then identification by using Vitek system.

3. Using the ELISA techniques, estimate the levels of Interleukins(IL-12,IL-13 and MIP-1α) in the serum collected from patients and controls.

4.Estimate the levels of White Blood cell Counts in patients and control blood.

5. Investigating the association between types of isolated bacterial species and Interleukins levels.

6. Determination the antibacterial of (Olive oil and Bitter Almond oil) on the most common bacterial isolates from patients with Otitis Media.

Chapter Two

literatures Review

2.Litreratures Review

2.1.Ear anatomy:

The ear is the organ of the body responsible for hearing and balance through the vestibular system. In mammals, the ear is divided into three parts: the outer ear, the middle ear, and the inner ear (Immordino *et al*.,2023). The outer ear is the visible part of the hearing organ, it consists of the ear (pinna) and the external auditory canal (Szymanski & Geiger., 2017). Middle ear air fills the cavity located in temporal bone and extends from the tympanic membrane to the oval window as shown in figure (2-1) (schilder et al.,2016).

1-Auditory ossicles is a small bone within the cavity that is connected by synovial joints.

a-. Malleus is outer most bone ,connected to the tympanic membrane and anvil.

b-Anvil-middle bone; connects to malleus and stapes.

c- Stapes-innermost bone; connects to the anvil and oval window.

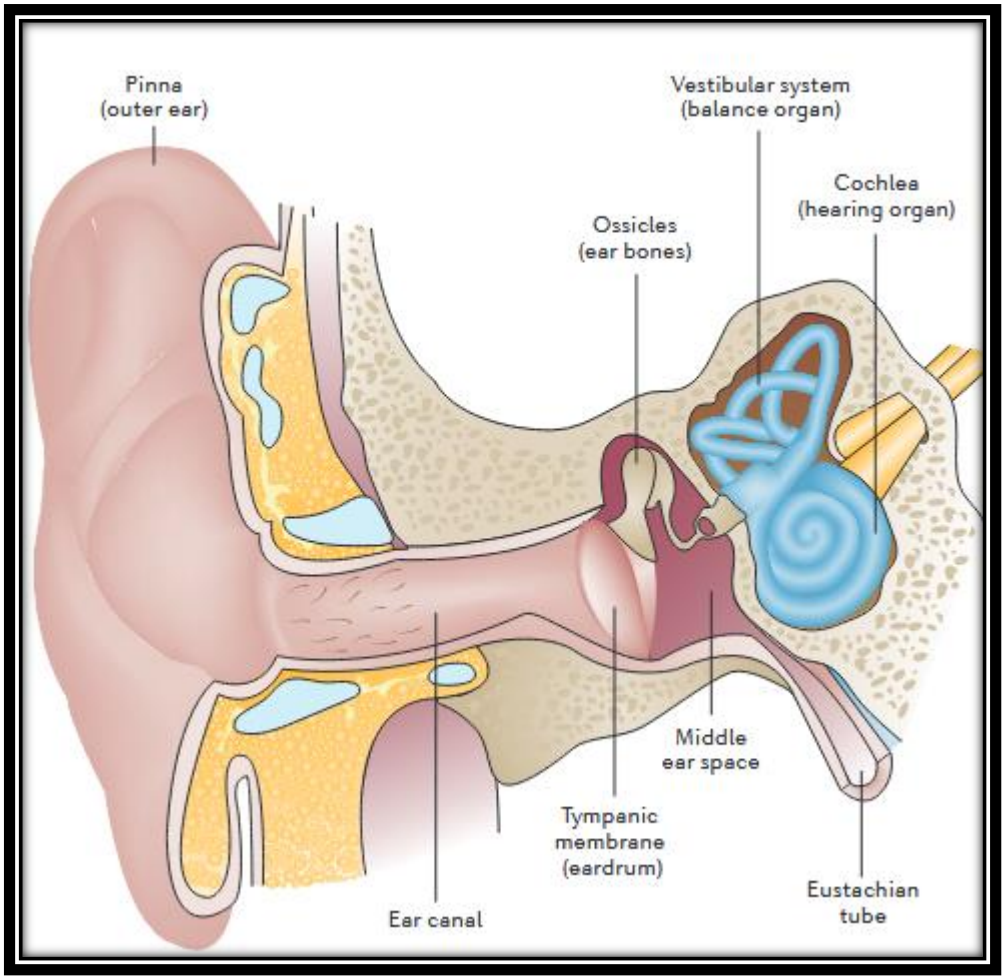
2-Oval window-membrane covered opening separating the middle and inner ear.

3-Round window-membrane covered opening between middle ear and cochlea.

4-Auditory tube (pharyngotympanic)or Eustachian tube –connects middle ear to the nasopharynx (part of throat near the nasal cavity) equalizes air pressure of middle ear with atmospheric air (Al-Obaidi.,2017).

The inner ear is the final and the most complicated part of the ear. It occupies a small bony cavity called the bony labyrinth (osseous labyrinth) that is located directly behind the medial wall of the middle ear, the inner ear consists of three

main anatomical parts: the semicircular canals, the vestibule, and the cochlea (Maroonroge *et al* ., 2000).



Figure(2-1): Ear anatomy (schilder *et al*.,2016).

2.2. Ear infections

Ear infection is an inflammation of the ear, and ear discharge is one of the most prevalent symptoms. It can take several forms, the most common of which is otitis media, which usually affects children ,in many low/middle-income nations, ear infections are among the primary causes of deafness. Unfortunately, most patients with ear infections in limited in resources instances avoid getting medical

treatment, resulting in adverse effects (Al-dhaher *et al* .,2018). *Pseudomonas aeruginosa* and *Proteus* species are the most frequently isolated bacteria from ear discharge cultures (Stubbs.,2005). Infection of the ear, which includes both human and animal ear disorders: Otitis Externa, Otitis Media, and Otitis Interna are the three types of infections(Jamal *et al*.,2022).

2.3.Otitis media:

Otitis media (OM), often known as middle ear inflammation, can manifest as acute otitis media (AOM), otitis media with effusion (OME) and chronic suppurative otitis media (CSOM) (Parmanand& Atfeh .,2023).

Among other conditions. OM is one of the most prevalent illnesses among young children around the world. It may spontaneously cure without any problems, but it can also cause hearing loss and have long-lasting effects, a previous research suggests that any type of otitis media and the subsequent hearing loss might have a severe impact on cognitive abilities, such as memory and attention as well as behavior and language (Ghazi *et al* .,2022).

2.3.1.Types of Otitis media

2.3.1.1.Acute otitis media (AOM) :

Acute upper respiratory tract infections frequently lead to acute otitis media which is characterized by fluid in the middle ear in addition to the warning indicators of a severe infection ,(scheldier *et al*.,2016). In young children, AOM is also among the symptoms that most frequently result in the prescription of antibiotics. The risk of developing an ear infection is increased by a variety of factors, including genetic ,microorganisms , immunologic, and environmental factors (Seid *et al*.,2013).

Lack of a link between clinical symptoms and the microorganisms that cause them complicates AOM diagnosis. As a result, AOM treatment is not always necessary and the prolonged misuse of antibiotics in AOM decreases treatment efficacy and puts patients at risk for drug-resistant infections (Grevers, G.,2010).

The most typical age range for acute otitis media is between 3 months and 3 years, while it can infect at any time. Between the ages of 6 and 11 months, the disease has a peak incidence, and its prevalence may be increasing (Mohammed&Younis.,2023). AOM is characterized by irritability or feeding difficulties, which may be the only indication of a septic focus while older children show persistent presence of fever, earache, or ear pulling and hearing loss (Ahmed.,2023). Middle ear infections have recently taken the lead in the global structure of childhood morbidity, according to the World Health Organization (WHO), roughly 15-20% of the world's adult population and children suffer with otitis media. In children, acute otitis media accounts for 50-59% of all clinical types of otitis (Umarov .,2023).

On the other hand ,the American Academy of Pediatrics estimates that more than 5,000,000 cases of acute otitis media (AOM) occur annually in American children, resulting in more than 10, 000, 000 antibiotic prescriptions annually and approximately 30, 000, 000 annual medical consultations annually, more than 700 million cases of AOM are diagnosed worldwide, with 50% of affected children being under 5 years of age (postma *et al*.,1997 ;Milucky *et al*.,2019).

2.3.1.2.Chronic otitis media(COM):

A tympanic membrane (TM) perforation causes chronic otitis media (COM), which is a recurring infection of the middle ear and/or mastoid air cells,aural fullness, otalgia, otorrhea, hearing loss, and occasionally vertigo are common

symptoms (Yang *et al*.,2023). When it lasts for longer than two months, a hole develops in the eardrum and a discharge from the ear canal continues as shown in figure (2-2) (Lusting *et al*.,2018). This chronic condition may have serious health repercussions, including intracranial problems and considerable morbidity among those affected ones (Khairkar *et al*.,2023). In addition, chronic otitis media often results in some degree of hearing loss, typically conductive and temporary, caused by a ruptured tympanic membrane or changes in the ossicular chain due to fixation or erosion resulting from the chronic inflammatory process (Yang *et al*.,2023).

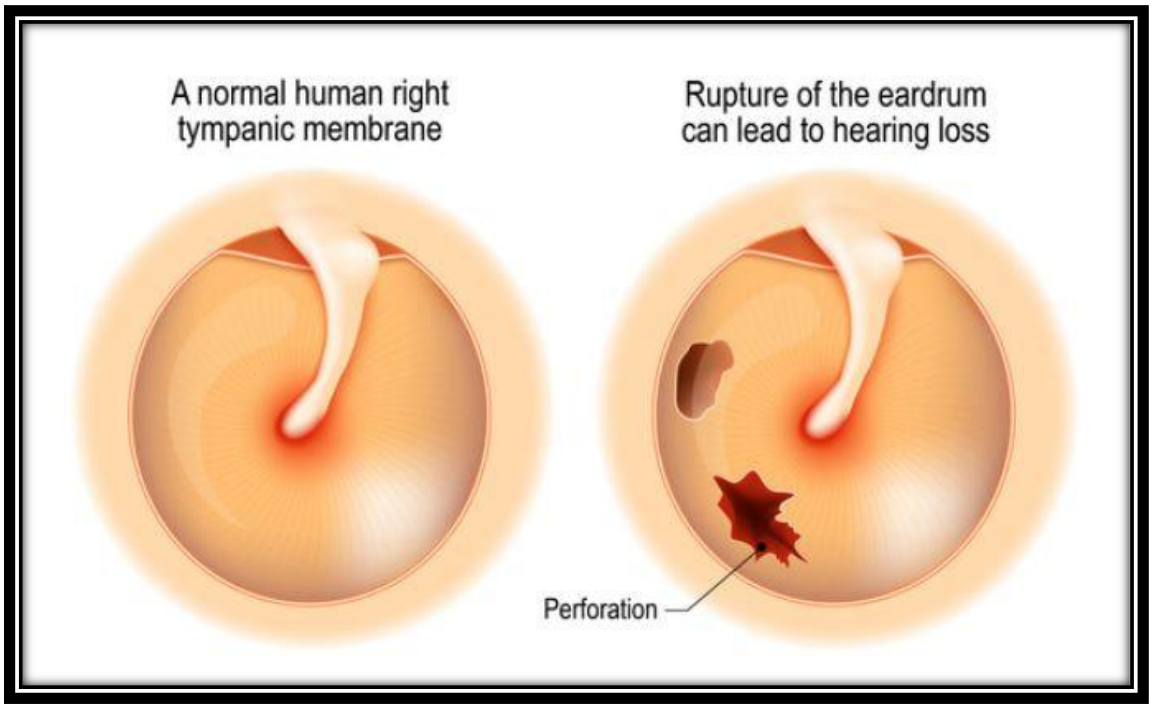


Figure (2-2) : chronic otitis media (Lusting *et al*.,2018).

Multiple environmental factors as well as genetic factors that lead to functional and anatomic differences in the middle ear that predispose to COM are the main causes of chronic middle ear inflammation ,the disease may be treated medically or surgically, depending on its stage and complications (Gökharman *et al*., 2023).

2.2.1.3.Otitis media with effusion(OME):

Otitis media with effusion is identified by a buildup of non-purulent fluid in the middle ear cavity for more than three months without the incidence of acute inflammation (Hassan e*t al*.,2023).

The tympanic membrane is pressed as a result of fluid accumulation in the middle ear and Eustachian tube. The pressure reduces sound conduction, which decrease the tympanic membrane's ability to vibrate properly, and thus reduce patient hearing. In order to qualify as having chronic otitis media, a patient must have a perforated tympanic membrane and have symptoms that have persisted for three months or longer on examination or tympanometry, according to some specialists (Searight *et al*.,2023).

The most frequent cause of childhood acquired hearing loss is OME, one of the most widespread infectious disorders in children . 20% to 50% of kids between the ages of 3 and 10 will have an OME episode (Rasheed *et al*.,2023). Early childhood is characterized by high prevalence of OME due to a developing immune system, frequent upper respiratory tract infections, shorter and more horizontal Eustachian tube than in adults (SAVAŞ .,2023).

2.4.Epidemiology of otitis media:

Otitis media is a worldwide condition that affects men slightly more than women. The exact number of instances per year is difficult to estimate due to a lack of reporting and varying incidences across many different geographical regions (Rijk et al.,2021).

Otitis media is most common between the ages of six and twelve months, and it gradually fades after that, approximately 80% of all children will have otitis media

at some point in their lives, and between 80% and 90% of all children will have otitis media with effusion before reaching school age. Otitis media is less prevalent in adults than in children, yet it is more common in some subpopulations, such as individuals with history of recurrent OM or cleft palate , immunodeficiency or immunocompromised status, and others (Danishyar & Ashurst.,2022).

OM prevalence in Nigeria was determined to be 9.2%, 10% in Egypt, 6.7% in China, 9.2% in India, 9.1% in Iran, and 5.1-7.8% in Russia (DeAntonio *et al*.,2016).

2.5.Clinical features of Otitis media:

The clinical symptom in most cases of acute purulent otitis media progresses in stages, depending on the stage and severity of the process, local and general symptoms of the disease manifest differently, Acute suppurative otitis media is divided into three stages: pre-perforative, perforative and reparative (Samieva & Olimjonova.,2023).

The infected ear in patient with acute otitis media is painful , with a red, bulging eardrum. Many people suffer from hearing loss. Infants may simply be grumpy or have trouble sleeping. In young children, fever, nausea, vomiting, and diarrhea are common. The bulging eardrum occasionally ruptures, allowing pus to drain from the ear and providing temporary relief from ear pain (Samieva *et al*.,2020).

An upper respiratory tract infection or an aggravation of seasonal allergic rhinitis frequently precedes the development of AOM in adults. Adults with AOM often have unilateral otalgia (ear discomfort) and reduced or muted hearing. The discomfort might be slight, moderate, or severe. If the tympanic membrane has ruptured, the patient may experience abrupt pain alleviation, which may be

accompanied by purulent otorrhea may exist, however it is rarely mentioned. Conductive hearing loss, which can occur as a result of middle ear fluid, is usually temporary. Other symptoms, such as high fever, acute ear discomfort, or facial paralysis, indicate unexpected problems (Limb *et al*.,2018).

Other clinical symptoms , such as nasal congestion, rhinorrhea, open-mouthed breathing, obstructive sleep apnea , snoring, and "adenoid face" caused by chronic airway obstruction, are important factors that can cause or worsen OME, Eustachian tube dysfunction , and acute/chronic nasal sinusitis in children (Chen *et al*.,2023).

Otitis media consequences are characterized as intracranial or extra cranial . They can develop as a result of acute or chronic otitis media with or without Cholesteatoma , the prevalence rates of extracranial and intracranial problems have been reported to vary from 0.69% to 5%, with an intracranial complications death rate of 8% (Watkinson& Clarke.,2018).

Otitis media complications are a major concern in underdeveloped nations (Yorgancılar *et al*.,2013), poverty, a lack of education, a lack of healthcare facilities, and a lack of knowledge regarding auditory symptoms may all be problems in developing nations. Complications in industrialized nations, on the other hand, it might be caused by antibiotic resistance, disguise symptoms with medications and changes in the virulence of pathogenic organisms (Dongol *et al*.,2020).

2.6.Etiology of Otitis media:

A variety of host, infectious, allergy, and environmental variables all have a role in the development of OM (Waseem *et al*., 2014). A bacterial infection,

particularly *S. pneumoniae*, is the most common, followed by *Haemophilus influenzae* and *Moraxella catarrhalis*, although viruses are responsible for one-third of cases (Darmawan *et al*.,2023).

The high incidence of OM results from exceedingly high burden of viral about 35% of viral URIS episodes are complicated by OM occurring mainly the first week of URI onset (Nokso-Koivisto *et al.*, 2015).

Superimposed fungal infection should be suspected in cases of OM that do not respond to local therapeutic medications, although culture investigations are regarded the gold standard for identifying etiologic agents, they are not always available or yield an optimal result (Juyal *et al*.,2014). True fungal infection is detected by the host's reaction in the form of inflammation, necrosis, or bleeding (Punia *et al* .,2019).

2.6.1.Bacterial agent:

Knowledge of the local microbiological flora in OM is important for beginning empirical therapy pending culture findings, making periodic monitoring of the microbiological profile and sensitivity pattern in OM mandatory. Furthermore, it is necessary to determine whether the microbial floras in bilaterally discharged ears are the same (Orji&Dike.,2015).

Many potential causes for OM have been identified in the literature, despite this *P. aeruginosa* and *Staph. aureus* are the most common (Taoussi *et al*.,2023). 87% of AOM samples detected just one pathogen, whereas 51% of COME positive samples contained numerous bacteria species (Nogues *et al*.,2020).

In the nasopharynx, the most common infectious pathogens colonize and multiply, including *S. pneumoniae, Haemophilus influenzae, Moraxella*

catarrhalis, *S. pyogenes*, *Staph. aureus*, and/or respiratory viruses, these microorganisms spread from the nasopharynx to the Eustachian tube and infect the middle ear, causing OM (Shah *et al*.,2023).

Study in Southern Ethiopia detected that (48.0%) of bacterial isolates were Gram positive and (52.0%) were Gram negative bacteria. The most frequently isolated pathogen was *Staph. aureus* (27.0%), followed by *P mirabilis* (12.5%), *Haemophilus influenzae* (9.2%), E.coli (7.2%), Enterococcus species (6.6%), *S. pneumoniae* (5.3%), *Klebsiella pneumoniae* (3.9%), and *Klebsiella ozeanae* (3.9%). The major part of the bacterial isolates (65.1%) were discovered in children under the age of five (Tadesse *et al*., 2019).

2.6.1.1*Staphylococcuse aureus:*

One of the most important human pathogens in both hospitals and communities for its viability to adapt and evade the immune system (Afzal *et al*.,2022), which is aspherical gram posative bacteria with a diameter ranging between 1-5.0 micrometers, arranged singly or in non-motile clusters, they do not form spores and are either aerobic or facultatively aerobic, also their colonies are white to yellow color and most of them are beta hemolytic when grown on blood agar medium et (Gnanamani *et al*.,2017).

As a multi host opportunistic pathogen, the organism has long evolutionary history. *Staph. aureus* colonizes roughly 20-30% of individuals, primarily in the nose, but also frequently in the skin, throat, axillae, groin, and intestine (Howden *et al*.,2023). Human-pathogenic *Staph. aureus* strains produce up to five distinct bicomponent toxins (leukocidins) that target and lyse neutrophils, innate immune cells that serve as the first line of defense against *S. aureus* infections (Berends *et al*.,2019). Immuno-compromised individuals, those having surgery, and anybody

with any type of indwelling medical device continue to face the greatest risks from *S. aureus*, also its sepsis alone has been responsible for about 20,000 deaths annually in the U.S. in recent years. Additionally, widespread resistance to several of the greatest anti-staphylococcal medications, including penicillin and methicillin (methicillin-resistant *S. aureus,* MRSA), sharply raises the mortality, morbidity, and financial burden associated with *S. aureus* infections (Otto.,2023).

