Ministry of Higher Education
And Scientific Research
University of Babylon
College of Medicine

Molecular and cytotoxic study of Candida albicans and Porphyromonus gingivalis isolated from patients with periodontitis

A Thesis
Submitted to the Council of the College of Medicine, University of Babylon, in Partial Fulfillment of the Requirements for the Degree of Doctor of philosophy in science /clinical Microbiology

By

Ali Awad Obais Abed

BSc. Pathological analysis/ 2012

M.Sc. microbiology/2019

Supervised by

Professor Dr. Maysaa S.M. Al-shukri **Professor Dr. Abeer Fauzi M. Al-Rubaye**

2023A.D **1444 A.H**

((وَعَلَّمَكَ مَا لَمْ تَكُنْ تَعْلَمُ وَكَانَ فَضْلُ اللَّهِ عَلَيْكَ عَظِيماً))

صَدَقَ اللهُ العَلِيُّ العَظيم

سورة النساء: الآية ﴿١١٣﴾

وزارة التعليم العالي والبحث العلمي
جامعــة بابـــل
كليــة الطـــب

دراسه جزيئيه وسميه للمبيضات البيضاء وبكتريا Porphyromonus gingivalis المعزوله من المرضى المصابين باللتهاب اللثه

دراسة مقدّمة إلى
مجلس كلية الطب / جامعة بابل
وهي جزء من متطلبات نيل درجة الدكتوراه فلسفه في
العلوم / الأحياء المجهرية الطبيه

من قِبل

علي عـــــــــواد عبيـــــس عبد
بكالوريوس تحــليلات مرضيـــــة \2012
ماجستير احياء مجهريه طبيه/2019

إشراف

الأستاذ الدكتور

ميساء صالح مهدي **عبير فوزي مراد**

1444هـ **2023 م**

الخلاصة

دراسة جزيئيه وسميه ل *Candida albicans spp* and *Porphyromonas gingivalis* المعزولة من المرضى المصابين بالتهاب اللثة المزمن

تم اخذ (150) مريض مصاب بالتهاب اللثه , حيث تم اخذ مسحة فموية ,لعاب paper point , من كل مريض ، الذين حضروا إلى المركز التخصصي للأسنان والعيادات الخارجية لطب الأسنان وخلال الفترة من (نيسان 2022 إلى أيلول 2022) و تتراوح أعمارهم بين (5 - 70) سنة.

شخصت الدراسة الجزيئية جينات الضراوة (ALS1و HWP1 و EAP1) في *C albicans* عن طريق تفاعل pcr باستخدام برايمرات محدده ذات حجم جزيئي (318 bp ، 572 bp ، 66 bp) على التوالي. أظهرت النتائج أن جين ALS1 اكتشف بمعدل 15 (22.05٪) بينما جين HWP1 بمعدل 19 (27.94٪) و جين EAP1 بمعدل 19 (76٪).

في هذه الدراسه تم استخدام ثلاث عزلات من كل نوع من المايكروبات المستخدمه في الدراسه وبتراكيز متعدده في الخط الخلوي (,normal oral epithelial tissue)حيث

أظهرت الدراسة النسيجية أن تأثير السمية الخلوية على 3 عزلات من *Candida albicans* تم تحديدها بواسطة MTT assy ضد الخط الخلية HGF-1 بعد 48 ساعة ،

أن C albicans أظهرت سمية خلوية انتقائية ضد خط الخلايا HGF-1 بتركيز مثبط 203.98 (IC50) ميكروغرام / مل ، 240 ميكروغرام / مل بينما كان IC50 لخط خلايا Candida-HGF-1 3 سلالة المبيضات 54.45 ميكروغرام / مل.

كشفت الدراسة الحالية أيضًا عن إمكانات السمية الخلوية لـ P.gingivalis.

كذالك تُظهر الدراسة الحالية أن إمكانات السمية الخلوية لمزيج من *P .ingivalis* و *Candida albicans* قد تم اجرائها بواسطة MTT assy ضد الخط الخلوي HGF-1 بعد 48 ساعة ، والذي يبدو أن المجموعة أظهرت سمية خلوية انتقائية ضد خط الخلايا HGF-1 بتراكيز مختلفه (تركيز 80.3699 (IC50) ميكروغرام / مل.

كذلك بعد الزرع الخلوي النسيجي وتحديد السميه وكذاك ال apoptosis

تم تحديد التعبير الجيني لجين H-Ras لتحديد دور وامكانية التأثير السرطاني لهذه الكائنات على الخلايا المستخدمه في الدراسه

باستخدام (real time-PCR) ، وأظهرت النتيجة أن هناك اختلافات كبيرة في التعبير الجيني لل RAS جين بين البكتيريا والمبيضات مقارنة مع control عندما عوملت العزلات بخط الخلية أظهرت النتائج التعبير الجيني المثبط مع المبيضات البيضاء أعلى من *P .ingivalis* و control. أظهرت النتائج الحالية وجود high significant (قيمة P 0.05٪) في بعض التراكيز والعزلات بينما لم يظهر الخليط بين *C albicans* و *P gingivalis* فرقا معنويا مع كل التراكيز.

كذلك استخدمت تقنية الاليزا للكشف عن مستوى IL-17 من لعاب مرضى التهاب اللثة ومقارنتها مع الأشخاص الاصحاء حيث اظهرت النتائج ليس هناك فرق معنوي 0.398) p value بينما اظهرت النتائج هنالك فرق معنوي في تركيز الانترلوكين بين الاشخاص المصابين في *C albicans* و *P gingivalis*. P value (0.001).

Summary

Atotal from 150 patients included in this study each patient sample oral swabs , Saliva and paper point were collected from periodontitis patients , attended to specific dental health center and outpatient clinics of dentistry in Al-Hillah city/ Iraq during the duration from (April 2022 to September 2022) . The age of patient range from (5–70) years.

The samples were collected by disposable cotton swabs and paper point . The swab were cultured on different media (Sabouraud's dextrose agar with chloramphenicol for selective isolation and culturing of *Candida albicans* and CHROM agar Medium) for isolation of *C. albicans* while the paper point culture on blood agar and selective media(*P. gingivalis* agar (P.GING)) for isolation of *P gingivalis* then diagnosis conform by molecular method. The result showed that out of 150 samples 25 (16.66%) *C albicans* isolated and 15(10%) *P.gingivalis* isolated from this fifteen isolate only 10(6.66) confirmed with molecular detection as *P.gingivalis.*

Molecular study for detection the *ALS1, HWP1,EAP1* virulence gene of *C albicans* by polymerase chain reaction by using specific primers with a molecular size (318 bp , 572 bp, 66 bp) respectively . The result showed that ALS1 gene detected in a rate 13(52%) while HWP1 gene detected in a rate 17(68%) and EAP1 gene in a rate 19(76%).

The cytotoxic effect were evaluated by MTT assay to 3 isolates of *Candida albicans* against HGF-1 cell line culture after 48 hrs., which appear that *C albicans* more exhibited selective cytotoxicity against HGF-1 cell line with inhibitory concentration (IC50) of the Candida strain-3 HGF-1 cell line was 54.45 μg/ml. Also the present study detect The cytotoxicity potentials to *P .gingivalis* in 3 isolates which found that they were evaluated by MTT assay against HGF-1 cell line culture after 48 hrs., which appear that *P*

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while the current study show that the cytotoxicity potentials for combination of *P .gingivalis* and *Candida albicans* were evaluated by MTT assay against HGF-1 cell line culture after 48 hrs., which appear that combination exhibited selective cytotoxicity against HGF-1 cell line with inhibitory concentration (IC50) 80.3699 μg/ml.

Gene expression of the H-*Ras* gene was determined to assess its role in the carcinogenic effect by using Quantitative-reverse transcription-polymerase chain reaction (qRT-PCR) the result indicated that there is a significant differences in *RAS* gene expression between bacteria and *candida albicans* with control. when the isolates is treated with cell line the results indicated inhibition gene expression with candida albicans higher than the *P .gingivalis* and control. The Current result showed that high significant difference (P value 0.05%) in concentration (75%) with the third isolated of bacterial (0.043) while the four isolates of bacteria show significant difference (0. 54%) in a concentration (100%) . Also the present study showed significant difference(0.043) in second concentration (50%) isolates of *C albicans* while the combination between *C albicans* and *P gingivalis* show no significant with all concentration

ELISA technique used for detection the level of IL-17 from saliva of periodontitis patient and compered with its level in control (health group) the result showed that there wase no significant p value (0.398) of IL-17 in saliva of periodontitis patient compared with control group while the result show there was a significant in IL-17 concentration when compered between the group that infect with *C albicans* alone and combination at P value (0.001).