2.6.1.2. Staphylococcus lentus

Staphylococcus lentus is a member of the *Staph. sciuri* family, *Staph. sciuri* is a coagulase-negative, novobiocin-resistant, oxidase-positive staphylococcus, the organism is primarily an animal bacterium species and is widely found on the skin and mucosal surfaces of a broad variety of pets, agricultural and wild animals (Dakic *et al*,2005;Osman *et al*,2017) .

It was extensively discovered in food-producing animals, including domestic fowl and cattle, as well as their products. Individuals who had interaction with animals were also shown to be *Staph.lentus* carriers. *Staph.lentus* has been associated to subclinical mastitis in dairy cows and, in rare occasions, has transferred diseases to humans. *S. sciuri* is capable of infecting people and causing serious illnesses such as septic shock, bacterial endocarditis, endophthalmitis, pelvic inflammatory disorders, urinary tract infections, incision and wound or abrasion infections (Alash & Mohammed.,2019).

2.6.1.3. *Psedomonas aeruginosa:*

Gram-negative bacteria that are straight or curved and non-spore forming, clever integrated flagella having a common diameter of (5.0-8.0) x (5.1-3) micrometers. Oxidase and Catalase, an optional aerobic test for the production of thermal range between 37 and 42 degrees Celsius (Adilabdulhady & Kadhim.,2022) and secreted pigments which are important in the clinical and laboratory diagnostics pyocyanin and pyoverdin (Levinson., 2014).

P. aeruginosa is an opportunistic pathogenic organism that causes severe infections, especially in hospitals, and it also plays a role in nosocomial infection because it prefers moist environments and patients suffering from malignant blood diseases, catheters, patients who take long-term treatments of immunosuppressant's , radiation, wounds, Burns , Otitis media , respiratory and urinary system diseases. Also, because they show high resistance to antibiotics, they play a major role in infections that occur around the world (Bassetti *et al*.,2018).

2.6.1.4.*Proteus mirabilis*:

Proteus mirabilis is a Gram-negative bacterium that is capsulated, spore-free, and pleomorphic. It belongs to the Enterobacteriaceae family. *P. mirabilis* is a widely distributed conditionally pathogenic bacterial species (Hao *et al.*,2023). Due to its ability to convert from microscopic rods into long, multinucleated swarmer cells with thousands of flagella ,*Proteus* has constantly interested scientists (Armbruster & Mobley., 2012). *P. mirabilis* is found in a variety of locations, including sewage, soil, and water, as well as animal and human gastrointestinal tracts (Alqurashi *et al*., 2022) .

This species' pathogenicity is enhanced by the expression of virulence factors such as adhesion molecules, urease, proteases, siderophores, and toxins (De Oliveira *et al*.,2021).

The microorganism are the third most common cause of urinary tract infections is *P. mirabilis*. It produces urease, which results in the formation of crystalline biofilm and is regarded as one of the most important pathogenicity properties of *P. mirabilis* strains, alongside their ability to swarm on solid surfaces (Mazyed & Al Atya.,2023).

2.6.1.5. *Serratia marcescens:*

Bacillus bacteria negative for Gram stain, non-spore forming. Negative for oxidase test, produces characteristic red pigment is Prodigiosin motile (Karnaker *et al* .,2023).

positive for catalase with white ,red or pink colonies (Grimont& Grimont.;2015).Also it an opportunistic nosocomial pathogen which can be located in the aerodigestive, respiratory, and urinary systems . It is connected with infections in the systems mentioned previously and can manifest as a rare and severe cause of necrotizing fasciitis (Khanna *et al* .,2013).

Ocular lens and ear infections, osteomyelitis, endocarditis, meningitis, and septicemia are all possibilities of *S. marcescens* infections. Acquired infections with *S marcescens* are more common in neonatology and intensive care units (ICU), where this microbe has been identified from catheters, oxygenation devices, prefilled syringes, needles, parenteral solutions, milk-drawers, sinks, nails, and health care workers' hands. It has also been discovered in disinfectant solutions and double-distilled water, demonstrating its incredible metabolic flexibility and

capacity to adapt and live in tough environments. Unfortunately, antibiotic resistance has become severe health care concern during the last decade; effective methods to manage and eliminate *S marcescens* resistance are urgently needed (Redondo-Bravo *et al.*, 2019 ;Prabhu *et al*, 2020 ; Tavares-Carreon *et al*,2023).

2.6.2.Viral agent:

Respiratory syncytial virus, rhinovirus, adenovirus, coronavirus, bocavirus, influenza virus, parainfluenza virus, enterovirus, and human metapneumovirus were the viruses most usually caused AOM symptoms (Marom *et al*.,2012).

According to Chinese studies, the virus was found in 86.2% of the children, the most frequently identified pathogens were human rhinovirus (38.3%), respiratory syncytial virus (32.1%), and parainfluenza virus type 3 (27.2%) (Yan *et al*.,2023). More than 90% of AOM patients develop this infection following a URI, confirming that a URI increases the risk of AOM (Jamal *et al*.,2022).

According to a recent study by (Heikkinen *et al.*,2003) the average RSV infection incidence rate among children under the age of three years was 275 per 1000 children per year, and 58% of these children with RSV developed AOM (Abdel-Razek *et al*., 2022).

2.6.3.Fungal agent:

In recent years, there has been a noticeable increase in infection cases pathogenic fungi, especially opportunistic ones, which prompted researchers to increase their efforts to prevent the spread of this type of infections , which often leads to death in many cases recorded globally (Abed *et al*., 2011) .

According to research Fungal infections of the ears are more common in the external auditory canal than in the middle ear (Vennewald *et al*., 2003). Invasive fungal infections occurring in the mastoid and middle ear are rare (Pichon *et al*., 2020).

In recent years, the incidence of invasive aspergillosis (IA) has gradually increased. Invasive aspergillosis occurring in the middle ear is relatively rare, and the entry point of fungal infection is mostly acute or chronic infection of the external ear or middle ear. Long term local administration of anti-inflammatory drugs and steroid combined with diabetes or immunodeficiency are the main risk factors for IA in middle ear (Liu *et al*., 2023).

Aspergilli are prevalent in airborne dust, and the increase is helped by cerumen and the ear canal's slightly acidic pH (Segers *et al*., 2023). Clinically, because it lacks the distinctive look of Aspergillus , it might present as otorrhea that does not respond to auditory antimicrobials and is frequently detected by fungal culture. This demonstrates the importance of fungal cultures in diagnosis (Ho et al.,2006).A study in Iraq showed that *Aspergillus niger* was the most present 31%, followed by *Candida albicans* 18%, then *Aspergillus flavus* 18% (Al-Ameri & Nasser.,2023).

Another study conducted in Iran showed that *A. Niger* (58.57%) was the foremost isolate, followed by *A. Flavi* (19.23%) and *Candida parapsilosis* (14.96%). The predominance of *Aspergillus* isolates had minimal in vitro sensitivity to tioconazole and nystatin (Roohi *et al*.,2023).

the mucor can cause an invasive fungal temporal bone infection that resulted in meningoencephalitis (Haruna *et al*.,1994) . It is important to understand that even

less virulent fungal isolates (including non albicans Candida) are becoming more prevalent in ear infections (Viswanatha & Naseeruddin.,2011).

Mucor is a weak pathogen that does not attack healthy people due to its weak virulence factors, but it can become a major threat when it attacks people with low immunity, particularly those with uncontrolled diabetes, as it causes Mucormycosis or black fungus During the spread of the Corona pandemic, the condition became well-known (Signh *et al*.,2021& Ghosh *et al*.,2022).

2.7.Immune response:

The immune system has developed to protect the host against thousands of harmful microorganism that are continually changing themselves. The immune system also assists the host in eliminating harmful or allergic substances that enter human body by the mucosal surfaces (Feld& Hyams., 2009).

The capacity of the immune system to identify self from non-self is critical to mobilizing a response to an invading virus, toxin, or allergy (Sutar.,2023). According to the research, the microbiota plays important roles in immunological development during childhood as well as the development of numerous medical conditions disorders such as allergies and asthma (Wypych *et al*.,2019).

T and B cells are used to provide immunity, there are two types of immune systems: innate and adaptive, innate system recognizes and reacts immediately to common medical conditions, but its specificity is restricted, it alerts the adaptive system, which supplies more targeted protection by B and T cells (Qin *et al*.,2020). Which are essential in determining the disease's prognosis, T cells could be the key facilitators to control the viral infection, Contagion with abnormally high levels of

T-cell activation have a bad prognosis (Moderbacher *et al.*,2020; Sette & Crotty.,2021).

2.7.1.Innate immunity:

The innate immune system is a non-specific first-line defensive mechanism which recognizes microbial permeation. The involvement of middle ear epithelial cells, neutrophils, macrophages, fibroblasts, mast cells, and natural killer cells in pathogen defense (Dayasena *et al* ., 2011) and uses pattern recognition receptors (PRRs) to recognize pathogen-associated molecular patterns (PAMPs).TLRs, NOD-like receptors (NLRs), retinoic acid-inducible gene (RIG-I)and C-type lectin receptors (CLRs) are among the PRRs (Sasai & yamamoto.,2013).

2.7.1.1.Epithelial cells:

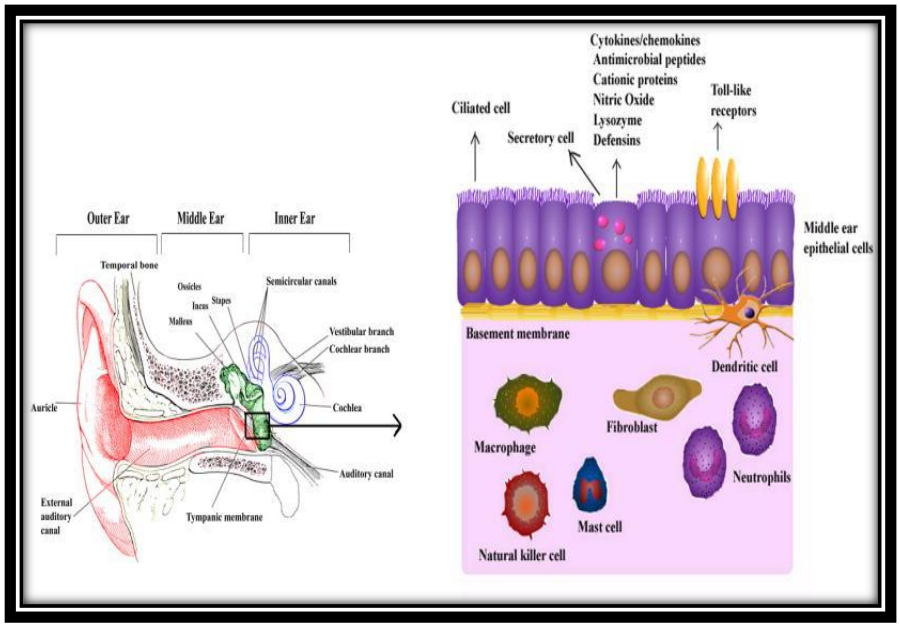
The physical barrier that guards against injury and infection is made up of closely similar epithelial cells, such as the skin and the sheet membranes that line the digestive tract, genitourinary tract, and respiratory tract (Medzhitov.,2021).

The epithelial surface also acts as a biochemical barrier by manufacturing and secreting compounds that capture or kill bacteria (epithelial chemicals) as shown in figure (2-3) (Mittal *et al*.,2014). Biochemical secretions like as mucus, sweating, saliva, tears, and earwax are able to trap and destroy potentially pathogenic microbes (Purnamasari&Hidayat.,2023).

The presence of the mucociliary apparatus, the trapping function of mucous glycoproteins and surfactants, the ability to secrete innate defense molecules such as defensins, interferons, lactoferrin ,nitric oxide, and antibody production via the adaptive immune response are all features of the middle ear epithelial lining.

Middle ear epithelial cells have PRRs similar to TLRs, which aid in pathogen detection via PAMPs (Jin *et al*.,2006).

The middle ear epithelial cells are principally responsible for the production of beta defenses, which are charged proteins having antibacterial activity against a wide variety of viruses, bacteria, fungi, and protozoa. The development of a hole within the microbial membrane is assumed to be their primary antibacterial mechanism. Some defenses, on the other hand, have been shown to induce pro-inflammatory cytokines/chemokines, operate as chemo attractants for neutrophils, mast cells, T cells, and dendritic cells, and directly block bacterial toxins (Yang *et al*.,2007&Patton.,2018).



Figure(2-3): Middle ear innate immunity (Mittal *et al*.,2014).

2.7.1.2.Lysozyme:

Various human airway cells, including surface epithelial cells, have been demonstrated to generate lysozyme, an antibacterial innate immune protein that destroys bacterial cell wall peptidoglycan (Parker& Prince., 2011).

It is present in the tubotympanum of animals and has an important function in OM. Lysozyme kill *S. pneumoniae* in a synergistic way, lysozyme M knock-out mice are more affected by *S. pneumoniae* infection, indicating that lysozyme plays a role in host defense (Lim *et a.l*,2000;Lee *et al.*,2004;Shimada *et al.*,2008).

2.7.1.3.Neutrophils :

Neutrophil participation in OM has received significant attention in recent years. The most prevalent leukocytes are neutrophils, also known as polymorph nuclear granulocytes (PMNs), which serve as the first line of defense against invading pathogens(Arazna *et al.*,2013).

Recent studies on acute OM induced by Nontypeable *Haemophilus influenzae* shown that neutrophil extracellular traps (NETs) are formed by PMNs in response to bacterial infection, by establishing a niche within the middle ear chamber (Simon *et al.*,2013).

NETs are theorized to influence bacterial persistence (rather than clearance),in the chinchilla infection model, NETs were found to be positively linked with greater bacterial burdens in middle ear fluids and surface-attached communities (Yipp&Kubes.,2013). Parameters such as neutrophil-lymphocyte ratio (NLR) and platelet-lymphocyte ratio (PLR) are used as inflammatory markers, they can be calculated by using the routine values in the complete blood count tests (Seo *et al*., 2015).

Recent studies reported that NLR and PLR values have both prognostic and diagnostic values in diseases accompanied by acute and chronic inflammation such as sudden hearing loss, cardiovascular diseases, systemic lupus erythematosus, acute appendicitis(Ha *et al*.,2019) .

Therefore, we hypothesized that changes in complete blood count parameters could be seen in OME, in which inflammatory etiology has played a role. Since NETs are unable to remove otopathogens, they may contribute to the establishment of persistent bacterial populations in the middle ear (Hong *et al*.,2009).

2.7.1.4.Macrophage :

Macrophages play an important part in the middle ear's innate immunity also macrophages have been found to be a prominent cellular component of human middle ear effusions (Davies *et al*.,2013) .

Middle ear macrophages isolated from human effusions release suppressive factors that cause lymphocytes to be less sensitive to a variety of mitogens ,this may contribute to the chronicity of OM by increasing weak bacterial clearance (Bhutta *et al*., 2020).

It has also been shown that macrophages recruited to the middle ear are functional, capable of discriminating phagocytosis and intracellular bacterial death (Mittal *et al*.,2014).

2.7.1.5. Cytokines:

Cytokines are tiny proteins that have a role in cell signaling , endothelial cells, macrophages, monocytes, and lymphocytes release them, and they can operate in an autocrine, paracrine, or endocrine way, cytokines are classified into several

families, which include interferons, interleukins, colony-stimulating factors, and tumor necrosis factors (Low *et al* .,2023).

Interleukins are also cytokines and their function is to communicate between immune cells (leukocytes) with each other so that their joint action is directed towards resistance to the pathogen or resistance to tumor cells. Interleukins initiate a response by binding to high-affinity receptors located on the surface of cells. Moreover, some types of interleukins, such as IL-1β and IL-6, along with TNF-α, fight infections and other processes in the immune system. These include, for example, raising the temperature, stimulating blood circulation, and increasing cell permeability to blood (Boyle., 2005).

Cytokines such as interleukin IL-6 are found at a very small rate in the blood in the picomole concentration range (10-12 M) and can increase by 1000-fold during illness or infection. Its spread is due to the fact that it is produced in cells distributed throughout the body, this is in contrast to the hormones secreted by certain organs in the body (such as insulin, which is secreted in the pancreas), and it appears that almost all cells with nuclei, including macrophages, have a high ability to produce Interleukin 1, Interleukin-6, and TNF-α (Brocker *et al*.,2010).

Increased levels of pro-inflammatory cytokines in the blood (e.g., interferon, interleukin (IL-1B, IL-6, IL-12) are linked to pulmonary inflammation and widespread lung involvement (Coperchini *et al*.,2020).

2.7.1.5.1. Interleukin-12:

IL-12 is a pro inflammatory cytokine that has a variety of impacts on both innate and adaptive immune responses also it can be produced by several different cell types including monocytes, macrophages, dendritic cells, neutrophils, and B cells

(Schwarz & Carson III .,2022). IL-12 (interleukin 12) was initially identified as a natural killer stimulating factor in 1989. IL-12 is a heterodimeric cytokine composed of a 35-kd light chain (p35 or IL-12A) and a 40-kd heavy chain (p40 or IL-12B) (Wojno *et al*.,2019).

In response to microbial infections monocytes/macrophages and dendritic cells generate interleukin-12 ,IL-12 is also vital in the biological control of lymphocytes (Yang *et al*., 2019). IL-12 is essential for resistance to infections, particularly those caused by bacteria or intracellular parasites, on which phagocytic cell activation is effective (Hamza *et al*.,2010).

The capacity of IL-12 to activate T and NK cells has made it a desirable option for overcoming immunosuppressive microenvironments found in several types of cancers (Zhao *et al*.,2019). Cases with genetic abnormalities related to IL-12 signal transduction are associated with an increased risk of bacillus Calmette-Guerin and environmental mycobacteria infection (Wang &Liao.,2023).

2.7.1.5.2. Interleukin -13:

IL-13 have a wide range of biological activity on mononuclear phagocytic cells, endothelial cells, epithelial cells, and B cells (Wu *et al*.,2020).IL-13 promotes mucus hyper secretion and nonspecific airway hyper reactivity (AHR) via a hormonal mechanism, and its production leads in the typical airway metaplasia of asthma, with the replacement of epithelial cells by goblet cells (Müller *et al*.,2024).

The success of IL-13-targeting treatments in this endotype supports the relevance of IL-13 in asthma presentations with a strong IL-13 signature. Both cytokines (IL-4&IL-13) have a common receptor component (the α subunit), their actions on immune cells are comparable, however IL-13 plays a more prominent

role in allergic inflammation by driving bronchial hyperreactivity and mucus overproduction (Foerster & Molęda .,2020).

IL-13 also stimulates B cell isotype flipping to create IgE. IL-13 is an essential mediator of allergic asthma in the mouse model ,and has been found to stimulate eosinophil migration to the airways (Lacy .,2017). IL-13 has also been linked to the development of OME (Smirnova *et al*.,2005).

Discovered this inflammatory cytokine in 25.9% (7/27) of effusions. It was also shown that IL-4 and IL-13 showed a strong positive connection with mucin content in MEEs. In an allergic airway inflammation model, it has been demonstrated that suppressing both IL-4 and IL-13 may be required to decrease mucus production and allergic inflammation (Donlan *et al* .,2023).