Acknowledgements

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Ali.A.O (2023)

Dedication

To the big heart…….my father

To the pure white heart…….my mather

To my sister and all my friends

Ali (2023)

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Abbreviations

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2	ALS1	Agglutinin-like protein gene
3	BHI	Brain Heart infusion
4	CaADH-1	Candida albicans Alcohol Dehydrogenase
5	CP	Chronic Periodontitis
6	CRS	Chronic rhininosinusitis
7	*DMSO*	Dimethyl sulfoxide
8	DPPIV	dipeptidyl peptidase IV
9	Eap 1	Enhanced Adherence To Polystyrene
10	ELISA	Enzyme Linked Immuno-Sorbent assay
11	EMT	Epithelial to mesenchymal transition
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13	GTPases	guanosine triphosphatase
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16	H-Ras	Harvey-Ras
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21	KGP	gingipain K
22	LPS	Lipopolysaccharide
23	MAPK	Mitogen-activated protein kinases
24	MMPs	Matrix metallo proteinase
25	MTT	Methyl Thiazolyl Tetrazolium
26	NF-κB	Nuclear factor –Kappa beta pathway
27	NLRP3	NLR family pyrin domain containing protein 3
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31	PMN	Polymorphonuclear leukocytes
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33	q RT-PCR	Quantitative Real Time PCR
34	RANKB	Receptor Activator nuclear kappa B
35	RGP	gingipain R
36	ROS	Reactive oxygen species
37	*Rpm*	*Revolution per minute*
38	RPMI	Roswell park memorial instituted
39	*Sap*	Aspartyl protease
40	SDA	Sabouraud Dextrose Agar
41	SPSS	Statistical Package for the Social Sciences
42	TLRs	Toll Like Receptors

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1. Introduction and Literatures Review

1.1. Introduction

Periodontitis is a chronic inflammatory disease characterized by the destruction of the supporting structures of the teeth. Its high prevalence and negative effects on quality of life make it one of the current problems in dentistry. *Porphyromonas gingivalis (P. gingivalis)* is the predominant periodontal pathogen that expresses a number of virulence factors involved in the pathogenesis of periodontitis : fimbriae, gingipains, haemagglutinin, and lipopolysaccharide (Oleinik and Goncharenko,2022).

Fimbriae that are encoded by fimA gene have been considered the main and the first virulence factor of this bacterium involved in adhesion, colonization, invasion, establishment and persistence within the host (Almaali, 2014). Invasion of *P. gingivalis* to host cell was relies on the ability of bacteria to produce gingipains (a trypsin – like cysteine proteinases) which support biofilm formation and regulate host defense response ((Ren *et al*., 2017).

The expression of several cytokines gingipains was modulate in multi cell kinds involved: gingival fibroblasts, endothelial cells, monocytes and T cells (Palm *et al*., 2015; Kariu *et al*., 2017).

Amongst the virulence factors of *P. gingivalis*, gingipains are the most important virulence factors which are responsible for damage of periodontal tissues inactivate and degrade a number of host defense and structural proteins, also it plays an essential role for *P. gingivalis* nutrient acquisition, colonization, immune subversion and signaling (Mahato *et al*., 2016). Initiation of periodontitis is relies mainly on embedding of microbes in subgingival dental plaque (biofilm) and interactions between microbes and host (Hajishengallis, 2015).

In addition to bacteria, fungi may also be related to oral mucositis. In immunocompromised individuals, *Candida albicans* frequently overgrows the microbial flora and causes infections and epithelial damage. *Candida* spp.,

particularly *C. albicans*, are associated with oral mucositis in patients with hematological malignancies(Sun *et al*., 2020).

C. albicans can induce carcinogenesis through its proinflammatory action, through induction of Th17 response, or through the production of direct carcinogene. *C. albicans* is capable of forming nitrosamines that act as carcinogens both alone and when combined with other chemical compounds. Their production leads to the activation of specific proto-oncogenes that can further support the formation of a carcinogenic lesion (Chang *et al*.,2019).

Interactions between *P. gingivalis* and *C. albicans* that can allow the cooperation of both microorganisms for mutual biofilm development and host invasion. For example, it was observed that *P. gingivalis* influences *C. albicans* morphology, enhancing germ tube formation (Guo *et al*.,2020). These findings were supported by the observed increased expression of genes encoding the *C. albicans* main adhesins, Als3 and Hwp1, and a secreted aspartic protease 6 (Sap6) that correlated with hyphal morphology (Bartnicka *et al*.,2019). However, some opposing effects have also been observed (Cavalcanti *et al*.,2016).

The mutual contact of both microorganisms was found to be based on direct interactions between the fungal adhesin *ALS3* and the adhesive domain of gingipain *rgpA* (Guo *et al*.,2020),. Another conductive interaction was also determined for the adhesion among both pathogens that induced the type 9 secretion system of *P. gingivalis* and increased the pathogenicity of the community (Hajishengallis *et al*.,2016).

On the other hand, the importance of a bacterial extracellular enzyme peptidylarginine deiminase (PPAD) for the mutual contact of both pathogens has been proposed (Karkowska-Kuleta *et al.*,2018). This enzyme converts protein arginine residues to citrullines, and this modification of selected surface-exposed *C. albicans* proteins was identified during the formation of

mixed biofilms by both microorganisms under hypoxic and normoxic conditions.

Aim of study:

This Case-control study aims to investigates effect of interaction of *Candida albicans* and *Porphyromonas gingivalis* in periodontitis at molecular level through following objectives:

1-Isolation and identification of *Candida albicans* and *Porphyromonas gingivalis* by using different media ,then identification of *P.gingivalis* by RT-q PCR.

2- Analysis of associated genes with important virulence factors include attachment and invasion gene by specific primers to *Candida albicans* .

3- Study the effect of *C.albicans* and *p.gingivals* alone and their combination on normal cell line (in vitro).

4-Study the effect of *Candida albicans* and *P*.gingivalis in tissue culture by detect specific biomarker by molecular method .

5-Detection of IL-17 level by ELIZA technique .

1.2. Literature Review

1.2.1 . Periodontitis

Periodontitis is a multifactorial chronic disease of the oral tissue, which is characterized by gingival inflammation and bone loss. More than 20 years ago, epidemiological studies reported that poor oral hygiene and tooth loss were significantly associated with oral squamous cell carcinoma OSCC, providing the first indication that oral bacteria might play a role in oral cancer development (Healy and Moran ,2019).

Clinically, periodontitis involves attachment loss around teeth, forming periodontal pockets, and bone destruction (Srivastava and Rana, 2022). Several studies have raised the heterogeneity of the microflora associated with periodontal disease. Indeed, various microorganism species, including Gram negative anaerobic bacteria organized in a complex biofilm, have been associated with the initiation and progression of periodontitis (Hussain *et al*.,2018).

Tezal *et al*.,(2009) reported that poor dental hygiene such as in the case of gingivitis and periodontitis are important risk factors for cancer inadequate poor oral hygiene is the primary cause behind periodontitis where accumulation of plaque and tartar stimulates production of inflammatory cytokines and prostaglandins to fight the infection.

Colonization of pathological bacteria is strongly correlated with inflammation and cancer progression. The immune system recognizes bacterial and viral invasion in periodontal pockets as foreign organisms damaging epithelial cells and attacks infected cells to remove the infection. Periodontitis is mainly caused by gram-negative anaerobic bacteria in dental biofilm which are responsible for secreting endotoxin substances destroying well-being of healthy epithelial cells (Peres *et al*.,2019).

In periodontitis, cellular migration and proliferation increases together with increased production of inflammatory cytokines, growth factors, enzymes

and some prostaglandins that are reported to be closely associated with cancer initiation and progression (Peres *et al*.,2019). Evidence indicates that chronic infection and inflammation are strongly associated with cancer (Peres *et al*.,2019).

A number of case-control studies describes the role of oral health and head and neck cancer and some cohort studies have explored the relationship between periodontitis and other types of systemic cancer reporting a significant positive association Tezal *et al*.(2007) .

A case-control study conducted in Beijing by Meyer *et al*.(2008)assessing the relationship between dentition and risk of oral cancer. This study recorded any missing tooth, gingivitis and periodontitis as a part of the oral exam. This study suggests that males and females suffering from periodontitis are more prone to develop oral cancerwhere males had a two to three-fold and females five to eight-fold increase in risk .

Sadighi Shamami *et al*. (2011) in a review of epidemiological research articles defines a clear association between tooth loss, periodontitis and carcinogenesis. Nine out of ten-case control studies reported significant increase in the risk of oral cancer in patients with periodontitis thereby indicating that there is a possible link between cancer and periodontal disease when modifying factors such smoking and drinking were controlled.

1.2.2. General Characteristic of *Candida* spp

Species of the genus *Candida* are found in their natural hosts, including humans. The genus *Candida* is distinguished by its oblong or oval shape, and it reproduces by bipolar bud (Alobaid *et al*., 2021). *Candida* grow on living tissue or culture media in the form of yeast or oval spherical cells and emerging yeast cells are cream-white in color range from 3 to 6 microns in length and may form what is known as pseudohyphae (Kadosh *et al* .,2020).

sometimes, this is called the case in the process of budding, identification methods have been developed for the purpose of diagnosing *Candida* species, such as the growth test on Chromagar *Candida* medium, where species grow on this medium in a range of different colors after an incubation period of 24 hours at 37°C (Rosiana *et al* .,2020), in addition to several differential tests Such as the formation of spores (Chlamydospores), which are characterized as large, thick-walled spores that contain a high content of proteins and fats, and the function of these spores remains opaque, which may represent a dormant stage of yeast, where these spores are formed in special conditions that differ from the normal state of growth such as Deficiency of Heat, Light, Nutrients and Oxygen, as well as *C. albicans* yeast is the main cause of fungal infection in humans, whether it is mucosal (Mucosal) or systemic (systemic) fungal infections (Villa *et al*.,2020).

The most common cause of superficial injuries to the nails and skin, a hot or humid environment of the body is important in the overgrowth of these yeasts because they stimulate their growth like skin folds in people with obesity, such as between the toes hand perineal and genitocrural (Webb *et al*., 2018).

The species of the genus *Candida* can be divided into two groups, *albicans* and non-*albicans*, which have the potential to cause infection, even partially in patients with somatic and immunocompromised (Webb *et al*., 2018).

Candida albicans is the main human pathogen among *Candida* yeast species. It is considered one of the yeasts that coexist in healthy people and is widespread in most environments as well as other species belonging to the genus *Candida* namely *C. krusei*, *C. glabrata, C. paraphsilosis, C. dubliniensis and C. tropicalis* (Thomaz *et al*., 2020).

Like the ability to Adhesion to cell walls (addition), which is the first step in the events of infection, and adhesion begins when a fibrous layer is

formed) of sugars on the surface of yeast cells, called cell adhesion, which helps to link yeast cells with carbohydrates and proteins. In the membranes of the host (Mourer *et al*.,2021).

This yeast is also characterized by its ability to form a germ tube, to which the mechanical strength is attributed to the resistance of immune cells or phagocytes in the host in addition to secreting enzymes that disintegrate the cells and tissues of the host and thus help the yeast to spread in the body host or extend between pus or chronic granulomas, or cause local infections suchas inflammation of the nails, skin, scalp, vagina, vulvitis and oral mucous membranes (Vila *et al*.,2020).

Among *Candida* species, *Candida albicans* is the most widespread yeast associated with healthy and pathologic oral conditions (d'Enfert *et al*.,2021). Indeed, this opportunist microorganism belongs to commensal microflora in the healthy human digestive tract, but can become pathogenic under the influence of general or local favorable factors. Periodontitis is a worldwide oral disease with a very complex etiopathogenesis, including dysbiosis and host immune responses that comaintain conditions for the occurrence of periodontal disease in susceptible individuals (Hajishengallis *et al*.,2017; , Lamont *et al*.,2018).

1.2.2.1.*Candida albicans*

It is classified as one of the most important types of opportunistic yeasts. These fungi form part of the normal flora of man. It turns into pathogens depending on the human immune status as a result of reduced cellular immunity and inhibition of the natural flora after antibiotic treatment that leads to the elimination of natural flora such as *lactobacilli* bacteria and exacerbation of *Candida* (Bohner *et al*., 2022).

This type of yeast is characterized by the presence of two dimorphic forms. The first form is single-celled yeast, and according to the environmental conditions in which it can be found, it grows in the form of yeast in a solid and

acidic medium that contains nitrogenous organic materials and sugar. As a carbon source, and another multicellular filamentous form (Multicellular filamentous form) (Rosiana *et al.*, 2020).

This species is characterized by the form of hyphae growing in acid culture medium that are neutralized with pH = 6.5 or more, media containing substances potato, dextrose, Or it may be pseudohyphae or the true hyphae, the transition from one form to another is due to intrinsic and extrinsic signals (Rane *et al*., 2019).

This process is of great importance in the pathogenesis of yeast, and this yeast can grow in different culture media such as blood agar, meat extract medium and Sabouraud dextrose cells, where the cells of this yeast appear under the microscope in an oval shape and may be irregular or elongated with a diameter ranging from It is between (10-12µ), and its soft and convex colonies may appear in a creamy white color and have a small yeast odor with a diameter of up to 0.5 mm in the case of growth for 18 hours, and when they remain for up to about a week with a diameter of 2 mm and may turn yellow, where It seems to have larger extensions and size, coarse and somewhat positive when using gram stain which is one of the most important diagnostic properties of white yeast, and the formation of the germination tube in the agricultural medium and under special environmental conditions, where the formation of this tube is an important diagnostic characteristic of these yeasts, as their ability to form squamous spores is another diagnostic feature of these yeasts (Lamoth *et al*., 2018).

1.2.2.2.Virulence Factors of *C. albicans*

A number of virulence factors support *C. albicans* to infect such complex host niches **(**Henriques, and Silva, 2021**)**. Virulence factors are found in many attributes like adhesive and invasive expression on the cell surface, biofilm production, secretion of hydrolytic enzymes and phenotypical switching (Staniszewska *et al*., 2020). Furthermore, *C. albicans* supports

virulence factors, colonize and avoid host defense acts through oral tissue adherence *C. albicans* (Fan *et al*.,2022).

Candida albicans must first react to environmental changes and transfer from the ordinary unicellular to invasive, multicellular filamentous forms to infect the host (Mundodi *et al*.,2021). Epithelial cell surfacing, germs and hydrolytic enzymes and hydrolytic materials are some of the essential factors , some of the hydrolytic enzymes are phospholipase, protease and lipase **(**Galocha *et al*., 2019).

Candida albicans produces a whole range of hydrolytic enzymes, which facilitates the adhesion of the pathogen to the cells host and is associated with the colonization of mucous membranes. This enables tissue penetration and digestion immune cells and antibodies (Yang *et al*.,2020; Dunker *et al*., 2021).

There are many reports in the literature that testify on the role of aspartyl protease (*sap*) in infections of *C. albicans* while examining the virulence characteristics of *C. albicans*, proved that that *sap* expression is associated with the formation of true hyphae, adhesion and phenotypic variability **(**Veni *et al*., 2022).

Hydrolytic enzymes (*sap*) coded by a family of ten *sap* genes catalyze hydrolysis peptide bonds (CO-NH) in proteins. *C. albicans* proteases digest cell membranes as well as immune system and antibodies it causes avoiding the host's defensive response in the process infections .*sap* proteins are key virulence factors having, participation in adhesion of *C. albicans* to host tissues (Permata *et al*., 2018).

Candida albicans adhesion to epithelial cells the mouth, esophagus, intestines and vagina is crucial stage of candidiasis development .Adhesion begins the process of *C. albicans* cell invasion into depth host tissues and this process follows that mannan is a component of the wall complex cellular containing highly glycosylated proteins, whose aspartic acid and serine/

threonine residues are joined sequentially by glycosidic bonds (N- and O- type) with sugar chains of different length (Mba and Nweze, 2020).

Mannan is detected on the outer surface of the cell wall *C. albicans*, and mannosebindings are responsible for the serospecificity of the strains ((Mba and Nweze, 2020). In adhesion to host cell surface are involved in *C. albicans* fimbrie , It has been shown that the main the structural subunit of fimbriae. According (Cavalcanti *et al*., 2017) . Mannoprotein sugar chains of *C. albicans* are involved in the adhesion process to cells the host. Cell surface hydrophobicity (CSH) is a virulence factor facilitating adhesion of *C. albicans* cells to tissues the host (Goswami, 2017). It has been proven that the inhibition of the process mannosylation increases the hydrophobicity cells and increased adhesion **(**Pérez-García *et al*, 2016).

Numerous authors have shown increased mannosylation external fimbriae proteins reduce adhesion *C. albicans* cells into epithelial tissues like also plastic medical tools (representing gateway to the development of disseminated candidiasis (Raška *et al*., 2007).

Hyphae forms have also been proven to adhere more strongly to the oral mucosa than the cells budding (blastoconidia) (Anderson *et al*., 2021).

The adaptability of *Candida* spp. is an important virulence determinant, as the fungus has to reproduce, respond to stress factors, and acquire nutrients in an efficient way to survive. Although *C. albicans* has been mostly considered an asexual fungus, thishas been denied by the discovery of a parasexual stage in its reproductive cycle. *C. albicans* can exist as sterile “white cells” and mating-competent “opaque cells” (Scaduto *et al*., 2014).

The two types of cells have the same genome, but they express different genes, and they have different metabolic preferences and different susceptibilities to antifungal drugs (Ciurea *et al*.,2020).

Sexual reproduction is inhibited by an acid pH and it is regulated by transcription factors encoded atthe mating-type loci (MAT) (Sun *et al*., 2015).

Mating competent/opaque cells communicate with mating incompetent white cells, enhancing the white cells' adherence and ability to form biofilms. This form of communication is conducted via pheromones (Dadar *et al*.,2018). *C. albicans*' response to stress is a factor of virulence, as it permits the yeast cells to survive and adapt. The response to stress is mediated by "heat shock proteins" (Hsps) (Jabra-Rizk *et al*., 2016).

The synthesis of Hsps-type proteins allows the survival of microorganisms at high temperatures, in environments with insufficient nutrients or under high oxidative stress conditions (Jabra-Rizk *et al*., 2016).

Heat-shock proteins (Hsps) 90, for example, is involved in the cellular dispersion inside biofilms, in *C. albicans* susceptibility to antifungals drugs, as well as in its temperature-dependent morphogenetic transition (de Aguiar Cordeiro *et al*., 2016).

The genetic exchange in *C. albicans* biofilms involves cell fusion and mating (Kowalski *et al* ., 2019). Inside a biofilm, sessile cells of *C. albicans* are less susceptible to antifungal drugs as compared with planktonic cells (free-floating cells). While forming biofilms in vivo¸ *Candida* spp. might use host components such as neutrophils, epithelial cells, proteins involved in inflammation (e.g., myeloperoxidase, alarmin S100-A9, Creactive protein) or site-specific proteins (Chong *et al*., 2018).

The interaction between *C. albicans* cells, for example, and the epithelium of the oral cavity is influenced by the presence of saliva. Some authors consider the possibility that proteins that are normally found in the saliva could act as bridging molecules between the hyphae and the epithelial cells and thus, they can facilitate the endocytosis of the yeast cells, but Sui *et al*.(2017) disproved this hypothesis. *In vitro* studies showed that even dead hyphae can adhere to

and are endocytosed by the epithelial cells found in the oral cavity, similar to live hyphae (Park *et al*., 2013).

Candida albicans exhibits a wide range of morphological phenotypes due to phenotypic switching and bud to hypha transition (Figure 1.1), (Gow and Yadav, 2017).