2.7.1.5.3. Macrophage inflammatory protein-1 alpha (MIP-1α):

MIP-1α is a macrophage-secreted chemokine. Its physiologic roles include attracting inflammatory cells, wound healing, stem cell repression, and effector immune response maintenance. It causes bone deterioration by activating bone resorption cells (Li *et al*,.2022) .

Because of its biological role of triggering an inflammatory response marked by neutrophil infiltration, this protein has been called macrophage inflammatory protein (MIP), Monocytes, T lymphocytes, B lymphocytes, neutrophils, dendritic cells, and natural killer cells are known to secrete MIP-1α (Bhavsar *et al* .,2015).

It has strong chemo attractant qualities for monocytes and lymphocytes and promotes histamine release from basophils and mast cells . MIP-1 was found in 9 of 11 MEE patients (Chonmaitree., 2004).

MIP-1 is anticipated to have a crucial role in the middle ear microenvironment, particularly in basophil function. This mediator, which is produced by monocytes and stimulates histamine release and monocyte chemotaxis, has the potential to be relevant in both acute and chronic OM (Juhn *et al*.,2008).

2.7.2.Adaptive Immunity:

The adaptive immune response is antigen-specific, resulting in pathogen-specific responses mediated by B and T cells. Thus, B cells participate in the humoral immune response, whereas T cells participate in the cell-mediated immunological response (Dabravolski *et al*.,2023).

T-cell receptors (TCRs) and B-cell receptors (BCRs), which are present on the surfaces of T-cells and B-cells, are important molecules in the adaptive immune response. These receptors are heterodimers (Minervina *et al*.,2019).

Actively acquired immunity is long lasting, although it can be modify by antigenic changes in the infecting microorganism, passively acquired immunity only provides transitory protection. Passive immunity may be passed to the fetus via the passage of maternal antibodies across the placenta (Clark *et al*.,2021).

The complexity of the upregulation of specific inflammatory cells and mediator secretion varies in response to the microbial otopathogens involved and the child's gender, with *Moraxella* and *Haemophilus* species tending to stimulate more inflammatory mediators in the middle ear (Enoksson *et al*.,2020).

2.8.Risk factor of Otitis Media :

Numerous host and environmental variables have a major impact on the risk of OM ,Young age, male sex, race and ethnicity, genetic factors and a family history

of OM, craniofacial anomaly such as cleft palate, atopy, immunodeficiency, upper respiratory tract infections (URIs) and adenoid hypertrophy (Todberg *et al*.,2014).

The laryngopharyngeal refluxare is a host factors that increase the risk of OM, low socioeconomic status, exposure to cigarette smoke, having elder siblings, day-care attendance, and usage of a pacemaker are also environmental variables that enhance the incidence of OM (De Hoog *et al*.,2014 ; O'Reilly *et al*,2015; Brennan-Jones *et al*,2015).

Breastfeeding is widely known to be beneficial in preventing OM, breastfeeding protects against OM over the first two years, and the protection is stronger for those who were exclusively breastfed and those who were nursed for an extended period of time (6 months) (Uhari *et a.l*,1996; Pamukcu *et al*.,2023; Gonzalez-Lamberth.,2023).

Current recommendations include avoiding tobacco smoke exposure, exclusively breastfeeding for 6 months, and discussing other lifestyle changes such as avoiding supine bottle feeding, reducing pacifier use, and considering alternative child care arrangements (for example, with smaller groups or using a child minder) (Nelson.,2012).

2.9.Antibiotic resistance:

Antibiotic resistance is a serious worldwide health problem currently, drug-resistant bacteria are quickly evolving and spreading, posing a danger to our capacity to treat common infectious microorganisms. One of the primary causes of drug resistance is the random use of commercial antibiotics(Acharjee *et al*.,2023).

Antibiotic-resistant bacteria pose a serious threat to public health and can result in increased morbidity, mortality, and medical care costs, when bacteria adapt and

survive in the presence of antibiotics, they acquire antibiotic resistance. The frequency with which antibiotics are taken is associated with the development of resistance (Pal *et al*.,2020).

Because several antibiotics are in the same class of drugs, resistance to one antibiotic agent can develop to resistance to the whole class, resistance that develops in one organism or area can spread quickly and unexpectedly, for example, through the interchange of genetic material between different bacteria, and can impact antibiotic treatment of a wide range of diseases and infections. Drug-resistant bacteria can spread in human and animal populations via food, water, and the environment, and transmission is impacted by commerce, travel, and both human and animal migration (Mirzayev *et al*.,2021).

2.10.Medical Plants:

Different plants components decoctions have been used to cure an infections (Ferdinand *et al*.,2023). Natural chemicals have been widely investigating in search of creative therapeutic improvements (Chandra *et al*.,2017).

Plants have been utilized as medicine for over 5000 years as a source of antibiotics, antineoplastic, analgesics, and cardio protective agents, among other things. Humans have recently used natural substances to fight against diseases. In poor nations, 70-90% of the population still uses ancient remedies based on plant extracts. Secondary metabolites are the most potent and promising constituents of plants, on which humans depend (Chen.,2015; Brown& Wright.,2016; Newman& Cragg.,2016).

2.10.1.Olive oil:

The olive family (Oleaceae) is a medium-sized group of woody plants comprising 28 genera and ca. 700 species, distributed on all continents (except Antarctica) in both temperate and tropical environments , Oleaceae is currently divided into five tribes, Myxopyreae, Jasmineae, Forsythieae, Fontanesieae and Olea europaea , the latter being subdivided into four subtribes (Oleinae, Fraxininae, Ligustrinae, and Schreberinae) (Dupin *et al*.,2020).

Olea europaea subsp. europaea is a distinctive element of the Mediterranean Basin (MB) flora and it can be found either as cultivated (subsp. europaea var. europaea) or as wild olive (subsp. europaea var. sylvestris)tree(Mariotti *et al*.,2023).

Olive belongs to the Family Oleaceae ,the Genus Olea and the Species Olea europaea (Hussain *et al* .,2021), extra virgin olive oil (EVOO)are popular Mediterranean diet ingredients because they have been linked to positive nutritional benefits,(Di Nunzio *et al*.,2018).

The majority of recent researches have focused on the health benefits of EVOOs. Several epidemiological studies have found a negative relationship between olive oil consumption and the development of certain conditions such as cancer or cardiovascular disorders. Other studies have linked EVOO consumption to a decrease in the age-related development of chronic inflammatory diseases like asthma and intestinal inflammation. Furthermore, EVOO bio phenols have been linked to the modification of the expression of atherosclerosis related genes. EVOOs also shown anti-inflammatory effect in preclinical tests, which was connected to the inhibition of COX enzymes,(celano *et al*,2018;Emma *et al*,2021).

Finally, the phenolic fraction of olive oils demonstrated chemo preventive effects against several types of cancer , while secoiridoids added to cancer therapy (in combination with other chemotherapy drugs) demonstrated synergistic effects in reducing tumor cell proliferation. Oleocanthal has lately been linked to a wide range of biological actions against breast cancer. This chemical has been studied as a possible therapeutic approach in the treatment of hormone dependent breast cancer, and it has been shown to reduce cell proliferation and tumor development in a number of cancer types,(Ayoub *et al*,2017.; Reboredo-Rodríguez *et al*,2018).

The olive oil have antibacterial activity against various pathogenic bacteria , effects of the oil against pathogens were associated with reduction of intracellular ATP concentrations, cell membrane depolarization, a decrease in bacterial protein content, and leakage of the cytoplasm (Gordon *et al*.,2001).

2.10.2.Bitter Almond oil:

Armeniaca sibirica (L.) Lam, sometimes known as wild apricot, belongs to the Rosaceae family's Armeniaca genus. It is a deciduous perennial tree that is mostly cultivated in Asia, Europe, and America,(Lim&Lim.,2012). The dried ripe seed of *Prunus armeniaca L., Armeniaca sibirica (L.) Lam var. sibirica, Prunus mandshurica (Maxim.) Koehne,* and similar species is known as bitter almond. Apricot seed has a wide variety of biological properties as a traditional eastern medicine, including antihyperlipidemia, anti-inflammatory, anticancer, antioxidant, antibacterial, antiasthmatic, analgesic, preventing heart disease, atherosclerotic, and much more (Abtahi *et al*.,2008; Korekar *et al*.,2011).

Bitter almond oil has been defined as a fatty oil and an essential oil; the former is composed mostly of unsaturated aliphatic acids, while the latter is composed of volatile components. The leftover (which includes the essential oil) is commonly

discarded as waste after pressing the fatty oil from the dry powder of bitter almond (Atapour& Kariminia.,2011). Furthermore, apricot has antibacterial action against dermatological diseases such as acne vulgaris, as well as antidandruff activity (Kamel *et al*.,2018).

2.10.3. Gas Chromatography:

Chromatography is the term used to describe a separation technique in which a mobile phase carrying a mixture is caused to move in contact with a selectively absorbent stationary phase. It also plays a fundamental role as an analytical technique for quality control and standardization of phyto therapeuticals (Zeki *et al*.,2020).

Gas Chromatography is used in the separation and analysis of multi component mixtures such as essential oils, hydrocarbons and solvents. Various temperature programs can be used to make the readings more meaningful; for example to differentiate between substances that behave similarly during the GC process. Intrinsically, with the use of the flame ionization detector and the electron capture detector (which have very high sensitivities) gas chromatography can quantitatively determine materials present at very low concentrations (Stettin *et al*.,2020).

Plants are a rich source of secondary metabolites with interesting biological activities. In general, these secondary metabolites are an important source with a variety of structural arrangements and properties. Gas chromatography - specifically gas-liquid chromatography - involves a sample being vapourised and injected onto the head of the chromatographic column. The sample is transported through the column by the flow of inert, gaseous mobile phase. The column itself

contains a liquid stationary phase which is adsorbed onto the surface of an inert solid (Rontani.,2022).

The principle of gas chromatography is adsorption and partition. Within the family of chromatography- based methods gas chromatography (GC) is one of the most widely used techniques. GC-MS has become a highly recommended tool for monitoring and tracking organic pollutants in the environment. GC-MS is exclusively used for the analysis of esters, fatty acids, alcohols, aldehydes, terpenes etc.(Zhu *et al*.,2021).

It is the key tool used in sports anti-doping laboratories to test athlete's urine samples for prohibited performance enhancing drugs like anabolic steroids. Several GC-MS have left earth for the astro chemistry studies. As a unique and powerful technology the GC-MS provides a rare opportunity to perform the analysis of new compounds for characterization and identification of synthesized or derivatized compound (Putri *et al*.,2022).

Chapter Three

Materials and Methods

3. Materials and Methods

3.1. Materials

3.1.1.Equipment and Laboratory apparatus:

Table(3-1): This table shows the laboratory tools used in this study and their manufacturer

Instruments and equipment	Company
Autoclave	Stermite-Japan
Bunsen burner	Reflecta –South Africa
Centrifuge	Hettich EBA20- Germany
Compact vitek2 system	Biomerieux -France
Conical flasks	BBL -U.S.A
Cylinders	Marienfeld - Germany
Electric oven	Memmert – Germany
ELISA washer and incubator	Biomatrix - India
Flasks	Marienfeld - Germany
Forceps	HiMedia - India
Graduate pipettes (5-10) ml	Marienfeld - Germany
Hot plate	Stuart- England
Incubator	Memmert, Germany
Light microscope	Olympus-Japan

Micropipette injector	**Eppendorf - Germany**
Microwave	**Gosonic-China**
pH. Meter	**K.G. – Germany**
Plastic rack	**Meheco-china**
Sensitive analytical balance	**Tartorus - Germany**
Spectrophotometer	**Tuder-Korea**
Stander wire Loop	**Himedia- India**
Stop watch	**Junahans - Germany**
Swelab Alfa Plus hematology analyzer	**Boule- USA**
Turbidity meter	**Lohand- China**
Vortex mixer	**Heidolph-Germany**
Water Distiller	**GFL -Germany**
Water bath	**Kottermann-UK**

3.1.2.Disposable Materials

Table(3-2):Single use material

Material	Company-source
Cotton	HDA-China
Cotton swap	Amies - China
Cotton swap with transport media	Amies – China
Cover slide	Sail Brand –China

Disposable loop	HiMedia – India
EDTA tube	AFNA-Dwaspo – Jordan
Eppendorf tube	Eppendrof-Germany
Filter paper	Schleizer and Schuell – Germany
Gel tube	Marienfeld –Germany
Latex Gloves	Broche-Malaysia
Micropipette tips	Slamd –Germany
Microscope slide	Sail Brand –China
Petri dish	Afco-Dipo-Jordan
Plain tube	AFco -Dwaspo - Jordan

3.1.3.Chemical materials:

Table (3-3) shows the chemical materials use in the current study

Chemical and biological material	Company
Hydrogen peroxide (H2O2) Oxidase reagent Glycerol	B.D.H-England
Chemical materials α-naphthol, KOH, methyl red, tetrametheyl-P-paraphenylene diamine dihydrochloride , HCL 99%ethanol,H_2O_2, *P*-dimethylaminobenzaldyde,	B.D.H.-England Fluka chemika-Switzerland

Gram's stain set: 1.Crystal violet 2.Ethyle alcohol 3.Iodin 4.Safranin stain	Crescent -Saudi
Kovas reagent	Himedia -India
Normal saline	Pioneer -Iraq

3.1.4.kits:

Table (3-4): The kits used in the study

Kits	Company-source
Human interleukin -12(IL-12)	Elabscience-USA
Human interleukin -13(IL-13)	Elabscience-USA
Human macrophage inflammatory protein 1 alpha(MIP-1 α)	Elabscience-USA
Vitek2gram positive **Vitek2 gram negative** **Vitek2 Antimicrobial susceptibility Test**	Biomerieux-France

3.1.5.Culture media:

Table (3-5):shows the cultures media which are used to grow and isolate bacteria in current study

Medium	Manufacturer (State)
Agar-agar	Mast Lab-England
Blood agar	Himedia-India
MacConky agar	Himedia - India
MR-VP broth	Diffco -USA
Muller Hinton agar	Himedia - India
Nutrient agar	Himedia - India
Nutrient broth	Himedia - India
Peptone broth	Mast Lab-England
Urea agar base	Himedia - India

3.1.6. Antibiotic discs:

The antibiotic discs listed in Table (3-6) were used in the antibiotic sensitivity test.

Antibiotic	Symbol	concentration	manufacturer
1.Tetracycline	TE	10 mg	BioMerieux - France
2.Ciprofloxacin	CIP	15 mg	BioMerieux - France
3.Ceftazidime	CTX	30	BioMerieux – France

4.Amikacin	AK	10	Bioanalyse-Turkey
5.Azithromycin	AZM	15	Bioanalyse-Turkey
6.Meropenem	MEM	10	BioMerieux – France
7.Levofloxacin	LEV	5	Bioanalyse-Turkey
8.Amoxicillin/Clavulanic acid	APX	30	BioMerieux - France

3.2.Methods:

3.2.1.Sampels collection:

3.2.1.1.Collection ear swab:

This study occurred on groups of patients 101 who had otitis media from E.N.T clinic for Al-Hassan , Al-Hussein medical city and outpatient clinic ranging in age from 1 to 70 years , of both sexes, male and female for the period of time March 2022 to May 2022. According to the questionnaire form in appendix No. 1, which covered the patient's name, age, sex, location, and chronic conditions , signs, symptoms . Then, Sterile cotton swab were used for the purpose of collecting ear fluid from the ear of the patients. The swabs were then grown on appropriate culture media and then incubated for 18-24 hours at 37°C (Parween *et al.*,2022).

3.2.1.2. Collection of blood samples :

A total of 5 ml of fresh blood was obtained from each patient through disposable syringes, 4 ml was transport to gel tube permitted to clot at room temperature, and then was spun at 2000 rpm for two minutes. Serum was isolated and pour into 0.5 mL aliquots in 4 Eppendorf tube before being stored under freeze at -20 C° until use. Another 1 ml of blood was put in EDTA tube for hematological testing as shown in figure (3-1) (Vaught.,2006).

3.2.1.3. Control group :

Blood samples and ear swabs were collected from 49 healthy individuals without any symptoms of otitis media ,chronic or acute infection, and immunological diseases as control group after the physical and medical examination.

3.2.1.4. Ethical Approval:

The study was conducted in conformity with the moral guidelines that the Helsinki Declaration served as the foundation for. In December 16, 2021, a local ethics committee examined and approved the research protocol using the reference number (4347) to receive this permission, also all patients information and their approval have been take through questionnaire forma in appendix one.

3.2.1.5. Exclusion Criteria:

All patients with one of the chronic diseases (such as high blood pressure and diabetes) and autoimmune diseases were excluded from this study.

3.2.1.6.Study design:

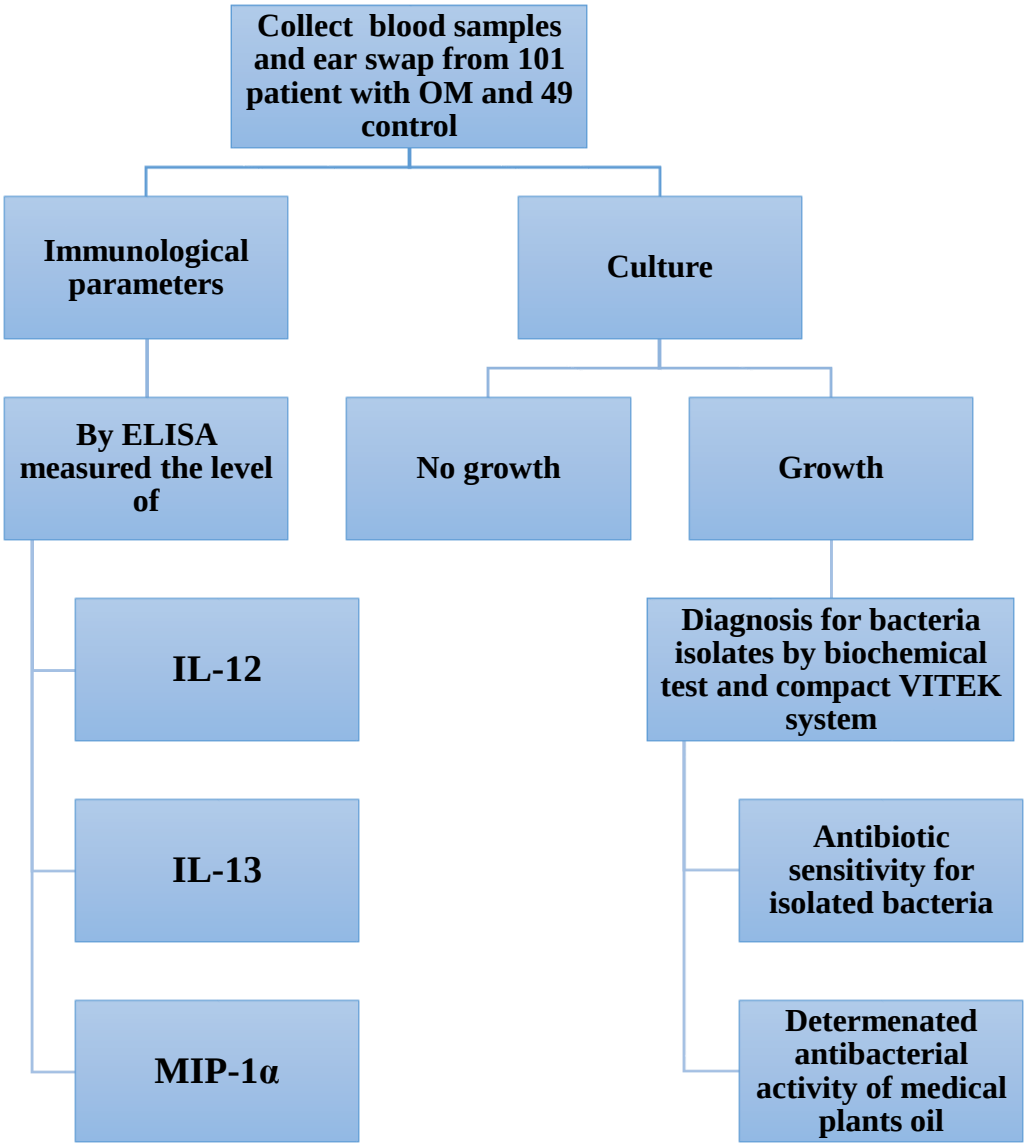


Figure (3-1): Study design

3.2.3.Preparation of Reagents and solution:

3.2.3.1. MacFarland solution 0.5

First add 0.5 mL of 1% dehydrated barium chloride solution to a 100 mL dish and then raise the volume to 100 mL with 1% sulfuric acid. The final barium sulfate solution is known as the 0.5 MacFarland standard opacity solution.This solution was used to arrive at an approximate number of bacterial cells estimated (0.5X 10) one cell per milliliter (Malekpourzadeh *et al* .,2023).