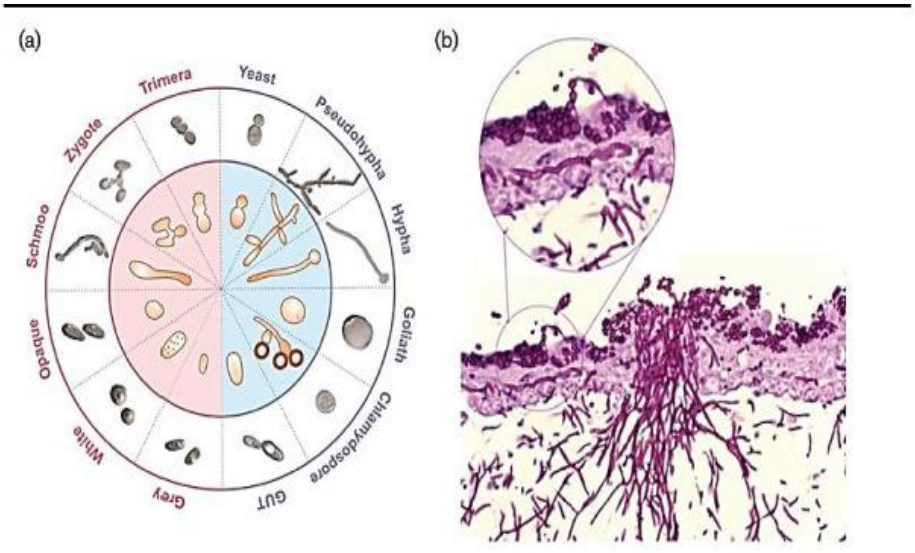


Figure (2.2): Morphology of *C. albicans*, showed (a): Morphotypes of *C. albicans*; blue sector represents the vegetative forms, whilepink sector represents the forms related to mating or changes in ploidy. (b): Superficial yeast colonization and invasion of chicken chorioallantoic membraneby *C. albicans* hyphal cells (Gow and Yadav, 2017).

Figure (1.1) :Morphology of *C albicans*(Gow and Yadav,2017).

The yeast-to-hyphae transition (filamentation) is a rapid process and induced by environmental factors, phenotypic switching is spontaneous, happens at lower rates, and in certain strains, up to seven different phenotypes are known **(**Kadosh *et al*., 2019).

Switching in *C.albicans* is often, but not always, influenced by environmental conditions such as the level of CO2, anaerobic conditions, medium used and temperature (Noble *et al*.,2017; Huang *et al*., 2019). In its yeast, form *C. albicans* ranges from 10 to 12 microns. Spores can form on the pseudohyphae called chlamydospores which survive when put in unfavorable conditions such as dry or hot seasons (Staib and Morschhäuser, 2007).

Unlike those of *Candida* species, *C. albicans* do not have a life cycle, but are either ovoid shaped budding yeast or ellipsoidally extended cells with restrictions of the sepsoidal tip, generally referred to as the pseudohyphae and as parallel wall-coated hyphae *C. albicans* have a period of life (Roselletti, *et al***,** 2019; Wijnants *et al*., 2021). Most pathogens are dimorphic, which helps them to exchange morphological states between the yeast and the germ tube **(**Kim *et al*., 2018). The fungal growth or invasion inside the host is often related to this exchange (Gow *et al*., 2017).

1.2.2.3.Pathogenesis

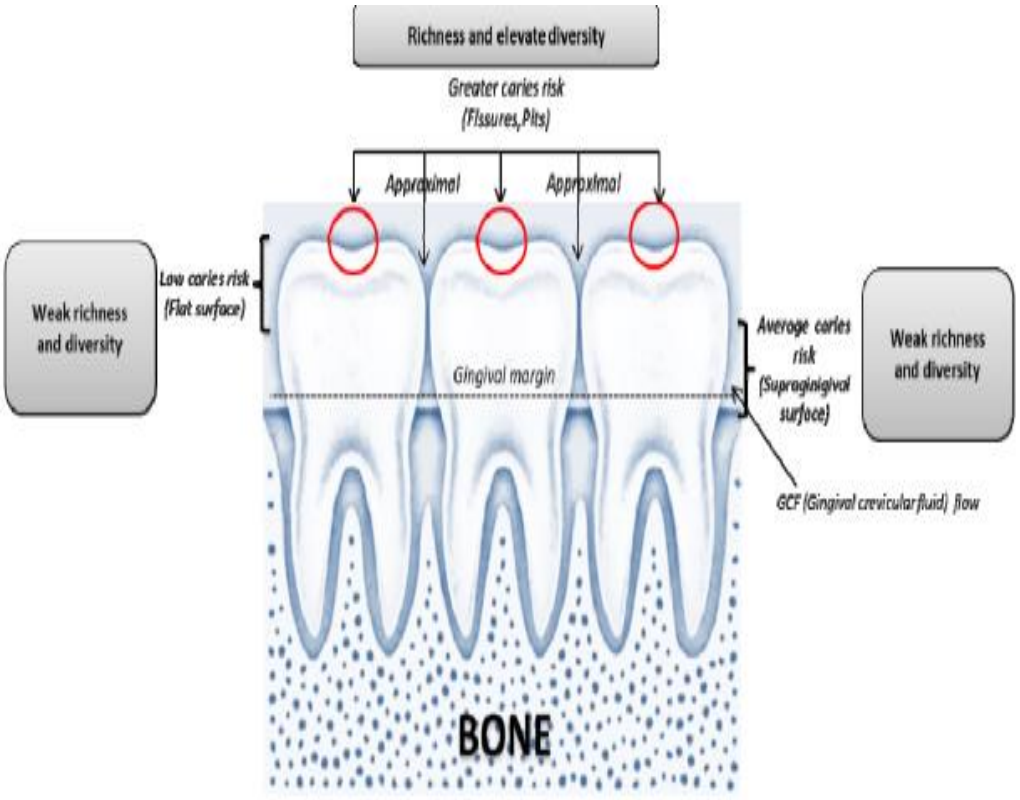
The formation of an oral candidiasis lesion is usually caused by the establishment of a complex biofilm containing *C. albicans* as well as other microorganisms (Contaldo *et al*.,2022). The biofilm adheres to oral surfaces, such as teeth, mucosa and dentures, and triggers an immune response heavy in neutrophils (McCall, *et al*.,2019). The biofilm is the perfect environment for the fungal cells to thrive, as the neutrophils cannot reach outside the body's own borders (Lalla *et al*.,2013).

C. albicans pathogenicity is linked to it phenotypic switching, between the commensal yeast form, and the invasive hyphal form (Gharaghani *et al* .,2021). Hyphae are elongations of the fungal cells, a tube without constrictions that can aid the pathogen in invading its host (Gharaghani *et al*., 2021).

C. albicans can invade the superficial layers of the oral epithelium, and cause proteolytic breakdown of E-cadherin (Lalla *et al*.,2013). E-cadherin is an important structural protein in the oral cavity, responsible for keeping the epithelium continuous, and a barrier against harmful substances. When E-cadherin is broken down, the tissue weakens and the protective barrier is compromised (Rouabhia *et al*.,2012). *C. albicans* uses this to migrate deeper into the tissue.

Interactions have been observed between *C. albicans* and *Porphyromonas gingivalis*, which have shown that cohesion caused by specific

proteins causes significant changes in gene expression by *P. gingivalis*, which could be used to increase infectivity. This means that interactions between different groups of microorganisms can trigger specific oral diseases such as the precancerous conditions. All the above show that the etiological flora of periodontal disease is not yet revealed and that several fungi can be a parameter responsible for the onset and progression of periodontal infection (Figure 1.2)(Sztukowska *et al* .,2018; Bottalico *et al*.,2016).



Figure(1.2). The risk associated with the areas that may be affected by caries and periodontal disease. Surfaces and locations with the highest variety and richness of oral microbial communities are more sensitive to caries and in the genital areas of periodontal disease. When tooth decay or periodontitis develops, the acidic environment reduces the variety and richness of local microbes.

1.2.2.4.The Biomolecular Mechanisms of *C. albicans*-Induced Oncogenesis

There are several hypothetical molecular mechanisms, that discuss the role of *Candida* cause dysplasia and malignant neoformations in the oral epithelium and can be mainly from (Patil *et al*.,2015; Sztukowska *et al.*,2018).

a. The production and release (via hyphal invasion) of nitrosamines, such as *N*-nitrosobenzyl-methylamine (e.g., caused by dysbiosis of the oral microbiota), which can lead to a tumor condition in mouse models (such as the Sprague-Dawley rats) (Sankari *et al.*,2015 ; Isacco *et al*.,2021).

b. An over-expression of P53, *Ki-67 labeling index*, and *Prostaglandin-endoperoxide synthase* 2 (COX-2) are some of the additional mechanisms by which *Candida* can affect malignant transformation into oral leukoplakia. P53 and Ki-67, which are markers of cell proliferation, have overexpression that is well established in malignant lesions, and COX-2, which is markedly increased in inflammation states and is associated with the release of prostaglandins, thus influencing cell proliferation, cell death, and tumor invasion (Warnakulasuriya, and Ariyawardana, ,2016 ; Alnuaimi, *et al.*,2016 ; Kiyoura and Tamai, 2015).

c. *Acid aspartyl proteinase* appears to be more present in oral lesions and therefore also in those with leukoplakia than in healthy subjects (Warnakulasuriya, and Ariyawardana, A ,2016).

The production of acid aspartylproteinase are putative virulence factors in candidiasis, and are why an acidic pH exists, thus degrading the sub endothelial extracellular matrix, as well as laminin 332 and E-cadherin. This induces dysplastic alterations and thus begins the *C. albicans* dissemination in the systemic circulation and therefore in the organs (Lim *et al.*,2017, Kiyoura and Tamai, 2015).

On the other hand, in a model of hyphal invasion (localized or uniform) of *Candida*, there is no difference between oral potentially precancerous disorders and oral squamous cell carcinoma. These biomolecular mechanisms

highlight the ability of *Candida* to influence malignant and cellular changes in oral leukoplakia (Kiyoura and Tamai, 2015).

d. Oral *Candida* infection is a cause of *up-regulation in proinflammatory cytokines* (interleukin (IL)-1α, IL-1β, IL-6, IL-8, IL-18, tumor necrosis factor (TNF)-α, IFN-γ, and GM-CSF), that influences the metabolic pathways and induces directly an endothelial dysfunction, playing a role in immune-related mechanisms with cancer development (Lim *et al*.,2017, Delaloye, and Calandra,2014; Jayachandran *et al*.,2016).

e. *C. albicans* can produce acetaldehyde (carcinogen due to mutagenic qualities in DNA) from precursors found in the oral cavity (metabolizing ethanol and glucose in high quantities, especially when associated with smoking and alcohol consumption)(Warnakulasuriya, and Ariyawardana,2016,Bombeccaria *et al*.,2016, Alnuaimi, *et al*.,2016). Thus, *Candida* can produce large quantities of acetaldehyde and acetyl-CoA synthetase (more in smokers) in cases of potentially malignant disorders and in oral carcinomas (concentrations of acetaldehyde and acetyl-CoA synthetase increase) compared to healthy individuals and those with ectodermal dystrophy and autoimmune polyendocrinopathy (with candidiasis) (Bombeccaria *et al.*,2016, Marttila *et al.*,2013).

However, the increase in the mutagenic amounts of acetaldehyde is more marked even in occupationally exposed workers to carcinogen and people with poor oral hygiene, than in healthy subjects, via the oral microbiota (*Streptococcus viridans* and resident fungi such as *Candida*) that can convert ethanol into acetaldehyde (possess the enzyme alcohol-dehydrogenase) (Gainza-Cirauqui *et al.*,2013). Indeed, the levels of acetaldehyde produced by *Candida* increase in proportion to the increase in alcohol consumption (Gainza-Cirauqui *et al*.,2013, O'Grady *et al.*,2020).

f. In oral squamous cell carcinoma, the reduction of β-defensins favors *Candida* superinfections. In chronic hyperplastic candidiasis, C. *albicans* is the

predominant species and is associated with high concentrations of alcohol dehydrogenase enzyme and P53 that suggests a dysplastic potential factor (Correia, *et al.*,2019). In fact, there is evidence that *Candida's* epithelial invasion can cause hyperplastic conditions (Figure 2.3) (Bombeccaria *et al.*,2016, Gupta, and Johnson, 2014).

g. The candidalysin (or 31-amino acid α-helical amphipathic peptide) is a cytolytic toxin of *C. albicans*. It is encoded by the ECE1 gene initially associated with fungal filamentation ability (release the toxin from the hypha) and host cell adhesion. Initially, ECE1 encodes 271 amino acid pre-proproteins that are cleaved by Kex8p enzyme into eight smaller peptides (Ece1-I to Ece1-VIII). Ece1-III6-93 is an epithelial immune activator and collaborates with the cytolytic activity of *C. albicans* (Moyes *et al.*,2016). Likewise, mmune activator and collaborates with the cytolytic activity of *C. albicans* (Moyes *et al.*,2016).

Likewise, candidalysin is an inducer for NF-κB and MAPK pathways. Candidalysin has been reported to excite granulocyte macrophage colony-stimulating factor GM-CSF, an essential molecule in carcinogenesis. After the macrophage death, the *C. albicans* can escape, survive, and outgrow other macrophages. On the other hand, it induces epithelial damage and elicits host inflammatory processes because it is a trigger for NLR family pyrin domain containing protein 3 (NLRP3) (Engku Nasrullah Satiman *et al.*,2020).

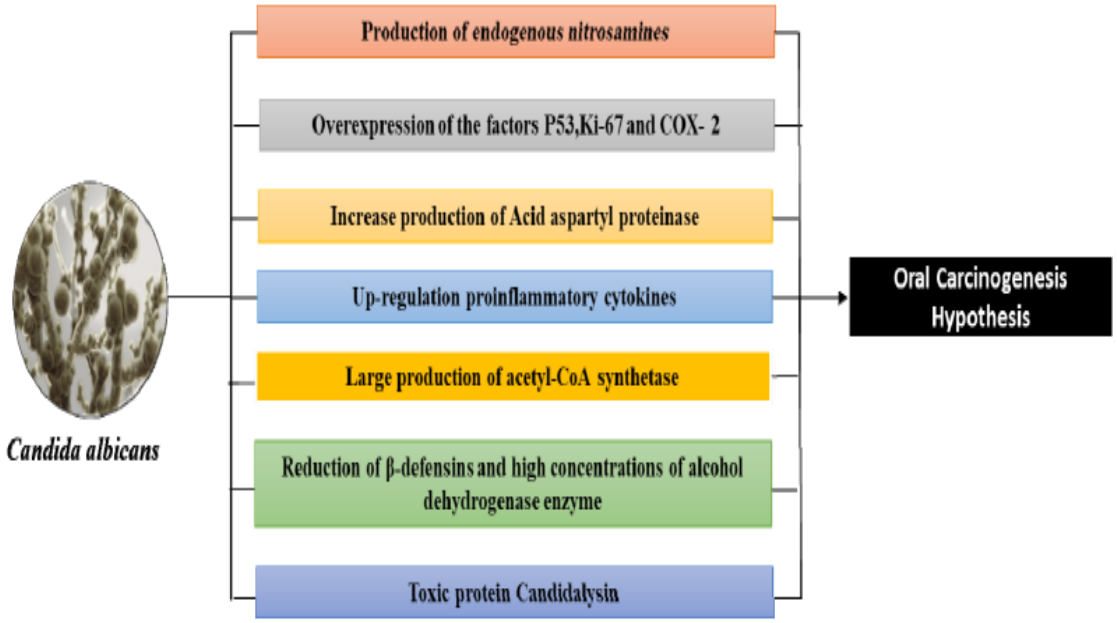


Figure 1. 3: The main hypothetical molecular mechanisms by which *C. albicans* can cause precancerous and malignant neoformations in the oral cavity.

1.3.1 Porphyromonas gingivalis

P. gingivalis is classified in the genus *Porphyromonas*, family *Porphyromonadaceae*, order Bacteroidales, class Bacteroides, phylum Bacteroidetes (Krieg *et al*.,2010). The bacterium is non-motile, Gram negative, rod-shaped, anaerobic, asaccharolytic and highly proteolytic. *P. gingivalis*, which is often found in deep periodontal pockets of humans, produces a broad array of potential virulence factors involved in tissue colonization and destruction as well as host defense perturbation (Silva and Cascales, 2021).

The major habitat of *P. gingivalis* is the subgingival sulcus of the human oral cavity. It relies on the fermentation of amino acids for energy production, a property required for its survival in deep periodontal pocket, where sugar availability is low (Bostanci and Belibasakis, 2012). Being an obligate anaerobe, *P. gingivalis* serves as the secondary colonizer of dental plaque, often adhering to primary colonizers such as Streptococcus gordonii and P. intermedia.

In the past few decades, *P. gingivalis* strains have been classified into invasive and non-invasive strains based on their ability to form abscesses in a mouse model. It has been demonstrated that the invasive strain of *P. gingivalis* possesses more pathogenic activities than the non-invasive strain both in vitro and in vivo (Dorn *et al*., 2000; Baek *et al*., 2015).

it was a member of the red complex (a group of three species including *P.gingivalis, Trepomema denticola* and *Tannerella forsythia*, which was strongly associated with each other and with periodontal disease site) and because it was the easiest of the three to grow and genetically manipulated, it became the most widely studied periodontal bacterium (Hajishengallis and Lamont,2012).

P. gingivalis can locally invade the periodontal tissues and evade the host defense system by utilizing a panel of virulence factors that cause disruption in the immune and inflammatory reactions. The potential virulence factors of *P. gingivalis* have been extensively described in several reviews (Mysak *et al*.,2014).

Porphyromonas gingivalis is strongly correlated with chronic periodontitis. Its chronic persistence in the periodontium depends on its ability to evade host immunity without inhibiting the overall inflammatory response, which is actually beneficial for this and other periodontal bacteria. Indeed, the inflammatory exudate (gingival crevicular fluid) is a source of essential nutrients, such as peptides and hemin-derived iron (Hajishengallis,2011).

Important features of *P. gingivalis*-mediated chronic periodontitis include the ability of the bacterium to adhere to and invade host cells, disseminate through host cells and tissues, and subvert host immunological surveillance and defense mechanisms(Carvalho-Filho *et al*.,2016).

Porphyromonas gingivalis rapidly adheres to the host cell surface followed by internalization via lipid rafts and incorporation of the bacterium

into early phagosomes. *Porphyromonas gingivalis* activates cellular autophagy to provide a replicative niche while suppressing apoptosis. The replicating vacuole contains host proteins delivered by autophagy that are used by this asaccharolytic pathogen to survive and replicate within the host cell. When autophagy is suppressed by 3-methyladenine or wortmannin, internalized *Porphyromonas gingivalis* transits to the phagolysosome where it is destroyed and degraded. For that reason, the survival of *Porphyromonas gingivalis* depends upon the activation of autophagy and survival of the endothelial host cell, but the mechanism by which *Porphyromonas gingivalis* accomplishes this remains unclear (Bélanger *et al*.,2006).

The harsh inflammatory condition of the periodontal pocket suggests that this organism has properties that will facilitate its ability to respond and adapt to oxidative stress. Because the stress response in the pathogen is a major determinant of its virulence, a comprehensive understanding of its oxidative stress resistance strategy is vital (Henry *et al*.,2012).

The ability of *Porphyromonas gingivalis* to cause adult periodontitis is determined by its arsenal of virulence factors. Biofilm formation and bacterial dipeptidyl peptidase IV (DPPIV) activity contribute to the pathogenic potential of *Porphyromonas gingivalis*. Furthermore, biofilm formation may enhance *Porphyromonas gingivalis* virulence through increased DPPIV activity. Because of their importance for bacterial colonization and growth, biofilm formation and DPPIV activity could present interesting therapeutic targets to tackle periodontitis (Clais *et al.*,2014).