3.2.3.2. Catalase reagent

The reagent was prepared by mixing 3 mL of hydrogen peroxide H2O2 in 100 ml of distilled water and using it to detect the ability of bacteria to produce catalase enzyme (Tille.,2015& Al-Rubaeaee *et al* .,2023).

3.2.3.3. Gram stain

This ready-made dye consisting of crystal violet, iodine solution and sufranine was used to study the phenotypic properties of isolated bacteria and for the purpose of differentiating bacteria into Gram-negative or positive dye (Tripathi& Sapra.,2020).

3.2.3.4. Methyl red reagent

Prepared by dissolving 0.1 of methyl red dye in 300 ml of 95% ethyl alcohol, then the volume was completed to 500 ml using distilled water and was used to detect the total decomposition of glucose sugar (MacFaddin.,2010).

3.2.3.5. Vogue's- Proskauer reagent

It consists of two solutions:

• Solution (A) KOH: It was Prepared by dissolving 40 g of the substance in90 ml of distilled water and then complete the volume to 100 ml

• Solution (B) Alpha-naphthol: It was Prepared by dissolving 5 g of the substance in 90 ml of ethyl alcohol at a concentration of 99% and then complete the volume to 100 ml using the same alcohol. This reagent was used to investigate the susceptibility of bacteria to the fermentation of glucose sugar and produced Acetyl Methyl Carbinol in Voges-Proskauer test (MacFaddin.,2000).

3.2.3.6. Oxidase reagent:

Prepared freshly by dissolving 1 g of tetraphenylene para Amine dihydrochloride in 100 ml of distilled water in clean, dark, sterile vial . it used to investigate the ability of bacteria to produce the oxidase enzyme (Tille.,2015).

3.2.3.7. Normal saline solution:

To prepare normal saline, 0.85 grams of sodium chloride were taken and dissolved in 90 ml of deionized water. After that, it was sterilized in an autoclave at 121 °C for 15 minutes and used to prepare a bacterial suspension (Suwansaksri *et al*., 2003).

3.2.4.Preparation of Culture Media:

All solution culture and diagnostic media were prepared according to the manufacture instructions and were sterilized by using autoclave at 121 for 15 minutes , after adjusting the PH for 2-7, additional solutions that are destroyed by heat were sterilized using 0.22 Mm Millipore filter paper.

3.2.4.1. Blood Agar Medium:

Forty gram of the medium powder was dissolved in a liter of distilled water, then it was sterilized in the autocleave and let it cool 40- 45C. then 5% of human blood was added, this enrichment medium was used to isolate bacteria and todetect the ability of bacteria to hemolysis red blood cell (Niederstebruch *et al*.,2017).

3.2.4.2. Urea agar:

This medium was prepared by dissolving 24 g of basic urea agar in a liter of distilled water and autoclaved, then left to cool at 45C and then 50 ml of Filter sterile urea solution (40%) was added and poured into sterile tubes. This medium was used to detect the susceptibility of bacteria to produce the urease enzyme, which decomposes urea into ammonia and carbon dioxide (McFadden., 2000).

3.2.4.3. MacConkey agar medium:

It was prepared by dissolving 51.5 g of the medium in 1 liter of distilled water, boiling it until completely dissolved, autoclaving it, and left it to cool to 40-45C and pouring it into Petri dishes. It was used to isolate and separate lactose fermenters from non-lactose fermenters gram-negative bacteria (Jacob *et al*.,2020).

3.2.4.4. Nutrient agar medium:

It was Prepared by dissolving 28 g of the media in 1 liter of distilled water, then autoclaved and left to cool to 45-50 C and poured into petri-dishes and then kept in refrigerator until use. This media use for conserving bacterial isolates (MacFaddin.,2000).

3.2.4.5. Nutrient broth:

It was prepared by dissolving 13 g of media in 1 liter distilled water, poured into tubes(5 ml/tube), then autoclaved ,let it cool down and kept in refrigerator until use. the purpose of used this media was activation of bacteria (MacFaddin.,2000).

3.2.4.6. Muller Hinton agar:

It was prepared by dissolving 38 g of the medium in 1 liter of distilled water, boiling it until completely dissolved, and autoclaving it, then pouring into Petri dishes. It was used to test the biological activity of bacteria (Uwizeyimana *et al*.,2020).

3.2.4.7. Motility medium:

It was prepared by adding 0.5 g of agar into 100 ml of the Nutrient broth , pour into tubes (5 ml/tube), autoclave and left to solidifies vertically . This medium used for detection of bacterial mobility (Jain *et al*.,2020).

3.2.4.8. Mannitol salt Agar:

Prepare this medium according to the company's instructions by dissolving 5.51 g of the medium inA liter of distilled water, sterilize it with an autoclave, then leave it to cool to 45-50C .Then pour it in sterilized dishes. This medium was used as a differentiation medium to isolate and diagnose Gram-negative bacteria through its ability to ferment lactose.(Jacob *et al*.,2020)

3.2.5.Identification the isolated bacteria

3.2.5.1. Morphologic and microscopic identification:

The properties of bacterial isolated have been studied by showing the shape, texture, and size of bacterial colonies. Single pure colonies were picked up and

stained with gram stain to examined by microscope to identify their response to this strain and observed the shape , length under oil immersion and distinguish between Gram positive and Gram negative bacteria (Collee *et al*., 2006).

3.2.5.2. Biochemical tests

3.2.5.2.1 Catalase test:

This test was performed by transforming a bacterial colony aged 18-24 to a glass slide and then a drop of a 3% hydrogen peroxide reagent was placed on top of it. the appearance of bubbles is an indication of a positive test and bacteria have the ability to produce the enzyme catalase, which decomposes the H2O2 reagent into oxygen and water (Reiner.,2010).

3.2.5.2.2 Oxidase test :

A drop of the oxidase reagent was put on a clean filter paper, and the bacterial colony was transferred to the filter paper using a wooden stick. The formation of a violate pigment within 10-20 seconds confirms that the isolate was capable of producing oxidase enzyme. (Tille.,2015).

3.2.5.2.3 Indole test:

Young testing bacteria were administered into peptone water and cultured for 24-48 hours at 37°C. A few drops of Kovacs reagent were added to each tube and gently shaken. The formation of a pink ring indicated a positive result(MacFadden., 2000).

3.2.5.2.4.Coagulase test:

0.5 ml of human blood plasma was placed in sterile tubes, then a bacterial colony aged 18-24 hours was transferred by a sterile wooden stick to the tubes containing

plasma, and after mixing them well, incubated at 37 C temperature for 4 hours, plasma coagulation is evidence of a positive test (Becker *et al*., 2014).

3.2.5.2.5. Methyl red test :

The MR-VP medium was inoculated with the bacteria to be examined, after incubated for 24 hours at 37 C, 5 drops of methyl red reagent were added. when the red color appears, evidence of a positive test and that the bacteria have the ability to ferment glucose sugar and produce acids (MacFadden., 2000).

3.2.5.2.6.Voges –Proskauer test:

The MR-VP medium was inoculated with the bacteria and then incubated for 24 hours at 37 C ,then 0.6 ml of alpha-naphthol reagent solution and 0.2 ml of potassium hydroxide solution were added and mixed together in the test tube . The transformation of the color of the medium from yellow to Pink is evidence of a positive test (MacFadden., 2000).

3.2.5.2.7. Urease test:

After being injected onto the urea agar slant, the bacteria were left to grow for 48 hours at 37 degree, after 24 hours, if the color of the purple pink changed from medium to purple pink, the urease test was positive(Dahlén *et al*.,2018).

3.2.5.2.8. Hemolysis production test:

The bacteria were planted on the blood agar by the planning method and incubated for 24 hours at 37C in aerobic conditions to assess an isolated bacteria’s capacity to hemolysis red blood cells and to determine the type of hemolysin present around the bacteria colonies (Collee *et al*.,2006).

3.2.6. Identification of bacteria

Using Vitek-2 Compact System Bacterial isolates were biochemically diagnosed according to the results report of the Vitek 2 automated system according to the manufacturer's instructions (Biomerieux Company / France). The bacterial suspension was prepared by transferring a quantity of bacterial colonies that has been purified advance age (18- 24) hours to 3 ml of sterile saline sodium chloride solution at a concentration of (0.45%), and the turbidity was adjusted using 0.5 McFarland tube for Gram negative bacteria and 0.63,McFarland tube for Gram-positive bacteria, as negative bacteria are diagnosed and used Gram-staining (Gram Negative Identifier) (GNID) and Gram-positive bacteria Gram Positive Identifier (GP-ID) is a completely enclosed system and no addition is required any auto reagents. The card is placed on a cassette designed for use with the Vitek-2 system, Place it in the machine, fill it automatically in a vacuum chamber, seal it, and incubate it at 35.5.°C, and is automatically subjected to a colorimetric measurement (with a new reading) every 15 minutes for a maximum incubation period of 8 hours. Data were analyzed using the Vitek-2 database, which allows identification of the organism actively starting 180 min after incubation initiation (Karagöz *et al*., 2015).

3.2.7.Preservation of Bacterial Isolate:

3.2.7.1. Short term storage:

The diagnosed bacterial isolates were kept in the nutrient medium after being inoculated with bacterial isolates by stabbing method and incubated at 37 C for 18 hours, then transferred to the refrigerator at 4 C and the preservation process was repeated in order to maintain the vitality of the isolates and avoid contamination every 3-4 weeks (Zhgun *et al*.,2020).

3.2.7.2. Long term storage :

For the purpose of preserving bacterial isolates for a long time without any damage or change in genetic traits, use a sterile brain - heart infusion broth with 15-20% glycerol , after inoculation with bacterial isolates, incubated for 4 hours, then sealed tightly with a parafilm and kept at a temperature of -20 for (4-6) months (Al-Mayyahi.,2018).

3.2.8.Antibiotic sensitivity test:

The disc diffusion method was used to test the sensitivity of bacterial isolates to antibiotics according to (Miller et al.,2014). The tubes containing nutrient broth medium were cultured and incubated at a temperature of 37 for a period of 2-8 hours or until the appearance of turbidity, and then a comparison was made between the tubes containing the bacterial culture and the standard McFarland tube (0.5), where a saline solution was used to adjust the density of the tubes of the bacterial culture until it equals the density of the McFarland tube. After that, the cells of the bacterial suspension are spread on the Mueller-Hinton medium using a cotton swab in a homogeneous manner, then left to dry for a few minutes, then the antibiotic disc were placed on the surface of the plate using sterile forceps at the rate of five disc per plate, then the plates were incubated at 37 degrees for 18 hours. The results were read by measuring the diameter of the inhibition zone and comparing it with the standard diameters of these previously determined antibodies by (Miller *et al* .,2014).

3.2.9.Measurement of the interleukin-12 , interleukin-13 and Human Macrophage Inflammatory Protein 1 Alpha levels in the blood of patient with otitis media:

3.2.9.1.Test principle:

This ELISA kit uses the sandwich-ELISA principle, the ELISA is aquantitative analytical method that show antigen –antibody reactions through the color change obtained by using an enzyme linked and enzyme substrate that serve to identify the presence and concentration of molecules in biological fluids .The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to Human IL-12/IL-13/MIP-1α samples (or standards) are added to the micro ELISA plate wells and combined with specific antibody. Then, a biotinylated detection antibody specific for Human IL-12/IL-13/MIP-1alpha and a Avidin-Horseradish Peroxides (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Human IL-12/IL-13/MIP-1alpha, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by addition of stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 ± 2nm. The OD value is proportional to the concentration of Human IL-12/IL-13/MIP-1alpha . You can calculate the concentration of human IL-12/IL-13/MIP-1alpha in the samples by comparing the OD of the sample to the standard curve.

3.2.9.2. ELISA Kit component of IL-12/IL-13/MIP-1alpha :

Table(3-7): ELISA Kit component

Items	Items Specifications
Micro ELISA plate (dismountable)	8 wells ×12 strips
Reference standard	2 vials
Concentrated biotinylated detection Ab (100x) **Concentrated HRP conjugate (100x)**	1 vial, 120 µL 1 vial, 120 Ml
Reference standard& sample diluent	1 vial, 20 mL
Biotinylated detection Ab diluent	1 vial, 14 mL
HRP conjugate diluent	1 vial, 14 mL
Concentrated wash buffer (25x)	1 vial, 30 mL
Substrate reagent	1 vial, 10 mL
Stop solution	1 vial, 10 mL
Plate sealer	5 pieces

Product description	1 copy
Certificate of analysis	1 copy

3.2.9.3. Reagent preparation of IL-12,IL-13and MIP-1 α :

1- Before use, all reagents were brought to room temperature (18-25°C).

2- Wash buffer: To make 750ml of wash buffer, 30 ml of concentrated wash buffer was diluted with 720 ml of deionized or distilled water. If crystals develop in the concentrate, heat up it in a 40°C water bath and gently mix it until the crystals are totally dissolved.

3- The standard working solution: was centrifuged at 10,000xg for 1 minute. Add 1 mL of reference standard and sample diluent, let it stand for 10 minutes, then gently flip it several times. After it has completely dissolved, carefully combine it with pipette. This reconstitution yields a 1000 pg/ml working solution for IL-12 and IL-13 but with MIP-1 α the reconstitution yields a 1500 pg/ml working solution (or add 1ml of reference standard and sample diluent, let it stand for 1-2 minutes, and then vigorously mix with a vortex meter set at low speed). Bubbles formed during vortexing might be eliminated by centrifuging at a low speed. Then, if required, produce repeated dilutions. The following is the suggested dilution gradient: 1000,500,250,125,62.5,31.25,15.63,0 pg/ml for IL-12 and IL-13 while the MIP-1 α the suggested dilution gradient: 1500,750,375,187.5,93.75,46.88,23.44,0 pg/ml.

Note:

The dilution process was as follows: 7 EP tubes were collected, 500 ml of the 1000 pg/ml(for IL-12 and IL-13)or 1500 pg/ml for MIP-1 α working solution was added to the first tube, and the mixture was mixed to generate a 10ng/ml working solution. Pipette 500 ml of the solution from the former tube into the latter, as directed. The graphic below is for reference purposes only. Take note that the last tube is treated as a blank. Do not pipette the solution into it from the previous tube. The operating diagram is included on the next page.

4- Biotinylated detection Ab working solution: the appropriate amount (100 ml/well) was estimated before to the experiment. It is best to prepare somewhat extra than is calculated. Centrifuge the concentrated Biotinylated detection Ab for 1 minute at 800 x g, then dilute the 100x concentrated biotinylated detection Ab to 1x working solution using biotinylated detection Ab diluent (1:99).

5- HRP conjugate working solution: the needed amount (100ml/well) was estimated before to the experiment. It is best to prepare somewhat extra than is calculated. Centrifuge the concentrated HRP conjugate for 1 minute at 800 x g, then dilute the 100 x concentrated HRP conjugate to 1 x working solution with HRP conjugate diluent (concentrated HRP conjugate to 1x working solution with HRP conjugate diluent = 1:99).

3.2.9.4. Assay procedure

1- Wells for diluted standard, blank and sample was determined . 100ml each dilution of standard, blank and sample was added into appropriate wells. Cover the plate with sealer provided in the kit. It was incubate for 90 min at 37 C.

Note: solutions should be added to the bottom of the micro ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.

2 - Each well's liquid was decanted; do not wash. Each well filled with 100 cc of biotinylated detection Ab working solution right away. Replace the sealer on the plate. It was incubated for 1 hour at 37 C.

3- After decanting the solution from each well, 350ml of wash buffer was added to each well. Soak for 1 minute before aspirating or decanting the solution from each wall and patting it dry with clean absorbent paper. Repeat the washing process three times. It should be noted that a microplate washer can be utilized in this and other wash phases. Make the tested strips available for use immediately following the wash process. Do not let wells dry out.

4- Each well filled with 100 mL of HRP conjugate working solution. Apply a fresh sealant on the plate. Incubate for 30 minutes at 37°C.

5- After decanting the solution from each well, the wash procedure was repeated five times as in step three.

6- 90 mL of substrate reagent was added to each well. Replace the sealer on the plate. Incubate for around 15 minutes at 37C. Keep the plate away from direct sunlight. The reaction time can be lowered or prolonged depending on the actual color shift, but it cannot exceed 30 minutes. Preheat the micro plate reader for about 15 minutes before measuring the OD.

7- 50 mL of stop solution was poured to each well. It should be noted that the stop solution was added in the same sequence as the substrate solution.

8- The optical density (OD) value of each well was measured at the same time using a micro-plate reader set to 450 nm.

3.2.10. Biological activity of medical plants:

3.2.10.1. Determination of active compounds in oils:

3.2.10.1.1. Total Antioxidants Capacity Assay: The CUPRAC Method

Principle: (Apak *et al*., 2007**)**

Total antioxidants + Cu^{+2} ⟶ Cu^{+}

Cu^{+} + 2,9-dimethyl-1,10-phenanthroline ⟶ complex (λ_{max} at 450 nm)

Reagents:

1. Copper(II) chloride solution at a concentration of 10^{-2}M was prepared from $CuCl_2 \cdot 2. H_2O$ weighing 0.4262 g, dissolving in H_2O and diluting to 250 ml with water.
2. Ammonium acetate (NH_4Ac) buffer pH = 7.0 was prepared by dissolving 19.27 g of NH_4Ac in water and completed the volume to 250 ml.
3. Neocuproine (Nc){2,9-dimethyl-1,10-phenanthroline} solution at a concentration of 7.5 * 10^{-3}M was prepared by dissolving 0.039 g Nc in 96% EtOH, the volume was completed to 25 ml with ethanol.
4. The standard solutions of sample antioxidants were prepared at 1.0 * 10^{-3}M Torolox.

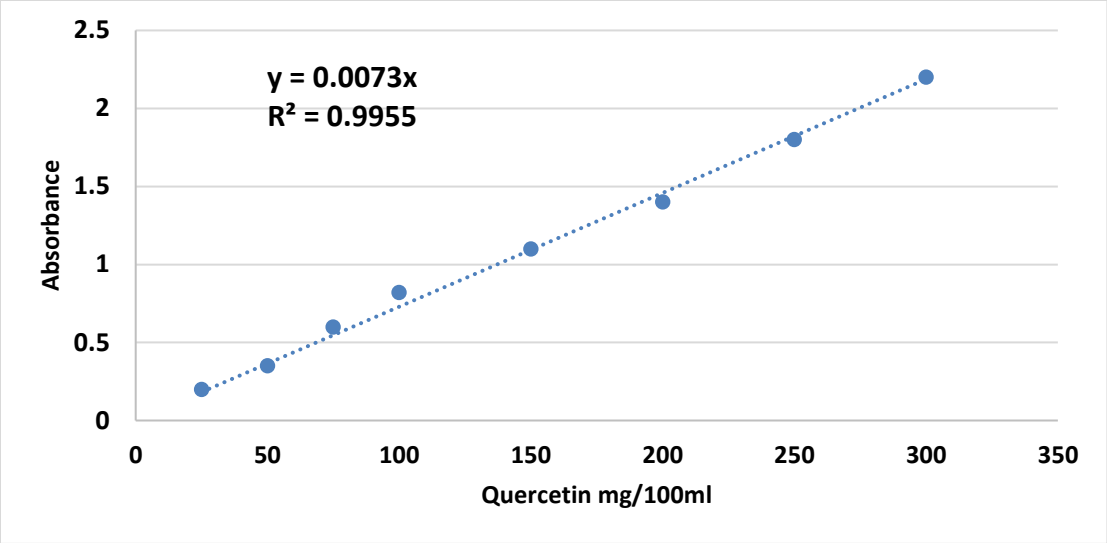
Reagents	Test	STD	Blank
Copper(II) chloride solution	1ml	1ml	**1ml**
Sample	50 µl	------	-------
Working standard solution	-------	50 µl	-------
D.W	-------	-------	**50 µl**
Neocuproine (Nc) solution	1ml	1ml	**1ml**
Ammonium acetate (NH_4Ac) buffer	1ml	1ml	**1ml**
Test tubes was mixed by vortex and incubated for 30 minutes at 37°C, after that the absorbance was read on a spectrophotometer at 450 nm.			

Calculation:

$$\text{Total antioxidants levels} = \frac{\text{A.test}}{\text{A.STD}} * Conc.of \quad STD \ \text{(mmol/l)}$$

3.10.1.2. Spectrophotometric determination of flavonoids

Quercetin was used to construct a calibration curve (standard solutions 25, 50, 80, 100, 150, 200, 250 and 300 mg/100ml in 80% ethanol (v/v)). The standard solutions or samples (0.5 ml) were mixed with 1.5 ml 95% ethanol (v/v), 0.1 ml 10% $Al(NO_3)_3$, 0.1 ml of 1 mol/l potassium acetate and 2.8 ml deionised water. In the blank, the volume of 10% $Al(NO_3)_3$ was substituted by the same volume of deionized water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm. Flavonoids were expressed as Quercetin (Ribarova.,2005&Sen.,2013).



Figure(3-2): stander curve for flavonoids

3.10.1.3. Determination of total phenolic content

The total phenolic content of the oils was estimated using Folin-Ciocalteu reagent. 1 mL of sample (1 mg/mL) was mixed with 1 mL of phenolfolin-ciocalteu reagent. After 5 minutes, add 10 ml of 7% sodium carbonate solution to the mixture, followed by the addition of 13 ml of deionized distilled water. Mix the solution well. The mixture was kept in the dark for 90 min at 23 °C, after which the absorbance was read at 760 nm. The total phenolic content was determined by extrapolation of the calibration curve prepared using gallic acid solution. TPC was expressed as Milligrams of gallic acid equivalents/g of oil sample.

3.10.1.4. Determination of total Tannin content:

Tannin contents were determined by the method of Broadhurst *et al*. 1978, using catechin as the standard compound. Where the volume was taken 400 µl of oil was added to 3 ml of vanillin solution (4%). in methanol) and 1.5 ml of concentrated hydrochloric acid. After 15 minutes of incubation the absorbance was read at 500 nm. Total tannin content was determined by extrapolation of the calibration curve prepared using catechin solution. Tannin was expressed as milligrams of catechin

equivalents/g of oil sample(Broadhurst.,1978; Paśko .,2019& Paramesha *et al.*,2023).

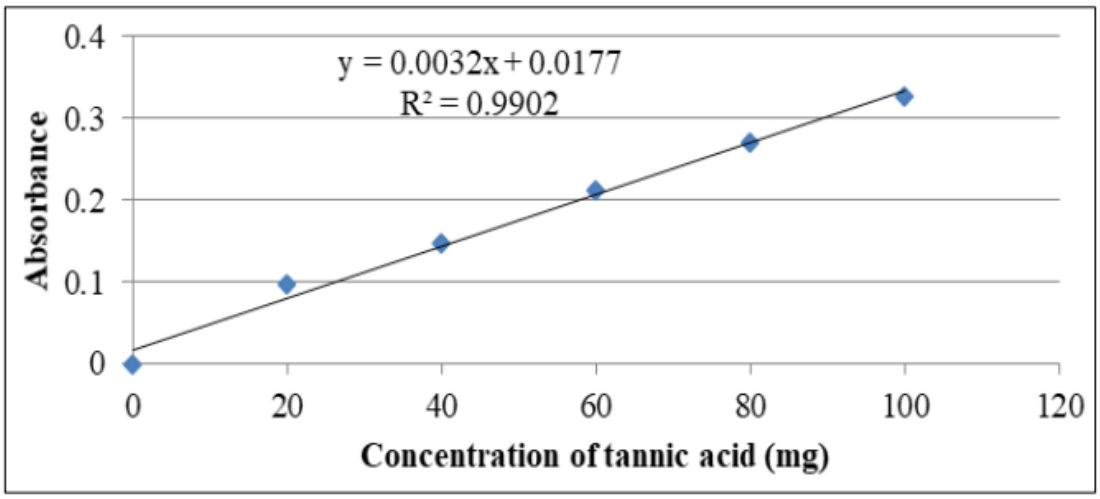


Figure (3-3): stander curve for Tannin

3.10.1.5. Determination of total Thiol

We offer a simple automated process for determining total thiols in oil based on Ellman's method. Thiol groups have been reported as the major responsible for antioxidative actions of plasmatic proteins. The conjugate base (R—S-) of a free sulfhydryl group is the target of DTNB in this reaction. TNB is the reaction's "hued" species, with a high molar extinction coefficient in the visible range. The molar extinction coefficient of TNB was originally reported to be 13,600 $M^{-1}cm^{-1}$ at 405 nm and pH 8.0.

Reagents:

1. Na_2HPO_4 was prepared by dissolving 0.5 g of Na_2HPO_4 in 100 mL of distilled water.
2. Ellman’s Reagent Solution :was prepared 1ml of DTNB in 50 ml of distilled water.

3. Glutathione (GSH) : prepared by dissolving 0.0307 mg of a GSH in a final volume of 50 ml of PBS .
4. Phosphate Buffer solution (PBS) : prepared by mixing 80 ml of D.W and 1.36 g of KH2PO4 and 0.14 g NaOH and completed to 100 ml of D.W.

Procedure:

Reagents	Test	Test Blank	Test Standared
Sample	10 µl	-------	-------
Na_2HPO_4	150 µl	150 µl	**150 µl**
Ellman'sReagent(DTNB)	50 µl	50 µl	**50 µl**
glutathione (GSH)	-------	-------	**10 µl**
D.W	-------	10 µl	------
Test tubes was mix and incubate at room temperature for 4 minutes. and then measure at 405 nm			

Calculation:

Calculate the amount and concentration of thiols in the sample from the molar extinction coefficient of TNB (14,150 $M^{-1}cm^{-1}$)(Costa.,2006).

$$Sample\ \ total\ \ thiol group\ \ levels = \frac{Absorbance}{d\ X \in} X\ D.F$$ (µmol/l)d = 1cm, ε =extinction coefficient = 14150 M-1cm-1, D.F = dilution factor = 11.2

3.10.1.6. Assay of total Tocopherol (vitamin E) in plant oils:

Principle:

Plasma total tocopherol was assayed by the method of(Quaife *et al*.,1949) it involves the Emmerie- Engel color reaction with ferric chloride and α,α-dipyridyl to give a red color (Christodoulou., 2022) as in method below:

Reagents:

1. Absolute ethanol.
2. α,α-dipyridyl : was prepared by dissolving 0.120 gm of α,α-dipyridyl in 100 ml of n-propyl alcohol.
3. Ferric chloride hexahydrate : was prepared by dissolving 0.120 gm of Ferric chloride hexahydrate in 100 ml of absolute ethanol. After the solution was kept in a dark brown or red glass bottle.
4. α-tocopherol standard (1µmol/L) was prepared by dissolving 2.0 mg of α-tocopherol in 100 ml of absolute ethanol.

Procedure:

Reagents	Test	STD	Blank
Absolute ethanol	0.6 ml	0.6 ml	**0.6 ml**
Sample	0.6 ml	------	-------
D.W.	-----	------	**0.6 ml**
STD	-----	0.6 ml	-------
Xylene	0.6 ml	0.6 ml	**0.6 ml**
Mixed well and centrifuged for 10 min at 3000 rpm.			
xylene supernatant layer	0.4 ml	0.4 ml	**0.4 ml**
α,α-dipyridyl was added and vortexed	0.4 ml	0.4 ml	**0.4 ml**
The 0.6 ml of this mixture was then pipetted into a cuvette and the absorption was measured spectrophotometrically at 460 nm against deionized water.			
ferric chloride	0.13 ml	0.13 ml	**0.13 ml**
Mixed thoroughly and absorption was again read at 520 nm spectrophotometrically exactly 1.5 min after addition of ferric chloride.			

Calculation:

$$\text{Conc. of test} = \frac{(\text{A}\,520 - 0.29\,\text{A}\,460)\text{test}}{\text{A}\,520\text{STD}} * \text{Conc. of STD}$$

3.10.1.7. Assay of ascorbic acid (vitamin C):

Principle:

Determination of ascorbic acid was carried out by the method of(Omaye *et al*.;1979) which involves a reduction of 2,6-dichlorophenolindophenol (DCIP) .

Ascorbic acid reduces the dye DCIP and causes a decrease in the absorption of the dye at 520 nm. The reaction was carried out within a pH range of 3.0–4.5.

Reagents:

1. TCA (5%)

i. Solution A: (0.1 M citric acid) Dissolve 21.01 g of citric acid in 1L of water.

ii. Solution B: (0.1 M sodium citrate) Dissolve 29.41g of $C_6H_6O_7Na_3.H_2O$ in 1L of water.

2. Citrate buffer (pH 4.2) (0.4 M): prepared by mixing 31.5 of solution A and 18.5 ml of solution B and dilute to 100 ml with D.W.

iii. citrate (pH 4.15): Dissolve 4.37 g of $C_6H_6O_7Na_3.H_2O$ in 100 ml of water.

iv. Dchlorophenolinddophenol (DCIP): Dissolve 0.1 g of Dchlorophenol-inddophenol in 100 ml of water.

3. Indophenols working solution: dilute 5 ml of (DCIP) to 50 ml with D.W. before using immediately.

Procedure:

Reagents	Test
5% TCA	**2 ml**
Sample	**1 ml**
Mix by vortex and centrifuge at 14,000 × g for 20 min	
Supernatant	**0.61**
citrate/ acetate buffer (pH 4.15)	**0.33 ml**
DCIP	**0.33 ml**

Calculation:

The absorbance was recorded against deionized water at 520 nm exactly after 30 s. A few crystals of ascorbic acid were added to bleach the dye by reducing it completely, and, the sample was read again. This value serves as the blank for the same sample. A standard curve, including a reagent blank, was constructed with the standards ranging between 0 and 20 µg of ascorbic acid per ml of 5% TCA. A change in absorbance (A) due to reduction of the dye by ascorbic acid in the sample was calculated from the following equation**:**

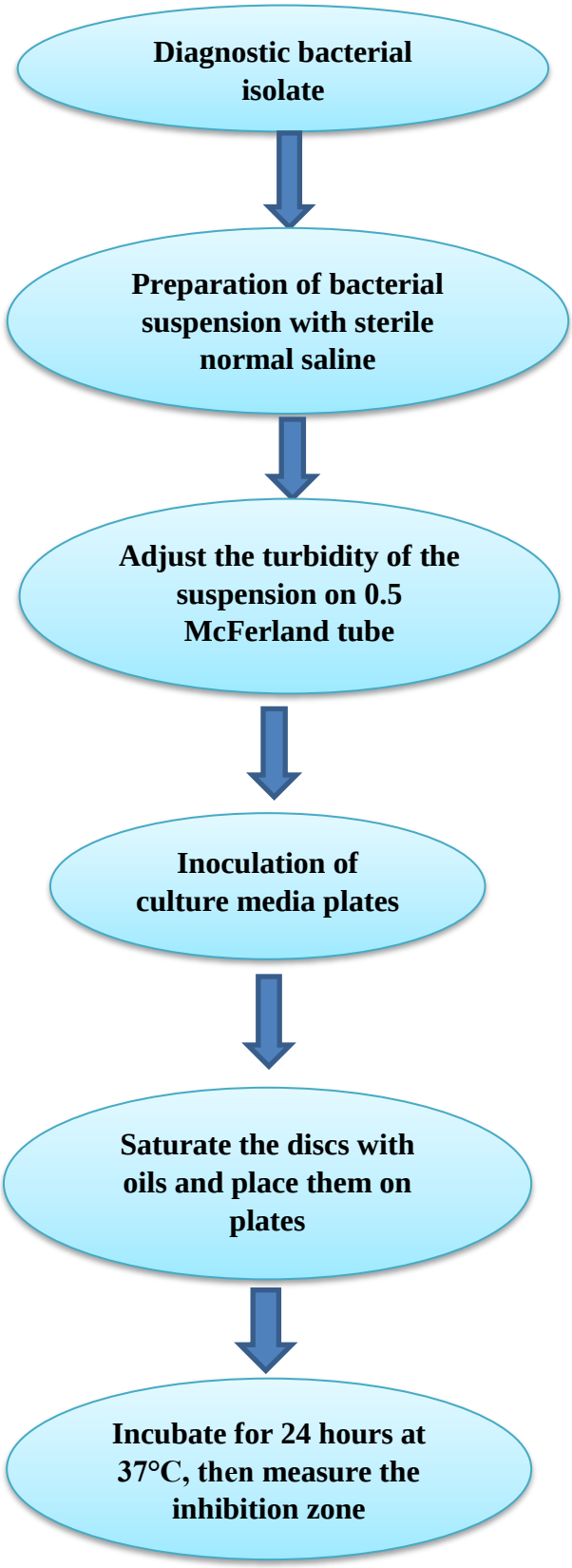
$$\Delta A = (RB - RBb) - (S - Sb)$$

Where RB is the absorbance of the reagent blank; RBb is the absorbance of RB after bleaching with ascorbic acid; S is the absorbance of the sample and Sb is the absorbance of S after bleaching with ascorbic acid. The concentration of the ascorbic acid in the sample was obtained by comparing ΔA with the standard curve and expressed as µg/ml of sample (Omaye *et al* .,1979& Okolie *et al*.,2014).

3.2.10.2.Biological activity of almond oil and olive oil:

The biological activity of almond and olive oil against bacterial isolates, were determined according to (Balouiri.;2016).The method of direct contact and spreading with discs was followed which suggested by (NCCLS) which can be summarized in the figure (3-7). To conduct this test, Muller-Hinten's medium is used with bacterial species. The medium is poured into Petri dishes with a diameter of 9 ml and a thickness of 4 mm at a rate of 20 ml for each dish, then left until it solidifies to be cultured on it. The homogeneous bacterial inoculum or suspension is then prepared from a fresh bacterial culture aged between 18 and 24 hours in sterile physiological water. The turbidity is then adjusted to McFerland 0.5, or by measuring the optical density of the suspension at a wavelength of 625 nanometers and setting it between 0.08 and 0.1. The inoculam must be administered within 15 minutes of preparation to avoid further bacterial growth. A sterile cotton swab is dipped in the bacterial suspension and then wiped with it over the entire dry medium in the form of contiguous lines, repeating the process three times by rotating the dish 60 degrees each time. Sterile discs with a diameter of 6 mm are saturated with 10 microliters of the raw plant extract, then the mixture consisting of oil. Basic diluted in DMSO to different concentrations (1/2, 1/5, 1/10, v/v), equivalent to 50%, 20%, 10%, respectively. Then, it was placed on cultured media, then a disk saturated with DMSO and an empty disk were placed as a control for the negative test. The study was also conducted on antibiotic tablets for comparison as a positive control. The antibiotics were chosen according to (NCCLS).The sensitivity of a strain is considered non-existent if the inhibition zone is less than or equal to 8 mm. It is limited when the diameter of inhibition ranges between 8 and 14, while it is average when the diameter of inhibition ranges between 14 and 20. However, bacteria are considered sensitive to the antibiotic

when the inhibition zone is greater than 20 mm (Schwalbe *et al*.,2007;Weinstein & Lewis.,2020).



3.2.11.Measurment of WBC:

To determine the increase and decrease in white blood cells levels

Procedure:

(2 ml) of venous blood was placed in anticoagulant EDTA tubes after transported to the laboratory, and a blood picture was taken using a blood cell counting machine, where the white blood cells were counted.

.32.12. Statistical Analysis

Data of studied specimens were entered and analyzed using the statistical

package for social sciences (S.P.S.S.) version 25. The outcomes were expressed as mean ± Standard deviation (Mean ±S.D.). Statistical analysis for the significance of differences of the quantitative data was conducted by using independent-sample T test.

The following statistical tests were used:

1- The square-Chi: test to evaluate the association between any two

categorical variables, provided that the number of cells is less of 20% is

expected to be less than 5 .

2- One way analysis of variance (ANOVA) to evaluate the difference in the

mean of numerical variables between more than two groups, provided that

these numerical variables are normally distributed, and one way ANOVA

was followed by the LSD post hoc test to assess individual differences in

values The mean between any two groups among the groups that were

primarily tested using One way ANOVA .

3- The t-test is a test used for hypothesis testing in statistics and uses the t-statistic, the t-distribution values, and the degrees of freedom to determine statistical significance.

Significance was determined at a P-value equal to or less than 0.05. The level of significance was considered high when the P-value is equal to or less than0.01

Chapter Four
Results and Discussions

4. Results and discussion

4.1. Distribution of Otitis Media according to age and sex:

The current results showed that there is no significant difference between patients depending on sex at ($p \leq 0.05$) as shown in table (4-1) where the number of males infected with Otitis Media were 51 (50.5%) while the number of females 50(49.5%).The result was similar to a study was conducted at Kirkuk General Hospital in the Ear, Nose, and Throat Unit. Men were more likely to be affected than women. Man's rate is 58% while women rate 42% (Hussen *et al*.,2023).This result is in agreement with others (Osazuwa *et al*.,2011).On the other hand the study disagree with(Egbe *et al*.,2010)which identified male gender as a major risk factor for otitis media. While (Kvestad *et al*.,2004) There was a significant pre-dominance of female cases compared to males .

In addition the results showed that there was non-significant difference between various age class this was similar to Iraqi study conduct at Central Pediatric Teaching Hospital (Al-Rawazq et al., 2013) while it disagree with (Kumari *et al*.,2023) where the predominant category was 11-20 years . In the recent study the age group (1-10 years)was 19(18.8%). This infection is more common in children likely due to the shorter Eustachian tube and is more horizontal (Weiner & Collison.,2003)the result agree with (Al-Ani *et al*.,2021).

while the age group (21-30) was 20 (19.8%) were the more frequent. This consistent with the preceding research of (Brook., 2003 ; Shyamala & Reddy, 2012 ; Oni *et al*., 2002)since these age groups were exposed to various risk factors for otitis media.