1.3. 2.Porphyrmonas gingivalis Virulence factors

P. gingivalis expresses four major virulence factors: fimbriae, capsule, gingipains and lipopolysaccharides. The main function of fimbriae is to mediate adhesion and invasion into host epithelial cells. *P. gingivalis* fimbriae modulate proinflammatory cytokine production and also induce T cell

activation in mice . There are two main types of fimbriae that can be expressed by this pathogen, the major fimbria (FimA) and the minor fimbria (Mfa) (Liang *et al.*, 2020; Hasegawa and Nagano, 2021.).

The capsule of *P. gingivalis* aids in immune evasion, promoting survival of the bacterium within host cells and increasing virulence (Jia *et al.*, 2019). Along with immune evasion, encapsulation also reduces the host immune response in a variety of ways. Phagocytosis is reduced, survival increases in the presence of host cells, dendritic cell maturation induced by *P. gingivalis* is reduced, and virulence of *P. gingivalis* is enhanced compared to non-encapsulated *P. gingivalis* strains (Jia *et al.*, 2019).

P. gingivalis gingipains are proteases that function to degrade proteins into peptides as a source of nutrients. *P. gingivalis* utilizes hemin, iron-containing Protoporphyrin IX, as the primary form of iron , *P. gingivalis* acquires hemin from hemoglobin via the enzymic activity of gingipains (Priyadarshini *et al.*, 2013).

Gingipains also contribute to evasion of phagocytosis by degrading serum opsonins and host tissues (Hočevar *et al*., 2018). There have been three cysteine proteases purified from *P. gingivalis* with site-specific hydrolysis.Two of the proteases hydrolyze peptide bonds after Arginine residues and one hydrolyzes peptide bonds after Lysine residues. The proteases have recently been referred to as RGP and KGP or "gingipain R" and "gingipain K" (Cherian *et al*.,2019). Gingipain R aids in intracellular invasion and evasion of the host immune response by mediating vascular permeability through bradykinin release, enhancing binding of fimbriae to fibroblasts, and destroying the proteins of the complement system (Hočevar *et al*., 2018). Gingipain K mediates similar activities and is currently described as the most potent fibrinogenase (Lee, 2022).

The LPS of *P. gingivalis* has unique properties. Most gram-negative bacteria interact with TLR4 as the main transmembrane receptor for

lipopolysaccharides, while TLR2 is the main receptor to yeasts and gram-positive bacteria. *P. gingivalis* is an exception in that it can interact with TLR2 (Ingram *et al.*,2019; Hsieh e*t al*., 2020). Surface components of *P. gingivalis* including the LPS, lipoproteins, and fimbriae interact with TLR2 expressed on the surface of host cells , TLR2 mediates the expression of genes responsible for inflammation (De Andrade *et al.*,2019). The activation of TLR2 by *P. gingivalis* LPS may allow the pathogen to be able to regulate the class of the immune response *in vivo*, favoring a humoral response and enhancing its survival (Bourgeois, *et al*., 2019).

1.3.3. Pathogenesis of *Porphyromonas gingivalis*

The pathogenicity of *P. gingivalis* has been widely studied, including its ability to colonize surfaces of oral tissues, interact with other oral bacteria, induce a destructive immune response, and invade host cells (Ho *et al.*,2016).

Fimbriae were found to increase the tissue invasiveness and pro-inflammatory ability of *P. gingivalis* (Olsen *et al*.,2016).

Cell invasion by *P. gingivalis* occurs in oral epithelial cells, gingival fibroblasts, aortic and heart endothelial cells, and vascular smooth muscle cells (Chaudhuri *et al*.,2014; Ho *et al*.,2016).

P. gingivalis invasion is believed to protect the bacteria against environmental challenges including innate immune surveillance systems and antibiotic treatment (Damgaard *et al*.,2015). This likely plays a pivotal role in chronic bacterial infection (Park *et al*.,2016).

Previous work has shown that binding of *P. gingivalis* to red blood cells (RBCs) restricts phagocytosis of the bacterium by monocytes and neutrophils. This has led to the hypothesis that RBCs may also affect *P. gingivalis*-stimulated release of pro-inflammatory cytokines and production of intracellular reactive oxygen species (ROS) by neutrophils (Damgaard *et al*.,2017).

The perturbation of epithelial cells by bacteria is the first stage in the initiation of inflammatory and immune processes which eventually cause destruction of the tissues surrounding and supporting the teeth which ultimately result in tooth loss (Kinane *et al.*,2008).

Porphyromonas gingivalis can locally invade periodontal tissues and evade the host defense mechanisms. In doing so, it utilizes a panel of virulence factors that cause deregulation of the innate immune and inflammatory responses (Bostanci *et al.*,2012).

Porphyromonas gingivalis rapidly adheres to the host cell surface followed by internalization via lipid rafts and incorporation of the bacterium into early phagosomes. *Porphyromonas gingivalis* activates cellular autophagy to provide a replicative niche while suppressing apoptosis. The replicating vacuole contains host proteins delivered by autophagy that are used by this asaccharolytic pathogen to survive and replicate within the host cell. When autophagy is suppressed by 3-methyladenine or wortmannin, internalized *Porphyromonas gingivalis* transits to the phagolysosome where it is destroyed and degraded. For that reason, the survival of *Porphyromonas gingivalis* depends upon the activation of autophagy and survival of the endothelial host cell, but the mechanism by which *Porphyromonas gingivalis* accomplishes this remains unclear (Bélanger *et al.*,2006).

The harsh inflammatory condition of the periodontal pocket suggests that this organism has properties that will facilitate its ability to respond and adapt to oxidative stress. Because the stress response in the pathogen is a major determinant of its virulence, a comprehensive understanding of its oxidative stress resistance strategy is vital (Henry *et al*.,2012).

The ability of *Porphyromonas gingivalis* to cause adult periodontitis is determined by its arsenal of virulence factors. Biofilm formation and bacterial

dipeptidyl peptidase IV (DPPIV) activity contribute to the pathogenic potential of *Porphyromonas gingivalis*. Furthermore, biofilm formation may enhance *Porphyromonas gingivalis* virulence through increased DPPIV activity. Because of their importance for bacterial colonization and growth, biofilm formation and DPPIV activity could present interesting therapeutic targets to tackle periodontitis (Clais *et al*.,2014).

Porphyromonas gingivalis is strongly correlated with chronic periodontitis. Its chronic persistence in the periodontium depends on its ability to evade host immunity without inhibiting the overall inflammatory response, which is actually beneficial for this and other periodontal bacteria. Indeed, the inflammatory exudate (gingival crevicular fluid) is a source of essential nutrients, such as peptides and hemin-derived iron (Hajishengallis *et al*.,2011).

Porphyromonas gingivalis contributes to the pathogenesis of aggressive periodontitis by inducing high levels of proinflammatory cytokines, such as IL-1β and IL-6 by peripheral $CD4^+$ T helper cells (Gonzales *et al*.,2014).

Porphyromonas gingivalis serotypes K1 and K2 but not others are associated with an increased production of the osteoclastogenesis-related factor RANKL. This important information suggests that these serotypes could elicit a greater bone resorption in vivo and have a significant role in the periodontitis pathogenesis. Destructive periodontitis is associated with a Th1-Th17-immune response and activation of RANKL-induced osteoclasts. In addition, *Porphyromonas gingivalis* K1 and K2 serotypes induce a strong Th1-Th17-response. These *Porphyromonas gingivalis* serotypes induce higher osteoclasts activation, by increased Th17-associated RANKL production and an antigen-specific memory T lymphocyte response (Vernal *et al*.,2014).

Chronic *Porphyromonas gingivalis* oral infection prior to arthritis induction increases the immune system activation favoring Th17 cell responses

which ultimately accelerate arthritis development. These results suggest that chronic oral infection may influence rheumatoid arthritis development mainly through activation of Th17-related pathways (Marchesan *et al*.,2013).

Salivary concentrations of metalloproteinase MMP-8, interleukin IL-1*β*, and *Porphyromonas gingivalis* are associated with various clinical and radiographic measures of periodontitis. The CRS index, combining the three salivary biomarkers, is associated with periodontitis. High salivary concentrations of metalloproteinase MMP-8, interleukin IL-1*β*, and *Porphyromonas gingivalis* have been associated with deepened periodontal pockets and alveolar bone loss and MMP-8 and IL-1*β* with bleeding on probing (Salminen *et al*.,2014).

The bacterium utilizes amino acids as energy and carbon sources and incorporates them mainly as dipeptides. Therefore, a wide variety of dipeptide production processes mediated by dipeptidyl peptidases (DPPs) could be beneficial for the organism (Ohara-Nemoto *et al*.,2014).

1.4.Role of interleukin-17 in Periodontal Disease

Periodontal disease is a multifactorial disease affecting the supporting tissues of the teeth and many etiologic factors are implicated in etiopathogenesis of periodontal disease. Although pathogenic bacteria have been the main causative factor in periodontal disease, host response accounts for the majority of periodontal destruction by releasing various inflammatory mediators, which has a negative impact on the periodontium. The host immune responses are regulated by various classes of T-cell subsets. Initially, periodontal disease can be explained with the T-helper 1/T-helper 2 paradigm. However, the discrepancies associated with it have led to the discovery of T-helper 17, which is responsible for the secretion of the cytokine interleukin-17 (IL-17) (Preeja, and Sivadas,2021).

IL-17 is a proinflammatory cytokine released from activated Th17 cells. Besides IL-17, TNF-α and IL-6 are released from Th17(Harrington *et al*.,2005).