Table (4-1): Distribution of patients and controls according to age and sex

Characteristic	Control	Patient	*p-value*
	***n* = 49**	***n* = 101**	
Age (years)			
1-10, *n* (%)	9 (18.4)	19 (18.8)	0.91 NS
11-20, *n* (%)	8 (16.3%)	17 (16.8)	
21-30, *n* (%)	10 (20.4)	20 (19.8)	
31-40, *n* (%)	8 (16.3)	17 (16.8)	
41-50, *n* (%)	7 (14.3)	13 (12.9)	
51-60, *n* (%)	4 (8.2)	9 (8.9)	
61-70, *n* (%)	3 (6.1)	6 (5.9)	
Mean ±SD	45.67±2.58	42.28±5.82	0.46 NS
Gender			***p-value***
Male, *n* (%)	25 (51.0)	51 (50.5)	0.82 NS
Female, *n* (%)	24 (49.0)	50 (49.5)	

4.2. Distribution of Otitis Media according to risk factors:

The current study shows that 63 patient with acute otitis media 35 of them with respiratory tract infection,7 were smoking and 41 with recurrent otitis media while 38 with chronic otitis media 9 of them with respiratory tract infection , 2 were smoking and 35 with recurrent otitis media .

The result showed significant difference between patient with Acute and Chronic otitis media according to respiratory tract infections the result came similar to another studies (Iftikhar *et al*., 2023 ;Sawada *et al*.,2019). Upper respiratory tract infection (URI) is a violent infections that affects the nose, Para nasal sinuses, pharynx, and larynx and is caused by a combination of microbial

load (viral and bacterial) and immunological response. Infants and young children are more susceptible to get upper respiratory tract infections, which commonly progress to bacterial issues, particularly acute otitis media. This is because germs can reach the middle ear via the Eustachian tube after inhabiting the nasopharynx (Bentivegna *et al* ., 2012).

Table (4-2) shows non-signinficant difference between smoker, nonsmoker with type of Otitis Media ,this result repel with prior study conducted by Athbi &Abed-Ali.,2020 in Karbala Pediatric Teaching Hospital in which smoking was highly significant risk factor associated with OM infection in infant.

The same table also shows a significant difference between recurrent otitis media and the type of otitis media in the study the result agree with (Zhang *et al*., 2014).

Table(4-2): . Distribution of Otitis Media according to risk factors

Risk factors	Acute *n* = 63	Chronic *n* = 38	*p-value*
Respiratory Tract Infection			
Positive, *n* (%)	**35 (55.6)**	**9 (23.7)**	**0.002 ****
Negative, *n* (%)	**28 (44.4)**	**29 (76.3)**	
Smoking			
Positive, *n* (%)	**7 (11.3)**	**2 (5.4)**	**0.477 NS**
Negative, *n* (%)	**55 (88.7)**	**35 (94.6)**	
Recurrent Otitis Media			
Positive, *n* (%)	**41 (65.1)**	**35 (92.1)**	**0.002 ****
Negative, *n* (%)	**22 (34.9)**	**3 (7.9)**	

4.3. Morphological and Biochemical Identification of isolated bacteria:

The isolated bacterial species were initially diagnosed using a number of basic criteria, such as the shape of the colonies and their color and appearance features on the general culture. Media of Blood agar, nutrient agar medium ,also the use of some differential media , such as MacConkey agar media, which exclusively aids in the growth and isolation of Gram-negative bacteria, as well as the use of several biochemical assays for the goal of initial identification of isolated bacterial species using the procedures outlined by (Collee *et al*., 2006).

Table (4-3): Biochemical test for *S.aureus* & *P.mirabilis*

Biochemical test	*S.aureus*	*P.mirabilis*
Gram stain	+	_
Oxidase	+	-
Catalase	+	+
Motility	-	+
Urease	+	+
Swarming	-	+
Hemolysis	β	-
Indole	-	-
Mythel Red	+	+
Vogase-Proskauer	+	-

4.4 .percentage of isolated bacteria :

One hundred and one ear swabs samples were collected from the patients with Otitis Media infection. Twenty-six of these samples showed negative bacterial culture, and the rest of samples were positive. The highest percentage and in the

lead were *Proteus Mirabilis* (22.66%), *S aureus* (21.33%), *Pseduomonas aeruginosa*(10.66%), *Staph.warneri*(8%), *Aeromonas sobria* (6.66%).

The study result was approaching into another study conducted at the ear, nose and throat (ENT) Department in Al-Diwaniyah Teaching Hospital (Mohsen & Jwad.,2020) and also it was close to (Al-Rawazq *et al*.,2013 and Nega *et al* ., 2013) while it disagree with (Emami *et al*., 2019) where the most prevalent infections were *Pseudomonas aeruginosa* and streptococcus pneumonia with 33.33% and 14.29% . Excess water in ear canal offers an environment for the *S. aureus* bacteria to develop, which is a major cause of staph infection in the ear. Where this is mainly caused by getting water in the ear while swimming, sweat or humid conditions can also cause the bacteria to become established. Also whenever you rip the skin of the ear during cleaning it with a cotton swab or scratching an itch, bacteria might enter through the break. Furthermore, infected devices such as hearing instruments or earphones may transfer the disease into the ear(Singh.,2017).

Table(4-4) : percentage of bacterial isolates

Type of Microorganisms	NO.(%) N=101	Acute n=55	Chronic n=46
Proteus mirabilis	**17(22.66%)**	**8(14.5%)**	**9(19.56%)**
S aureus	**16(21.33%)**	**13(23.63%)**	**3(6.52%)**
Pseduomonas aeruginosa	**8(10.66%)**	**2(2.63%)**	**6(13%)**
Staph.warneri	**6(8%)**	**2(2.63%)**	**4(8.7%)**
Aeromonas sobria	**5(6.66%)**	**4(7.27%)**	**1(2.1%)**
Enterococcus.faecalis	**4(5.33%)**	**2(2.63)**	**2(4.3%)**
Staph.lentus	**4(5.33%)**	**1(1.8%)**	**3(6.52%)**
Enterobcteriacaea	**3(4%)**	**2(2.63%)**	**1(2.1%)**
Serratia marcescens	**3(4%)**	**2(2.63%)**	**1(2.1%)**

Raoultella.planticola	**3(4%)**	**1(1.8%)**	**2(4.3%)**
Providencia stuartii	**2(2.66%)**	**1(1.8%)**	**1(2.1%)**
Leuconostoc.mesenteroide	**1(1.33%)**	**1(1.8%)**	**0**
Pantoea ssp	**1(1.33%)**	**1(1.8%)**	**0**
Rhizobium radiobacter	**1(1.33%)**	**1(1.8%)**	**0**
Citrobacter freundii	**1(1.33%)**	**0**	**1(2.1%)**
No growth	**26(25.74%)**	**14(25.4%)**	**12(26%)**

* Significant differences ; A : one way ANOVA, NS: non significant at P≤ 0.05

4.5.Percantage of bacterial isolates according to risk factor :

The results of the study have been shown that *P mirabilis* 8(50%) the first cause of URI infection in the current study while the second was *S aureus* 6(35.3%).

Recurrent otitis media was highly associated with *S aureus* infection where the number of patient with *S aureus* was 14 (82.4) while *P mirabilis* 11(68.8%) of the bacterial infection this result confirm previous study (Madana *et al*.,2011). There was non-significant relation between URI and type of bacterial infection in OM episodes ,the study result disagree with (Hassooni *et al*., 2018) which was conducted in Baquba teaching hospital at the Faculty of medicine, Diyala University which suggested that OM and URIs are clinically and microbiologically related .

Secondly the result suggested that smoking was unrelated with type of bacterial infection in otitis media this result correspond with (Tarhun .,2020 and Paneru *et al*.;2021).

Table (4-5) : Type of bacterial infection and risk factor

Features	*P mirabilis* *n*= 17	*S aureus* *n*= 16	*Pseudo aerogenoza* *n*= 8	P- value
URIs				
Positive, *n* (%)	**6 (35.3%)**	**8 (50.0%)**	**4 (50.0%)**	**0.36 NS**
Negative, *n* (%)	**11 (64.7%)**	**8 (50.0%)**	**4 (50.0%)**	
Smoking				
Positive, *n* (%)	**0 (0.0)**	**2 (12.5%)**	**2 (20.0%)**	**0.016****
Negative, *n* (%)	**17 (100.0%)**	**14 (87.5%)**	**6 (80.0%)**	
ROM				
Positive, *n* (%)	**14 (82.4%)**	**11 (68.8%)**	**3 (37.5%)**	**0.014****
Negative, *n* (%)	**3 (17.6%)**	**5 (31.3%)**	**5 (62.5%)**	

n= number of cases; T: Independent sample T test ; * significant at P< 0.05; NS= non-significant differences

4.6.White Blood cells Count in Patients and control group :

There was a significant difference in the level of WBCs between patients and controls group at $p \leq 0.0001$ this result approach to the study of (Somuk *et al.*;2014) which show non-significant difference between many CBC parameters and Otitis Media infection. On the other hand, the result correlated with (Arikan & Kelles.;2019) which shows a significant difference in WBCs count between patients and control, The objective criterion (WBC count) might assist clinicians in treating AOM correctly by preventing the inappropriate use of antibiotics without creating severe clinical complications. This might also help to lessen the negative effects of antibiotics as well as the growing bacterial resistance to conventional antibiotics.

The results also showed a significant difference between patients and control in the level of Lymphocytes and Granulocyte count the mentioned result concur with (Trzpis *et al.*,2014 ; Yukkaldıran *et al.*,2021; Yıldız *et al.*,2022). The inflammatory is the most often associated factor in OME etiology (Vanneste & Page.,2019). Continuing inflammation in the middle ear caused by viral and bacterial infections, along with Eustachian tube dysfunction, results in fluid production from the middle ear epithelium (Atan *et al.*,2016)This effusion has an excessive concentration of protein and inflammatory mediators (Kubba *et al.*,2000).

Table (4-6): Levels of WBC parameters in both control and patients

WBC parameters	Control *n* = 49	Patient *n* = 101	
WBC			
Mean ± SD	**7.52 ± 1.23**	**9.31 ± 2.06**	**0.0011 T *****
Range	**4.20 – 12.30**	**4.00 – 18.00**	
Lymphocyte count			
Mean ± SD	**2.68 ± 0.74**	**3.12 ± 1.12**	**0.0091 T ****
Range	**0.10 – 0.70**	**0.10 – 2.10**	
Granulocyte count			
Mean ± SD	**4.58 ± 1.10**	**5.75 ± 2.44**	**0.0024 T ****
Range	**2.60 – 7.50**	**1.30 – 14.80**	

n= number of cases; T: Independent sample T test ; * significant at P< 0.05; NS= non-significant differences

4.7. Association of WBC Levels with type of bacterial infection in OM patients:

The recent study shows a high level of granulocyte (3.9± 0.55)associated with *p mirabillis* infection ,while the levels of WBCs increased (11.45± 0.022)

with *Pseduomonas aeruginosa* , (4.1±0.51) was the higher levels recorded in this study which was related to *S warneri* .

The table (4-7) show a significant difference between various bacterial infection and WBCs count the result coincide with (Li *et al* .,2020).While there is non-significant difference between various type of bacterial infections with Granulocyte count the result disagree with (Korppi *et al* .,1993& Pavare *et al*., 2018).

Table (4-7) also show non-significant difference in Lymphocyte count in various bacterial infection associated with OM, this outcome correlate with (Yang *et al*.,2019).According to Hartmann *et al.*, the level of Lymphocyte count increase after 30min of the infection after that their level return to normal value while the granulocyte level increase after 60 min after infection and then after 24 their level begin to decrease , From this, the patients' delay in visiting a specialist doctor has contributed to making the association non-significant, also the lack of symptoms at the beginning of the infection has also contributed in this result.

Table (4-7): Level of WBC parameters with various bacterial infection in OM patients

Type of Microorganisms	NO.	Mean ±SD WBCs	Mean ±SD Lymphocyte count	Mean ±SD Granulocyte count
P mirabilis	17	8.6± 0.041	3.6± 0.83	**3.9± 0.55**
S aureus	16	7.8± 0.088	3.3± 0.56	3± 0.41
Pseduomonas aeruginosa	8	**11.45± 0.022**	3.5± 0.014	2.82 ± 0.16
S warneri	6	8.8±1.73	**4.1±0.51**	3.6± 0.42
Aeromonas sobria	5	9.4± 0.094	2.4± 0.36	2.7± 0.45
S lentus	4	6.3± 0.061	3.1± 0.43	4.5± 0.39
Enterococcus.faecalis	4	7.4± 0.041	2.6± 0.32	3.7± 0.28

Enterobcteriacaea	3	6.4± 0.093	2.4± 0.47	2.9± 0.32
Raoultella.planticola	3	8± 0.092	**4.1± 0.84**	3.4± 0.43
Serratia marcescens	3	6.2± 0.07	2.7± 0.61	3± 0.2
Providencia stuartii	2	7.3± 0.055	4± 0.76	3.5± 0.13
Leuconostoc.mesenteroide	1	9.2	3	**3.9**
Pantoea ssp	1	6.7	3.3	3.7
Rhizobium radiobacter	1	8.4	3.5	3.1
Citrobacter freundii	1	7.9	2.8	**3.9**
P value	**-**	**0.035 A ***	**0.052 A NS**	**0.062 A NS**

* Significant differences ; A : one way ANOVA NS: not significant at P≤ 0.05

4.8.Levels of Interleukins (IL-12,IL-13and MIP-1 α):

Both Karyia *et al* and Lioa *et al* suggested that Interleukins level increase during OM infection ,their conclusion compatible with the recent result which shows that IL-12 and IL-13 which recorded higher levels in the serum of those infected compared to the control group. Where the level of IL-12,IL-13 and MIP-1α in patient group were 33.69, 48.11 and 168.48 respectively in compare to control group were 20.55,30.23 and 106.26.

The pathophysiology of OME has also been linked to IL-13. A research by Smirnova *et al*. discovered this inflammatory cytokine in 25.9% of effusions. Additionally, it was shown that there was a strong positive association between the amount of mucin in MEEs and IL-4 and IL-13. In an allergic airway inflammation model, it has been demonstrated that suppressing both IL-4 and IL-13 may be required to lessen the production of mucus and allergic inflammation (Perkins *et al* .,2006). Further investigation is required to comprehend the roles of IL-13 and IL-4 as pro-inflammatory cytokines during OM .On the other hand IL-12 is important in early immunity because it stimulates innate resistance and generates a immune

response. It is believed to form a link between innate and adaptive immunity. Phagocytic cells produce IL-12, a pro-inflammatory cytokine, including neutrophils, macrophages, and Langerhans cells. These cells emit a lot of IL-12 when they are activated by bacteria or bacterial toxins like LPS(Juhn *et al*.,2008).

Table(4-8): Levels of Interleukins (IL-12,IL-13and MIP-1 α) in patients and controls

Interleukins	Patient *N* = 101	Control *N*= 49	*P-value*
IL-12			
Mean ± SD	33.69 ± 9.13	20.55 ± 4.22	0.034 *
IL-13			
Mean ± SD	48.11 ± 8.97	30.23 ± 5.39	0.027 *
MIP-1 α			
Mean ± SD	168.48 ± 13.79	106.26 ± 7.55	0.040 *

The previous table show significant difference in serum levels of MIP-1 α between patients and control, where its level in control range (18.41-135.36) while in patients (50.91-248.01) the result dis agree with (Tong *et al*.,2003). MIP-1 α appears to have a crucial role in the middle ear microenvironment, particularly in basophil function . This mediator, which is produced by monocytes and stimulates histamine release and monocyte chemo taxis, is believed to be relevant in both acute and chronic OM (Juhn *et al*.,2008) .

4.8. Association of Levels of Interleukins and Type of Bacterial genus in OM patients :

The study showed that the higher levels of IL-12 registered with *S.aureus* (26.6pg/ml),while the lower levels recorded with *S.warneri* bacteria(10.4 pg/ml) ,

the higher levels of IL-13 was recorded with *Aeromonas sobria* (37.9± 5) whil the higher level of MIP-1α was (138.6± 11.8) which was related to *S aureus* infection.

The table (4-9) showed that there were a significant differences between the levels of interleukin 12 with different types of bacterial infection in OM patients, which corresponds to (Himi *et al*.,2000 & Liao *et al*.,2022) .While it contradicted with (Lee *et al* .,2013). IL-12 has significant therapeutic applications in treating and preventing bacterial infections as a strong inducer of Th1 immune response and an essential mediator between innate and adaptive immunity(Hamza *et al*.,2010)**.**

Table (4-9): Interleukins level and type of bacterial isolates

Type of Bacterial isolates	NO.	**Mean ±SD IL-12**	**Mean ±SD IL-13**	**Mean ±SD MIP -1α**
Aeromonas sobria	5	21.4± 3.7	**37.9± 5**	102.4± 30.6
Enterococcus.faecalis	4	15.4± 2.1	30.7± 1.2	102.9± 46.4
Leuconostoc.mesenteroide	1	24.8	33.6	126.1
Pantoea ssp	1	14.9	32.5	103.6
Proteus mirabilis	17	21± 3.1	32.5± 1.2	117.2± 21.7
Enterobcteriacaea	3	13.5± 3.6	36.9± 4.6	110.4± 33.1
Raoultella.planticola	3	10.2± 4.5	32.8± 4.6	107.1± 37
Rhizobium radiobacter	1	23.3	37.5	116.2
Serratia marcescens	3	12± 2.6	36.1± 4.5	138.4± 48.8
Staph.aureus	16	**26.6± 3.8**	30.5± 2.7	**138.6± 11.8**
Staph.lentus	4	11.7± 3	34.3± 4.6	110.5± 44.4
Staph.warneri	6	10.4± 4.8	31.5± 3.2	112.2± 39.4
Pseduomonas aeruginosa	8	25.2± 4.5	30.3± 2.1	103.7± 3.8
Providencia stuartii	2	23.6±1.3	36.5± 2.8	130.7± 46.7
Citrobacter freundii	1	12.4	33.5	103
P value	-	**0.036 ***	**0.075**	**0.028***

The previous table also showed no significant differences in IL-13 consideration various type of bacterial infections in OM which disagree with(Liao *et al* .,2022). The cytokine IL-13 enhanced bacterial load in highly differentiated human airway epithelial cells, indicating that IL-13 plays a negative function in epithelial defense against pathogenic bacteria. The processes by which IL-13 increases bacterial burden on airway epithelium, on the other hand, are mainly unexplored (Simon *et al*.,2011).

While the MIP-1α levels in patients serum was positively associated with different bacterial infections during OM ,this statically product accord with (Svanberg *et al*.,2020). MIP-1 is anticipated to have a crucial role in the middle ear microenvironment, particularly in basophil function. This mediator, which is produced by monocytes and stimulates histamine release and monocyte chemotaxis, has the potential to be relevant in both acute and chronic OM (Patel *et al*.,1995).

4.10.Antibiotics susceptibility test of bacterial isolates :

Antibiotic sensitivity test was evaluated according to CLSI standards(Humphries *et al*.,2019). The most common isolated bacteria in gram positive was *S.aureus* , and *P.mirabilis* was the higher percentage of gram negative bacteria .different type of antibiotics disc was used (Tetracycline 10 mg ,Ciprofloxacin 15 mg, Ceftazidime 30 mg, Amikacin 10 mg, Azithromycin 15 mg ,Meropenem 10mg ,Levofloxacin 5mg ,Amoxicillin/Clavulanic acid 30 mg).

Table(4-10): Antibiotic sensitivity test for *S.aureus*

S.aureus N=16	Resistance NO.%	Sensitive NO.%
APX 30	0	100%
LEV 5	18.75%	81.25%
CTX 30	0	100%
CIP 15	6.25%	93.75%
AK 10	6.25%	93.75%
MEM 10	0	100%
AZM 15	18.75%	81.25%
TE 10	50%	50%

The susceptibility test showed that *S aureus* isolates was highly sensitive to (APX30, CTX30,MEM10, LEV 5, AK 10, AZM 15,CIP 15).This result is concur to a study conducted in Nepal (Basnet *et al* .,2017) ,also the result was close to study conducted in Tikrit, Iraq (Mahmood *et al.*,2019). The antibiotic (AZM)Which belongs to the macrolide class by suppressing protein synthesis in the bacterial cell via binding to the 50S ribosomal subunit. This binding mechanism suppresses the activity of the Peptidyl enzyme. Transferase is an enzyme that prevents amino acid transfer during protein translation and assembly(Halfon *et al* .,2019).The Tetracycline inhibited bacterial growth via its reversible binding to the 30s ribosomal subunit, it inhibits protein translation (Rose &Rybak.,2006).

Table(4-11): *P.mirabilis* susceptibility test for different antibiotics

P.mirabilis N=17	Resistant No.%	Sensitive No.%
VA 30	4(23.53%)	**13(76.47%)**
AZM 15	9(52.94%)	**8(47.05%)**
CN10	1(5.88%)	**16(94.11%)**

TE10	12(70.58%)	**5(29.42%)**
CIP10	0	**17(100%)**
LEV5	4(23.53%)	**13(76.47%)**
L 10	1(5.88%)	**16(94.11%)**
CFM5	17(100%)	**0**
AMC30	17(100%)	**0**

(PBPs) PBP1a, PBP2, or acquiring of various antibiotic resistance genes, including carbapenemase genes. Furthermore, resistance to non-β-lactams is common, including compounds used to treat UTI infections (e.g., fluoroquinolones, nitrofurans). The emergence and dissemination of multidrug resistant *P. mirabilis* isolates, such as those generating ESBLs, AmpC cephalosporinases, and carbapenemases, is becoming increasingly common. The several genetic pathways involved in the acquisition of resistance genes to numerous antibiotic classes, transforming *P. mirabilis* into dangerous pandrug resistant bacteria with difficult to treat (Girlich *et al* .; 2020).

4.11. Estimation of the biologically active compounds of vegetable oils used in the study (olive oil, pitter almond oil):

The chemical composition of almond oil and olive oil were examined by capillary GC-MS and interpreted based on standard mass spectrum data in order to increase the degree of comprehensive usage and encourage the creation of functional products, and biological activities had been evaluated. Protein, vitamins, minerals, fatty acids, oxalic acid, phosphorus, thiamine, folic acid, nicotinic acid, riboflavin, salt, potassium, magnesium, iodine, copper, chlorine, and sulfur are all present in almonds.Almond oil and olive oil have nine components that have been investigated.

Table (4-12) : The biological active compounds in Olive oil and Almond oil

Components	Almond oil	Olive oil	Unite
DPPH %	6.354009	30.8796	**µg/ml**
Total peroxide	8.066667	13.66667	**µmole/g**
VITAMIN C	1.75	1.88	**mg/g**
VITAMIN E	1.75	2.686	**mg/g**
FRAP	16.6055	6.146789	**µmole/g**
CUPRAC	6.761468	18.26605	**µmole/l**
Total thiol	49.26471	31.10294	**µmole/g**
Phenols	71.49	49	**mg/g**
Flavonoids	9.9428	9.80	**µg/g**

According to table (4-12) the almond oil had high concentration of phenols ,while the lower concentration were vitamin C and vitamin E. On the other hand the higher concentrations were phenols and Thiol ,otherwise the lower concentration was vitamin C.

4.12. Antibacterial of study oils:

4.12.1.Antibacterial activity of Almond oil against *S aureus* :

One usage for almond oil is to treat kidney and neurological discomfort. Almonds are a vital component of a balanced diet that includes dietary fiber, vitamin E, riboflavin, and certain minerals like phosphorus, magnesium, copper, and manganese . Due to their anti-stress, antioxidant, immune-stimulating, lipid-lowering, and laxative properties, pharmacologists were said to possess this quality

. It greatly enhances the strength and vitality of the mussels. Its antioxidant qualities are excellently utilized (Barreca *et al* .;2020).

Table (4-13) : Antibacterial activity for Almond oil against *S.aureus*

Con. Of Almond oil	%100	%50	%25	%12.5	%6.25	%3.125	Meropenem	Negative control
S.areus 1	30	0	0	0	0	0	24	0
S.areus 2	26	25	0	0	0	0	20	0
S.areus 3	20	20	0	0	0	0	18	0
S.areus 4	22	20	0	0	0	0	19	0
S.areus 5	30	0	0	0	0	0	33	0
S.areus 6	20	0	22	0	0	0	30	0
S.areus 7	30	22	25	0	0	0	22	0
S.areus 8	26	25	0	0	0	0	20	0
S.areus 9	0	0	0	0	0	0	24	0
S.aureus10	20	21	0	0	0	0	20	0
S.aureus11	0	19	0	0	0	0	18	0
S.aureus12	25	25	0	0	0	0	19	0
S.aureus13	23	20	0	0	0	0	33	0
S.aureus14	30	25	0	0	0	0	30	0
S.aureus15	20	0	0	0	0	0	22	0
S.aureus16	22	20	0	0	0	0	20	0
Mean ±SD	21.5±2.3	15.12 ±1.8	0	0	0	0	23.25±2.31	0
P value	0.0032 **	0.0078**	-	-	-		0.057	-

The table (4-13) show the higher concentration (100, 50) were the effective and the relation was significant while the other concentrations (25 ,12.5 ,6.25 ,3.125),where non-effective. The maximum inhibition zone at the first concentrations was (30mm) at the isolates (S1,S5,S14),whereas the minimum inhibition zone was 19 mm at the isolate (S11).The isolates (S1,S5,S9,S15) were resistance for the concentration (50).

The result was near to study conducted in Brazil by(Machado *et al.;*2019) ,who found that In some infectious bacterial disease, the use of oils giving a synergistic

effect of antibiotics is a potential option against bacterial resistance .The oils' synergistic activity can manifest itself in a variety of ways, including a reduction in antibiotic accumulation within the bacterial cell, either through a decrease in outer membrane permeability or by drug efflux to the extracellular environment (Nobre *et al* .;2019).

The potential of the oil to serve as an antibacterial may be connected to fatty acid detergent properties against the amphipathic structure of the bacterial cell membrane. Almond oil detergent's propensity to solubilize membrane components (lipids and proteins) may induce split in this structure, affecting metabolic activities necessary to the bacterial cell's energy production, such as the electron transport chain and oxidative phosphorylation. These membrane defects can also impair nutritional absorption, limit enzyme function, and cause hazardous peroxidation. Furthermore, the presence of hydrophobic chemicals in fixed oils may make the cell more permeable to antibiotics, boosting their efficacy and decreasing the lowest concentration necessary to damage the bacterial cell (Chan *et al*.;2015).

4.12.2.Antibacterial activity for Olive oil against *S.aureus* :

Olive oil consider as an important source of nutritionally and healthfully compounds, so that it is considered as a real functional food, with the ability of protective effects in treating many chronic diseases, such as cancer and cardiovascular illnesses(Watson *et al*.,2018).

The table(4-14) showed there was a significant difference at P-value ≤ 0.05 in the crowed olive oil which it concentration was 100 for the different isolates of *S.aureus* .The maximum inhibition zone were for the isolates (S1,S14,S16) , whereas the minimum inhibition zone were for (S2,S6,S12) .Also the concentration 50 had a significant difference at p-value < 0.01,the higher inhibition zone was for the isolate S1 with diameter 29mm , while the minimum inhibition

were (15,16)mm, on the other hand the isolates (S11,S14) were resistance. The concentrations (25,12.5,6.25,3.125)failed to show any activity against *S.aureus* The result is approaching to (Nazzaro *et al* .,2019& Guo *et al*., 2020). Polyphenols are probably one of the most important groups of minor polar components present in the EVOO. The biological importance of polyphenols gives rise from their numerous ascertained biochemical activities, such as the prevention of oxidation reactions to fatty acids. In addition, for this reason they contribute to the stability of the oil over time, delaying rancidity. Polyphenols are also capable of preventing and inhibiting radical-type reactions in the human body, thus limiting the formation of anomalous molecules that might alter the smooth functioning of cell membranes (Gorzynik-Debicka *et al* ., 2018).

Table (4-14): The activity of olive oil against *S.aureus*

Con. Of Olive oil	100	50	25	12.5	6.25	3.125	Meropenem	Negative control
S.aureus 1	35	29	0	0	0	0	24	0
S.aureus 2	25	22	0	0	0	0	20	0
S.aureus 3	30	20	0	0	0	0	18	0
S.aureus 4	30	15	0	0	0	0	19	0
S.aureus 5	35	20	0	0	0	0	33	0
S.aureus 6	25	21	0	0	0	0	30	0
S.aureus 7	32	22	0	0	0	0	22	0
S.aureus 8	30	25	0	0	0	0	20	0
S.aureus 9	30	20	0	0	0	0	24	0
S.aureus 10	29	22	0	0	0	0	20	0
S.aureus 11	30	0	0	0	0	0	16	0
S.aureus 12	25	20	0	0	0	0	19	0
S.aureus 13	33	21	0	0	0	0	33	0
S.aureus 14	35	0	0	0	0	0	30	0
S.aureus 15	30	15	0	0	0	0	17	0
S.aureus 16	30	16	0	0	0	0	20	0
Mean±SD	30.25± 0.82	18.12±1.9	0	0	0	0	22.81±2.31	0
P value	0.041*	0.008**	-	-	-	-	0.057	-

4.12.3Antibacterial activity of Almond oil against *P.mirabilis*:

The almond oil failed to show any inhibition zone against the bacteria *P.mirabilis* isolates. *P. mirabilis* isolates have been described with multiple acquired resistance genes encoding narrow spectrum β-lactamases TEM, also epidemiological studies report a dramatic increase in ESBL producing *P. mirabilis* isolates (Huang *et al*.,2015).

4.12.4Antibacterial activity of olive oil against *P.mirabilis*:

P.mirabilis isolates showed a high resistance to olive oil concentration ,this might be because of it acquired resistant mechanisms .Also The mobility that *P. mirabilis* possesses is due to its flagella; this aids in colonization and has also been linked to the organism's capacity to build biofilms and resistance to some drugs and host defenses(Wang *et al*., 2020). *P.mirabilis* can also cause infections from colonized skin and oral mucosa in patients and staff in hospitals and long-term care facilities, though they are less frequently the source of nosocomial infections(Potron *et al*.,2019).

Chapter Five

Conclusions and Recommendations

Conclusions

1. The *P mirabillis* and *S aureus* bacteria were tha most common isolated bacteria from patients with Otitis media.
2. There were significant association between recurrent infection and Upper Respiratory Tract infection with development of ear infection.
3. There were significant interaction in Interleukins levels (IL-12,IL-13 and MIP-1α) during Otitis Media.
4. There were high antibacterial activity of high concentration of almond oil and olive oil against bacteria genus *S aureus* isolated from patients with Otitis Media.
5. *P mirabilis* was highly resistance for the high concentrations of olive and almond oil.

Recommendation

1. Determined the level of Immunological biomarkers that associated with development of Otitis Media infection.
2. Extraction and purification of active components of medical oil and detect it is effect against most isolate that cause ear infections.

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Appendix

Karbala University
College of Science
Department of Biology

Questionnaire for the patients with AOMs & the control

1. Name: No. The date:
2. Gender: Male Female Age:

Culture (register types of bacteria and other pathogens)	**Discharges associated with the infection (describe colour and texture)**

3. Status of cigarette-smoking Yes No
4. Alcohol consuming Yes No
5. Recurrent AOM Yes No

   Time duration between the recurrent AOMs

6. Relatives having recurrent AOM Father Mother Sister
7. Types of antibiotics have been taken as a medicine:
8. Other infections around the body associated with ear disease :

Appendix 2

bioMérieux Customer: Microbiology Chart Report Printed July 8, 2022 8:15:09 AM AST

Patient Name: Patient ID:
Location: Physician:
Lab ID: ab907 Isolate Number: 1

Organism Quantity:
Selected Organism : Staphylococcus aureus

Source: **Collected:**

Comments:	

Identification Information	**Analysis Time:**	4.82 hours	**Status:**	Final
Selected Organism	98% Probability	**Staphylococcus aureus**		
	Bionumber:	010402023763231		
ID Analysis Messages				

Biochemical Details

2	AMY	-	4	PIPLC	-	5	dXYL	-	8	ADH1	+	9	BGAL	-	11	AGLU	-
13	APPA	-	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	+
20	LeuA	-	23	ProA	-	24	BGURr	-	25	AGAL	-	26	PyrA	+	27	BGUR	-
28	AlaA	-	29	TyrA	-	30	dSOR	-	31	URE	-	32	POLYB	+	37	dGAL	-
38	dRIB	+	39	ILATk	+	42	LAC	-	44	NAG	+	45	dMAL	+	46	BACI	+
47	NOVO	-	50	NC6.5	+	52	dMAN	+	53	dMNE	+	54	MBdG	+	56	PUL	-
57	dRAF	-	58	O129R	+	59	SAL	-	60	SAC	+	62	dTRE	+	63	ADH2s	-
64	OPTO	+															

Page 1 of 1

bioMérieux Customer:

Microbiology Chart Report

Printed July 8, 2022 8:13:56 AM AST

Patient Name:
Location:
Lab ID: ab906

Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Staphylococcus aureus

Source:

Collected:

Comments:	

Identification Information	**Analysis Time:**	8.00 hours	**Status:**	Final
Selected Organism	**Bionumber:**	**Staphylococcus aureus** 010002023763231		
ID Analysis Messages				

Biochemical Details

2	AMY	-	4	PIPLC	-	5	dXYL	-	8	ADH1	+	9	BGAL	-	11	AGLU	-
13	APPA	-	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	-
20	LeuA	-	23	ProA	-	24	BGURr	-	25	AGAL	-	26	PyrA	+	27	BGUR	-
28	AlaA	-	29	TyrA	-	30	dSOR	-	31	URE	-	32	POLYB	+	37	dGAL	(-)
38	dRIB	+	39	ILATk	+	42	LAC	-	44	NAG	+	45	dMAL	+	46	BACI	+
47	NOVO	-	50	NC6.5	+	52	dMAN	+	53	dMNE	+	54	MBdG	+	56	PUL	-
57	dRAF	-	58	O129R	+	59	SAL	-	60	SAC	+	62	dTRE	+	63	ADH2s	-
64	OPTO	+															

bioMérieux Customer:

Microbiology Chart Report

Printed July 8, 2022 8:10:04 AM AST

Patient Name:
Location:
Lab ID: ab900

Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Enterobacter cloacae complex

Source:

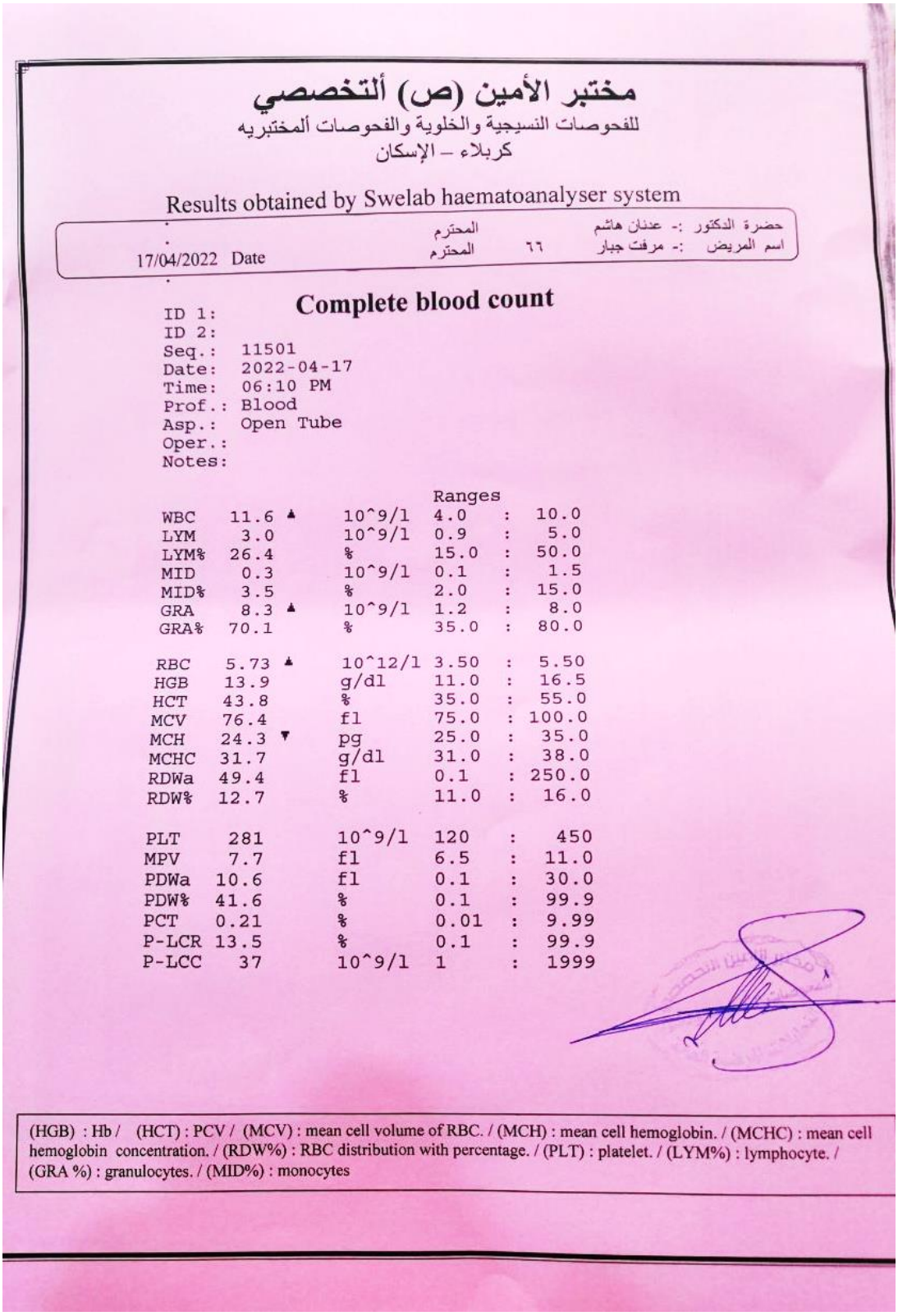
Collected:

Comments:	

Identification Information	**Analysis Time:**	5.85 hours	**Status:**	Final
Selected Organism	98% Probability **Bionumber:**	**Enterobacter cloacae complex** 0627734553532010		
ID Analysis Messages				

Biochemical Details																
2	APPA	-	3	ADO	-	4	PyrA	-	5	lARL	-	7	dCEL	+	9	BGAL	+
10	H2S	-	11	BNAG	+	12	AGLTp	-	13	dGLU	+	14	GGT	+	15	OFF	+
17	BGLU	+	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	+	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	+	29	TyrA	+	31	URE	-	32	dSOR	+
33	SAC	+	34	dTAG	-	35	dTRE	+	36	CIT	+	37	MNT	+	39	5KG	-
40	lLATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	+	44	AGAL	+	45	PHOS	-
46	GlyA	-	47	ODC	+	48	LDC	-	53	lHISa	-	56	CMT	-	57	BGUR	-
58	O129R	+	59	GGAA	-	61	lMLTa	-	62	ELLM	-	64	lLATa	-			