IL-17 plays a major role in immune response because it stimulates the secretion of various chemokines resulting in recruitment of neutrophils and macrophages causing the subsequent clearance of pathogens, IL-17 mediates the actions of adaptive and innate immune systems resulting in proper regulation of immune response. Immune responses if not properly orchestrated can adversely affect the host which is manifested as bone destruction in periodontal disease. Evidence from various studies(Zwicky *et al*.,2020) substantiated that the destruction of bone in periodontal disease is mainly by immune response of the host rather than due to infectious microorganisms.

The interplay between osteoblasts and osteoclasts in bone remodeling has a crucial role in maintaining bone homeostasis. The differentiation of osteoclasts is under the control specific factors released from B and T lymphocytes (Jolink *et al*.,2017).

The occurrence and development of periodontitis involves a series of immune and inflammatory reactions. Periodontal tissues damage caused by periodontitis is mainly attributing to the host's immune response to infected microorganisms and their toxic products, not just directly caused by the infected microorganisms. The innate and adaptive immune defense and inflammatory defense that occur when the body prevents microbial invasion and diffusion will damage the local periodontal tissues. Therefore, the protective and destructive mechanism of the host immunity is an important link in the progression of periodontitis. When periodontal tissue was infected by pathogens such as *Tannerella forsythia*, *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans* and *Prevotella intermedia*, neutrophils and macrophages phagocytize and kill pathogens, which not only

play an immune activation and regulatory role, but also promotes the local inflammatory response of periodontal tissue (Nussbaum and Shapira, 2011; Kinane *et al*.,2017).

Activated B cells secrete antibodies while plasma cells secrete TNF-α, IL-6, IL-10, TGF-β and MMPs (Berglundh *et al*.,2007). Local infiltrating plasma cells during periodontal inflammation may be an important reason for the imbalance between MMPs and its blocker TIMPs. This lone-term chronic inflammatory response results in the absorption of alveolar bone by osteoclasts and the degradation of periodontal membrane fibers by MMPs. The ratio of Th17/Treg cells in gingival tissue and peripheral blood of patients with chronic periodontitis was significantly higher than that of healthy people (Okui *et al*.,2012), and the number of γδT cells in gingival tissue was also higher than that of healthy people (Barel *et al*.,2022).

As what mentioned before, both Th17 cells and γδ T cells are the cell sources of IL-17A, suggesting that the expression level of IL-17A in periodontal region of patients with chronic periodontitis is significantly higher than that of healthy people, which has been confirmed by many studies in recent years (Zenobia and Hajishengallis,2015 ; Kang *et al.*,2021). Furthermore, it has been reported that the expression level of IL-17A mRNA in gingival tissue of patients with chronic periodontitis was higher than that of patients with gingivitis (Thorbert-Mros *et al.*,2019).

Although IL-17 has a major role in periodontal pathogenesis whether they have a protective or destructive role in periodontal disease is still a matter of controversy (Xiong *et al.*,2019)

A previous study done by Yu *et al*(2007) found that the IL-17 has a bone protective effect in periodontal disease due to its crucial role in neutrophil

regulation. This can be explained due to the fact that neutrophils form the first line of defense and they play an active role in controlling periodontal infection; hence, impairment of neutrophil function can lead to increased microbial load. In sterile inflammatory conditions such as rheumatoid arthritis or other autoimmune disorders, IL-17 can cause tissue destructive effects.

However, in infectious inflammatory conditions such as periodontal disease, IL17 is having tissue protective actions due to its key role in the recruitment of neutrophils and other immune cells, thereby limiting the spread of periodontal infection.,Thus, Th17 cells play a key role against protection from bacteria and fungi which are not adequately dealt with Th1-mediated immunity.(Liu *et al*.,2021). However, there are various studies reporting elevated levels of IL-17 in the destructive phase of periodontal disease(Kumar *et al*.,2021).

Another previous study implicated a destructive role of Th17 cells due to the fact that Th17 acts as an osteoclastogenic lymphocyte that links T-cell activation to bone resorption , Therefore, IL-17 is a proinflammatory cytokine with dual action in various inflammatory disorders and further studies should be done in this field to evaluate the predominant role played by IL-17 in periodontal disease (Sato *et al*.,2006).

outer membrane protein of Porphyromonas gingivalis can induce the production of IL-17 in patients with periodontitis, and after its stimulation, it is detectable in patients with periodontitis than in those with gingivitis((Kumar *et al*.,2021) .

Another study investigated for the presence of IL-17 in periodontal lesions and to determine the effect of IL-17 on IL-6 production in human gingival fibroblasts. They found that IL-17 is produced in periodontal lesions and is involved in Th1 modulation and also enhances inflammatory reactions through mediators from gingival fibroblast in periodontal disease(Takahashi *et al*.,2005).

Bartemes and Kita (2018) evaluated for the presence of IL-17 in gingival crevicular fluid (GCF) and from cell cultures of gingival tissue in patients with periodontitis and reported its presence in GCF and gingival cell cultures suggesting a role for IL-17 in periodontal pathogenesis.

Another study done by Chen *et al.*(2015) investigated Th1/Th2/Th17 cytokine levels in plasma and GCF in chronic periodontitis patients and healthy controls. The results showed a stronger correlation between IL-17/IL-4 and IL-17/IL 10 in periodontitis patients than in healthy controls.

Cheng *et al.(*2014) in a review proposed the possible role of IL-17, and IL-17 producing CD4+ T cells (also called Th17 cells) in inflammatory periodontal disease.They suggested the crucial role of IL-17 and Th17 cells in periodontal disease and also the proof from animal studies, indicating the potential role in gingival inflammation and bone resorption in periodontitis.

1.5.Cell Line Culture

A cell culture that is derived from one cell or the set of cells derived from the same type and in which under certain conditions the cells will proliferate indefinitely in the laboratory. Cells are separated in two classes : Eukaryotic and Prokaryotic Cells.The clone or clones of cells derived from a small piece of tissue develope in culture. Cell is the basic structural unit of life that are bounded by plasma membrane i.e divided on the basis of the presence of cell or plasma membrane. Cell lines were the clones of animal or plant cells that grow on a suitable nutrient media in the laboratory which has various applications in the field of biochemistry and cell cell biology and biotechnology (Chaudhary& Singh, 2017).

Origin of cell line-1950-55. Hela cell, is a cell type in an immortal cell line that was used in scientific research. It is the oldest cell and most commonly used human cell line(Rahbari *et al.*,2009).Early, the primary cell line said to be named after a "Helen Lane" to conclude the fact that cells were taken without her knowledge or consent by Gey. Despite this attempt, her name was used by

the press within a few years of her death. These cells were treated as cancerous, as they are obtained from a biopsy taken from a lesion on the cervix as part of diagnosis of cancer. A conflict still continues on the classification of these cells.Culture that were derived from main tissue is known as primary culture. A primary culture has becomes a cell line when it is transferred into the other culture vessel. Adherent cultures, the cells were separated using a protease, such as trypsin, and/or a chelating agent, such as EDTA, and subdivided — that process was known as passaging(Marx .,2014).

For cells that will grow in suspension, the culture was split into new culture vessels. Under these circumstances the specialized culture conditions are used, within a few passages a relatively uniform population of proliferative cells was selected. This population was probably representative agent of the cells that divide when the tissue of origin is suffered, and will carry on growing until the end of the natural proliferative lifespan were reached and senescence occurs. As far as the cells proliferate, they show little or no law of tissue-specific differentiation. However, given the suitable signals, they can regenerate a functional tissue. Culture derived from primary subculture is known as cell lines and from continous culture or derived from passage of cell lines is known as subclones (Luong, *et al.*,2011).

The term cell line refers to the propagation of culture after the first subculture. In other words, once the primary culture is sub-cultured, it becomes a cell line. A given cell line contains several cell lineages of either similar or distinct phenotypes. It is possible to select a particular cell lineage by cloning or physical cell separation or some other selection method. Such a cell line derived by selection or cloning is referred to as cell strain. Cell strains do not have infinite life, as they die after some divisions(Marx .,2014).

The gingiva, both anatomically and functionally, is a unique structure with gingival fibroblasts (GFs) as the predominant cells of the gingival connective tissue. The existence of various subpopulations of GFs has been

reported (Fournier *et al*., 2013, Fournier *et al.*, 2010) However, these subpopulations are phenotypically different, sharing fibroblast-like structures and requiring identical growth conditions *in vitro* (Fournier *et al*., 2013).

A distinct property of the gingival cells is their role during scarless wound healing. Upon damage to oral tissues, the inflammatory response is manifested by a unique cytokine response from the GFs. At the same time, the GFs display fetal fibroblast-like properties including those related to migration and the production of migrating stimulation factors (David *et al*., 2014, Haekkine*n et al*., 2000). This healing capacity of the gingiva and its regenerative capacity has resulted in extensive research to identify the resident stem cell population within the gingiva with the ability to self-renew (Politis *et al*., 2016).

Gingival tissue represents an ideal source of tissue biopsies and GFs due to its accessibility and significantly reduced donor site morbidity compared to other dental tissues (Jin *et al*., 2015, Mostafa *et al*., 2008, Zhang *et al*., 2009). The literature offers overwhelming evidence to support the hypothesis that a subgroup within the GF cell population possesses mesenchymal stem cell (MSC) properties – and are thus called gingival mesenchymal stem cells (GMSCs) (Gardin *et al.*, 2016). Whether sorted (enriched) or unsorted, several studies have demonstrated that these GMSCs are able to differentiate into more than one lineage *in vitro* including osteogenic, chondrogenic, and adipogenic (Fournier *et al.*, 2010, Marynka-Kalmani *et al.*, 2010, Mitrano *et al.*, 2010).