Appendix 3



مختبر الأمين (ص) ألتخصصي

للفحوصات النسيجية والخلوية والفحوصات ألمختبريه

كربلاء – الإسكان

Results obtained by Swelab haematoanalyser system

حضرة الدكتور :- عدنان هاشم

اسم المريض :- مرفت جبار ٦٦ المحترم

17/04/2022 Date المحترم

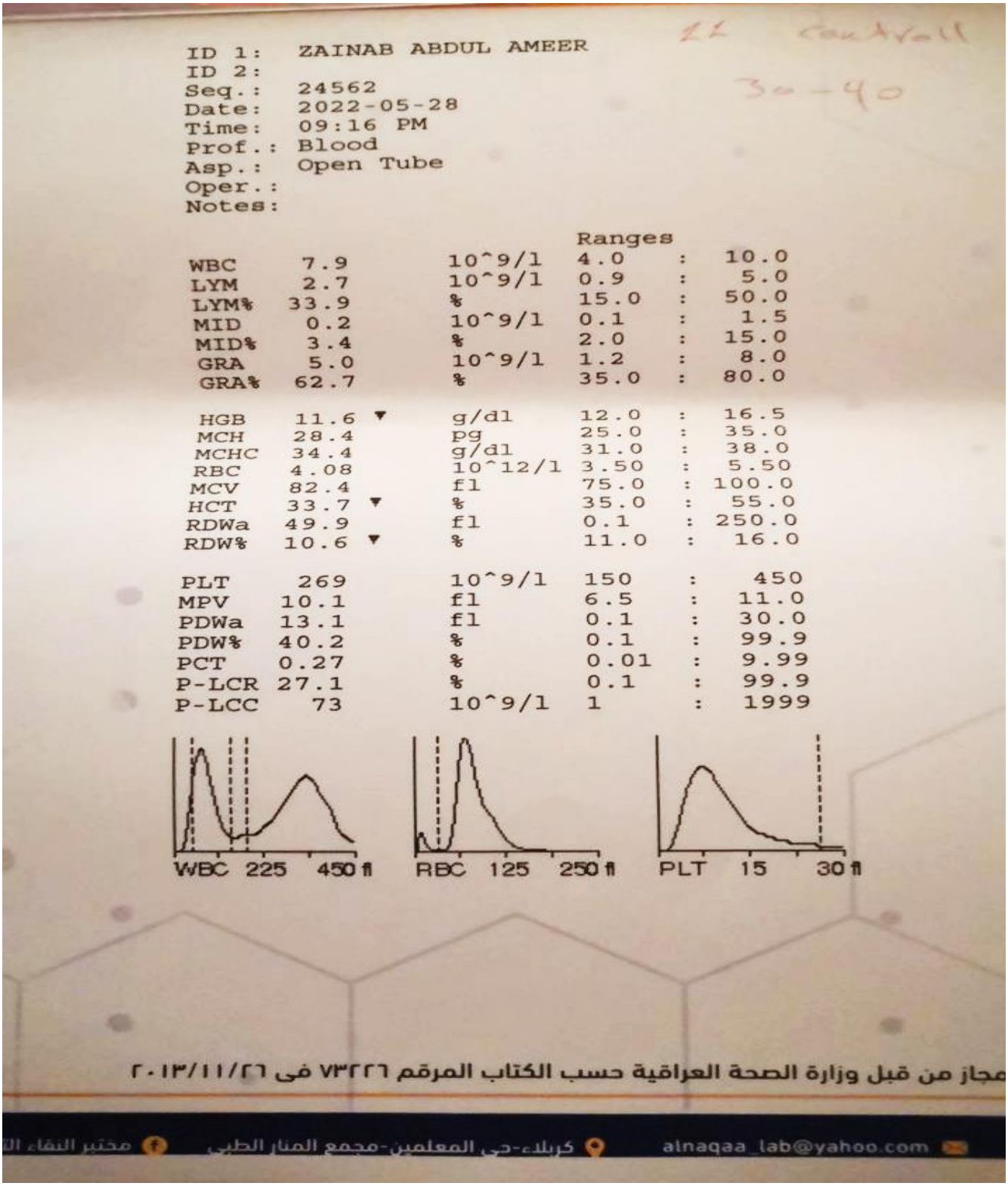
Complete blood count

ID 1:
ID 2:
Seq.: 11501
Date: 2022-04-17
Time: 06:10 PM
Prof.: Blood
Asp.: Open Tube
Oper.:
Notes:

			Ranges		
WBC	11.6 ▲	10^9/l	4.0	:	10.0
LYM	3.0	10^9/l	0.9	:	5.0
LYM%	26.4	%	15.0	:	50.0
MID	0.3	10^9/l	0.1	:	1.5
MID%	3.5	%	2.0	:	15.0
GRA	8.3 ▲	10^9/l	1.2	:	8.0
GRA%	70.1	%	35.0	:	80.0
RBC	5.73 ▲	10^12/l	3.50	:	5.50
HGB	13.9	g/dl	11.0	:	16.5
HCT	43.8	%	35.0	:	55.0
MCV	76.4	fl	75.0	:	100.0
MCH	24.3 ▼	pg	25.0	:	35.0
MCHC	31.7	g/dl	31.0	:	38.0
RDWa	49.4	fl	0.1	:	250.0
RDW%	12.7	%	11.0	:	16.0
PLT	281	10^9/l	120	:	450
MPV	7.7	fl	6.5	:	11.0
PDWa	10.6	fl	0.1	:	30.0
PDW%	41.6	%	0.1	:	99.9
PCT	0.21	%	0.01	:	9.99
P-LCR	13.5	%	0.1	:	99.9
P-LCC	37	10^9/l	1	:	1999

(HGB) : Hb / (HCT) : PCV / (MCV) : mean cell volume of RBC. / (MCH) : mean cell hemoglobin. / (MCHC) : mean cell hemoglobin concentration. / (RDW%) : RBC distribution with percentage. / (PLT) : platelet. / (LYM%) : lymphocyte. / (GRA %) : granulocytes. / (MID%) : monocytes

Appendix 4



ID 1: ZAINAB ABDUL AMEER
ID 2:
Seq.: 24562
Date: 2022-05-28
Time: 09:16 PM
Prof.: Blood
Asp.: Open Tube
Oper.:
Notes:

			Ranges		
WBC	7.9	10^9/l	4.0	:	10.0
LYM	2.7	10^9/l	0.9	:	5.0
LYM%	33.9	%	15.0	:	50.0
MID	0.2	10^9/l	0.1	:	1.5
MID%	3.4	%	2.0	:	15.0
GRA	5.0	10^9/l	1.2	:	8.0
GRA%	62.7	%	35.0	:	80.0
HGB	11.6 ▼	g/dl	12.0	:	16.5
MCH	28.4	pg	25.0	:	35.0
MCHC	34.4	g/dl	31.0	:	38.0
RBC	4.08	10^12/l	3.50	:	5.50
MCV	82.4	fl	75.0	:	100.0
HCT	33.7 ▼	%	35.0	:	55.0
RDWa	49.9	fl	0.1	:	250.0
RDW%	10.6 ▼	%	11.0	:	16.0
PLT	269	10^9/l	150	:	450
MPV	10.1	fl	6.5	:	11.0
PDWa	13.1	fl	0.1	:	30.0
PDW%	40.2	%	0.1	:	99.9
PCT	0.27	%	0.01	:	9.99
P-LCR	27.1	%	0.1	:	99.9
P-LCC	73	10^9/l	1	:	1999

WBC 225 450 fl RBC 125 250 fl PLT 15 30 fl

مجاز من قبل وزارة الصحة العراقية حسب الكتاب المرقم ٧٣٢٢٦ في ٢٠١٣/١١/٢٦

مختبر النقاء الـ كربلاء-حي المعلمين-مجمع المنار الطبي alnaqaa_lab@yahoo.com

Appendix 5

Correlation between Immunological markers with patients infected with *S aureus*

Number of patient N=16

Correlation *Staphyloccus areus*	MIP	IL-13	IL-12
MIP	1		
IL-13	0.58	1	
IL-12	-0.62	0. 47	1

Correlation between Immunological markers with patients infected with *P mirabilius*

Number of patient N=17

Correlation *Proteus mirabilius*	MIP	IL-13	IL-12
MIP	1		
IL-13	0.62	1	
IL-12	-0.73	0. 33	1

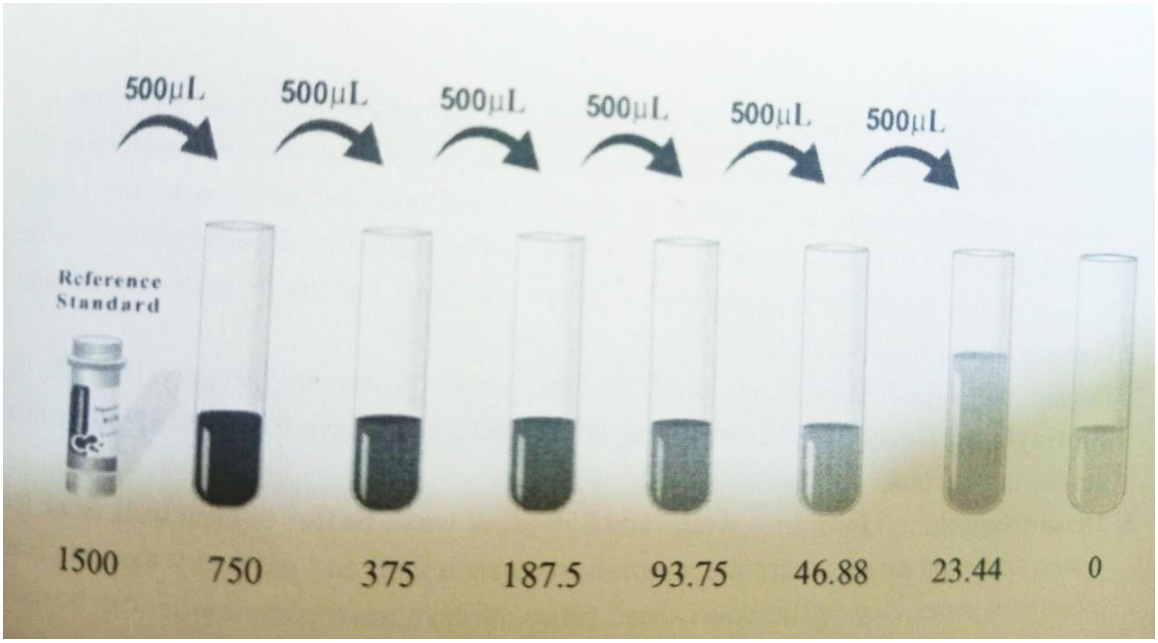
Correlation between Interleukins with WBC

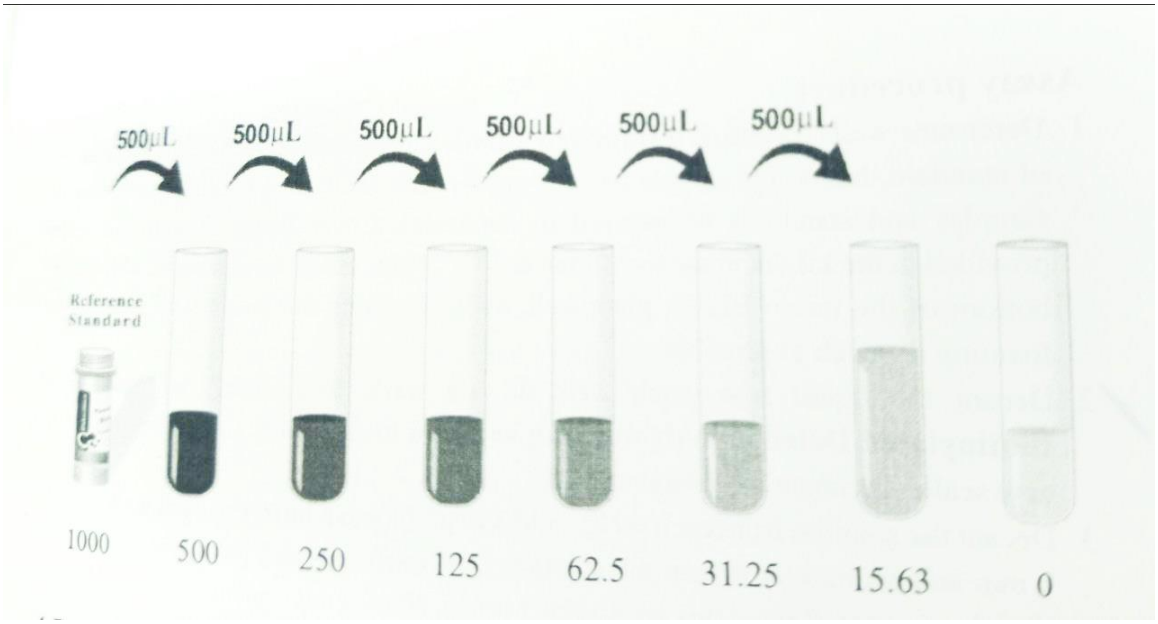
	WBCs	Lymph	Gran.	MIP	IL-3	Il-12
WBCs						
Lymph	0.685					
Gran.	-0.406	0.267				
MIP	0.143	0.222	0.685			
IL-13	-0.836	-0.036	-0.041	0.038		
Il-12	-0.070	0.758	-0.067	-0.105	0.088	

Red color positive correlation

Yellow color negative correlation

Appendix 6


500µL
500µL
500µL
500µL
500µL
500µL
Reference Standard
1500
750
375
187.5
93.75
46.88
23.44
0


500µL
500µL
500µL
500µL
500µL
500µL
Reference Standard
1000
500
250
125
62.5
31.25
15.63
0

جامعة كربلاء

كلية العلوم

قسم علوم حياة

دراسة مناعية وتشخيصية لمسببات خمج الأذن الوسطى والتأثير التثبيطي لبعض المستخلصات النباتية والمضادات الحياتية لها

رسالة

مقدمة الى مجلس كلية العلوم / جامعة كربلاء

وهي جزء من متطلبات نيل درجة الماجستير في علوم الحياة

من قبل

ضي عبد الجليل كامل

بكالوريوس علوم / علوم حياة جامعة كربلاء 2014

بإشراف:

الأستاذ الدكتور	الأستاذ المساعد الدكتور
وفاء صادق الوزني	علي احمد الميالي
كانون الثاني / 2024	رجب / 1445

الخلاصة:

التهاب الأذن الوسطى هو عدوى في الأذن الوسطى يمكن أن تلحق الضرر بالهياكل المحيطة بالأذن وكذلك الجهاز السمعي. ونظراً للبنية التشريحية ونمو جهاز المناعة، فهو من أكثر الأمراض التي تصيب الأطفال الصغار كل عام، وخاصة من هم في سن السادسة تقريباً.

خلال الفترة الممتدة ما بين أذار2022 إلى أيار 2022، تم جمع مسحات الأذن وعينات الدم من 101 مريضاً مصاباً بالتهاب الاذن الوسطى و49 من الأصحاء، تراوحت أعمارهم بين سنة واحدة إلى 70 سنة. . تم جمع العينات في مكانين (مستشفى الامام الحسين التعليمي والعيادة الخاصة). تم التعرف على النمو البكتيري باستخدام نظام .Vitek-2 تم قياس مستويات كل من الانترلوكين 12 والانترلوكين 13 والبروتين الالتهابي البلعمي -1 α بواسطة جهاز الاليزا. وأظهرت النتائج أن معدلات الإصابة لدى الذكور (50.5) أعلى من الإناث (49.5). ومن ناحية أخرى أعلى معدل إصابة كانت الفئة العمرية 21-30 بينما كانت الفئة العمرية الأدنى 61-70. من بين 101 مسحة أذن، أظهرت 75 عينة (74.25%) نمو بكتيري إيجابي، في حين لم تظهر 26 (25.75%) أي نمو. وكانت نتائج العزلات البكتيرية هي (17/75) *Proteus Mirabilis* 22.66%، (16/75) 21.31% *Staphylococcus Aureus*، *Pseduomonas aeruginosa* (10.66%)، *Aeromonas sobria* (6.66%). *Staph.warneri* (8%)

كانت هناك فروق معنوية عند ($P\leq 0.05$) في مستويات عدد كريات الدم البيضاء في الدم بين المرضى (9.31 ملم^3) ومجموعة السيطرة (7.52ملم^3). علاوة على ذلك، كان عدد الخلايا الليمفاوية معنوياً عند ($P\leq 0.05$)وكان عدد الخلايا المحببة معنوياً أيضاً عند ($P<0.025$) وكانت مستويات الإنترلوكينات معنوية أيضاً بين المرضى ومجموعة السيطرة حيث سجل IL-12 في المرضى (33.69بيكوغرام/مل) وفي مجموعة السيطرة (20.55بيكوغرام/مل).كما كانت هناك اختلافات معنوية عند ($P\leq 0.05$) في مستويات IL-13في المرضى (48.11بيكوغرام/مل) بينما في المجموعة السيطرة (30.23بيكوغرام/مل)ومن ناحية أخرى كانت هنالك فروق معنوية في MIP-1α عند مستوى ($P<0.05$) في المرضى (168.48بيكوغرام/مل) بينما في مجموعة السيطرة (106.26 بيكوغرام/مل).

كما كانت هناك فروق معنوية بين مستوى الإنترلوكينات والعزلات البكتيرية عند مستوى ($P\leq 0.05$) حيث ارتبط المستوى الأعلى للإنترلوكين 12 مع المكورات العنقودية .بعدوى المكورات العنقودية الذهبية (26.6 ± 3.8 بيكوغرام / مل)، ارتبطت المستويات الأعلى من IL-13 ببكتيريا *Aeromonas sobri* ± 37.9)

5 بيكوغرام / مل) بينما تم تسجيل تركيز أعلى من MIP-1α أيضًا مع البكتيريا الذهبية (138.6 ± 11 بيكوغرام / مل) .(كانت حساسية عزلات Staph.aureus للمضادات الحيوية عالية حيث كانت حساسة لكل من (أموكسيلين/حمض الكلافونيك 30 ملغ، سيفتازيميد 30 ملغ، ميروبينيم 10 ملغ، سيبروفلوكساسين 15 ملغ، أميكاسين 10 ملغ .(بينما البكتيريا *p. mirabilis* حساس للغاية لـ (سيبروفلوكساسين 10 ملغ، جنتاميسين 10 ملغ .(أظهرت كروماتوغرافيا الغاز الكتلي لزيت الزيتون واللوز المر وجود مركبات نشطة بيولوجيا مختلفة مثلDPPH%) ، البيروكسيد الكلي ، فيتامينC ، فيتامينE ، FRAP، CUPRAC، الثيول، الفينولات، الفلافونويدات) ومستويات هذه المركبات في أما زيت الزيتون فكان (30.8796، 13.66667، 1.88، 2.686، 6.146789، 18.26605، 31.10294، 49، 9.80) على التوالي .بينما أظهر زيت اللوز (6.354009، 8.066667، 1.75، 1.75، 16.6055، 6.761468، 49.26471، 49.71، 9.9428) على التوالي .أظهر زيت المخزون لكلا (زيت الزيتون وزيت اللوز المر) اختلافات معنوية عند $P\leq0.05$ ضد نمو بكتريا*S. aureus* ، في حين كانت *P. mirabilis* مقاومة لكلا الزيتين.