1.6. Harvey-Ras gene expression

Rat sarcoma virus (RAS) is a protooncogene and its three family members are Harvey-Ras (H-Ras), Neuroblastoma Ras (N-Ras) and Kristen Ras (K-Ras having isoforms A and B) (Murugan *et al*.,2012). This family encodes Ras proteins having inherent guanosine triphosphatase (GTPase) activity and stimulates downstream signaling cascade via Raf-MEK-ERK, PI3K/AKT or c-Jun N-terminal kinase (JNK) pathways involved in cellular

proliferation, migration, adhesion and differentiation after growth factor stimulation such as EGFR (Krishna *et al*.,2018).

This gene and its signaling pathway is frequently mutated in oral cancer and mostly the mutations (T81C, Q61R, G12V and G13R) are reported in H-Ras (Akiyama *et al.*,2016). These mutations are mostly reported in smokers, betel quid chewers and also show ethnic variations (Krishna *et al*.,2018).

The studies have highlighted the role of H-Ras mutations in treatment failure or development of resistance to EGFR tyrosine kinase inhibitors such as cetuximab and erlotinib in oral cancer patients (Hah *et al.*,2014). The proposed mechanism of therapeutic resistance to EGFR TKIs include constant stimulation of downstream signaling pathways by mutated RAS gene in oral cancer via special group of genes such as CCND1, c-MYC, BCL-XL and BCL-2 (Rampias *et al*.,2014).

Harvey-Ras (H-Ras) is an important guanosine triphosphatase protein for the regulation of cellular growth and survival. Altered Ras signaling has been observed in different types of cancer either by gene amplification and/or mutation. The H-Ras oncogene mutations are well reported, but expression of the H-Ras gene is still unknown (Krishna *et al.*,2018).Ras gene family members play a relevant role in cancer, especially when they are mutated (Sciacchitano *et al*.,2021).

A number of transforming cellular oncogenes have been identified and isolated from different types of human tumor. Categorization of these oncogenes provides an understanding of cancer at the molecular level. In this context, attention has focused on the Harvey-Ras (H-Ras) gene in oral cancer. The RAS gene family consists of three functional genes, H-Ras, Kristen Ras (K-Ras: isoform A and isoform B) and Neuroblastoma Ras (N-Ras) encoding four highly similar, small and conserved Ras proteins (or p21 proteins), which located on the inner surface of the plasma membrane(Han, *et al.*,2017).

The Ras proteins have intrinsic guanosine triphosphatase (GTPase) activity that transduces the growth signal from the cell surface to intracellular effectors through mitogenic activating protein kinase (MAPK), c-Jun N-terminal kinase (JNK) and p38-kinase pathways, which regulate normal cell proliferation function(Krishna *et al.*,2015). The RAS GTPases activation regulates through cycle between GDP bound inactive and GTP bound active state with the help of guanine nucleotide exchange factors and GTPase-activating proteins (GAPs)(Kratz *et al.*,2007).

Previous research has improved the understanding of the structure, processing and signaling pathways of RAS in cancer cells and opened up new avenues for inhibiting RAS function (Ferrer *et al.*,2018;Marín-*Ramos et al.*,2019).

Abnormally activated RAS proteins regulate the function of major signaling pathways involved in the initiation and development in one-third of human cancers, RAS proteins act as a cellular switch that is turned on by extracellular stimuli, resulting in the transient formation of an active, GTP-bound form of RAS that activates various signaling pathways which regulate basic cellular processes (Khan, *et al.*, 2019; Li *et al.*,2018; Lindsay *et al.*,2018).

2.1. Subjects of the Study

This study involved (150) patients were sample collected from periodontal patients, the samples include saliva ,cotton swab and paper point from each periodontitis patients were admitted to specific dental health center and outpatient clinics of dentistry in Al-Hillah city/ Iraq during the duration from (April 2022 to September 2022).These patient were diagnosed by the dentist. Each patient were underwent-detailed history regarding age (the age of patients ranged from 5 to70 years) including both males and females., symptoms of infection .

2.1.1 Ethical Approval

The necessary ethical approval from ethical committee of the hospitals and patients and their followers must obtained. Moreover, all subjects involved in this work are informed and the agreement required for doing the experiments and publication of this work is obtained from each one prior the collection of samples.

2.2.Materials

2.2.1. Laboratory Apparatuses and Instruments

The main scientific apparatus, and technical instruments with disposable materials respectively, those were employed during the course of this study listed down in Tables (2-1) and (2-2).

Table (2-1): Scientific Laboratory Apparatus.

N	Item	Company	Country
1	Autoclave	Herayama	Japan
2	Bacteriological cabinet	Labogene	Denmark
3	Benson burner	Membrane	Germany
4	Camera	Nikon	Japan
5	Candle – jar	Memmert	Germany
6	Centrifuge	Kokusan	Japan

7	Deep freezer 20-°C	Jermaks	Germany
8	Electrophoresis	Biometra	USA
9	Incubator	Memmert	Germany
10	Light microscope	Stermite Olympus A &D	Japan
11	Micro centrifuge	Hamburg	Germany
12	Micropipettes(different size of Micropipettes)	Gillson Instruments	France
13	Nano drop	Biometra	USA
14	Oven	GS	Taiwan
15	PH meter	Orient	USA
16	Refrigerator	Concord	Italy
17	Sensitive Balance	Kern	Germany
18	Thermocycler	BioRad	USA
19	UV transilluminater	Wised	Korea
20	Vortex	IKA	USA
21	Water path	Polyscience	USA
22	ELISA Reader and Washer	BioTek	USA

Table (2-2): Technical Instruments and Disposable Materials.

N	Item	Company	Country
1	Eppendrof Tube	Biobasic	Canada
2	Glass Slides	Sail brand	China
3	Medical Gloves	Broche	PRC
4	Microscopic Cover Slide	Gitoglas	China
5	Parafilm	Bemis	USA
6	Pettri dishes	Himedia	India
7	Plastic Test Tubes 10ml	Afco	Jordan

8	Sterile Swabs	Afco	Jordan
9	Syringes	Dolphin	Syria
10	Test Tube Rack	Himedia	India
11	Tips	Afco	Jorden
12	Wooden Sticks	Supreme	China

2.2.2. Chemical Materials

Itemized down in **Table (2-3)**, the main chemicals utilized in this study.

Table (2-3): Chemical Materials and Reagents.

N	Item	Company	Country
1	Agarose	iNtRON	Korea
2	**Agar**	**Himedia**	**India**
3	**Dextrose**	**Himedia**	**India**
4	Ethanol 70%	GCC	England
5	Ethidium Bromide	Biotech	Canada
6	Glycerol	B.D.H	England
7	Gram stain kit	Sigma	Germany
8	KOH	Schuchariot	Germany
9	Normal Saline	Pharmaline	Egypt
10	Nuclease Free water	Bioneer	Korea
11	TB-Buffer	Biotech	Canada
12	Tetramethyl–p–phenylen Diamin Dihydrochloride	Fluka	UK
13	Phenol	BDH	England
14	Proteinase k	Promega	USA
15	Sodium chloride	BDH	England
16	Tris – Hcl	BDH	England
17	Interleukin(17) kit	BT-Lab	Korain

2.2.3. Biological Material

The main biological materials utilized in this study was listed in table(2-4).

Table (2-4): Biological Materials.

N	Item	Company	Country
1	Blood Agar Base	Oxoid	UK
2	CHROM agar candida	Liofilchem	Italy
3	Brain Heart Infusion Broth	Oxoid	UK
4	Columbia Agar	Himedia	India
5	Sabouraud Dextrose Agar	Hi media	India
6	Potato dextrose agar	-	Prepare in laboratory
7	*P. gingivalis* agar (P.GING)	-	Prepare in laboratory

2.2.4. Polymerase Chain Reaction Kits

In table (2-5) chemical materials used in PCR experiment in this study with their companies and countries of origin are listed.

Table (2-5): PCR Kits with their Remarks.

No.	Kit	Company	Country
1	**Genomic DNA Extraction Kit (Bacteria)**	**iNtRON**	**Korea**
	Buffer CL		
	Buffer BL		
	Buffer WA		
	Buffer WB		
	Buffer CE		
	Spin column/ Collection tube 2ml		
	Proteinase K 22mg/ml		
	RNase A 10mg/ml		

2	**Maxime PCR PreMix kit**	**Bioneer**	**Korea**
	Taq DNA polymerase		
	dNTPs (dATP, dCTP, dGTP, dTTP)		
	Tris-HCl pH 9.0		
	KCl		
	$MgCl_2$		
	Loading dye		
3	**TransSript Green One step qRT-PCR Super mix**	TRAN	
	TransSript Green One step RT\RI Enzyme Mix		
	2x PerfectStar Green One step qPCR Super Mix		
	Passive Reference Dye(50x)		
	RNase- free water		
4	DNA Lader(100bp)	NEXmark™	Korea
5	**Master Mix**	Promega	USA

2.3. Methods

2.3.1. Sample Collections

After obtaining the permission from the patients for examination and sampling the proper specimens collected for bacteriological and fungal analysis are described below. These specimens were collected in proper ways to avoid any possible contamination under use Rubber dam.

Samples were obtained from the necrotic pulp or most diseased sites with individual sterile paper points for *P.gingivalis* ,and cotton swab for

C.albicans,.paper point which were placed in necrotic pulp for 15 sec, In the present study three paper point were used for the collection sample (Singamaneni *et al* ;2010).

Then sterile paper points placed in tube contain 5 ml of BHI broth, after that cultured on Blood agar plates and on selective media anaerobically ;in the anaerobic incubator with using jar and gas back at 37°C for 7-14 days plus (10% Co_2).Then subjected to identification according to the cultural properties, microscopic examination and Biochemical test (MacFaddin,2000 and Forbes *et al* .,2007).The cotton swab were streaking on on S. agar & chromo agar for isolation *C.albicans* as shown in Figure(2.1).

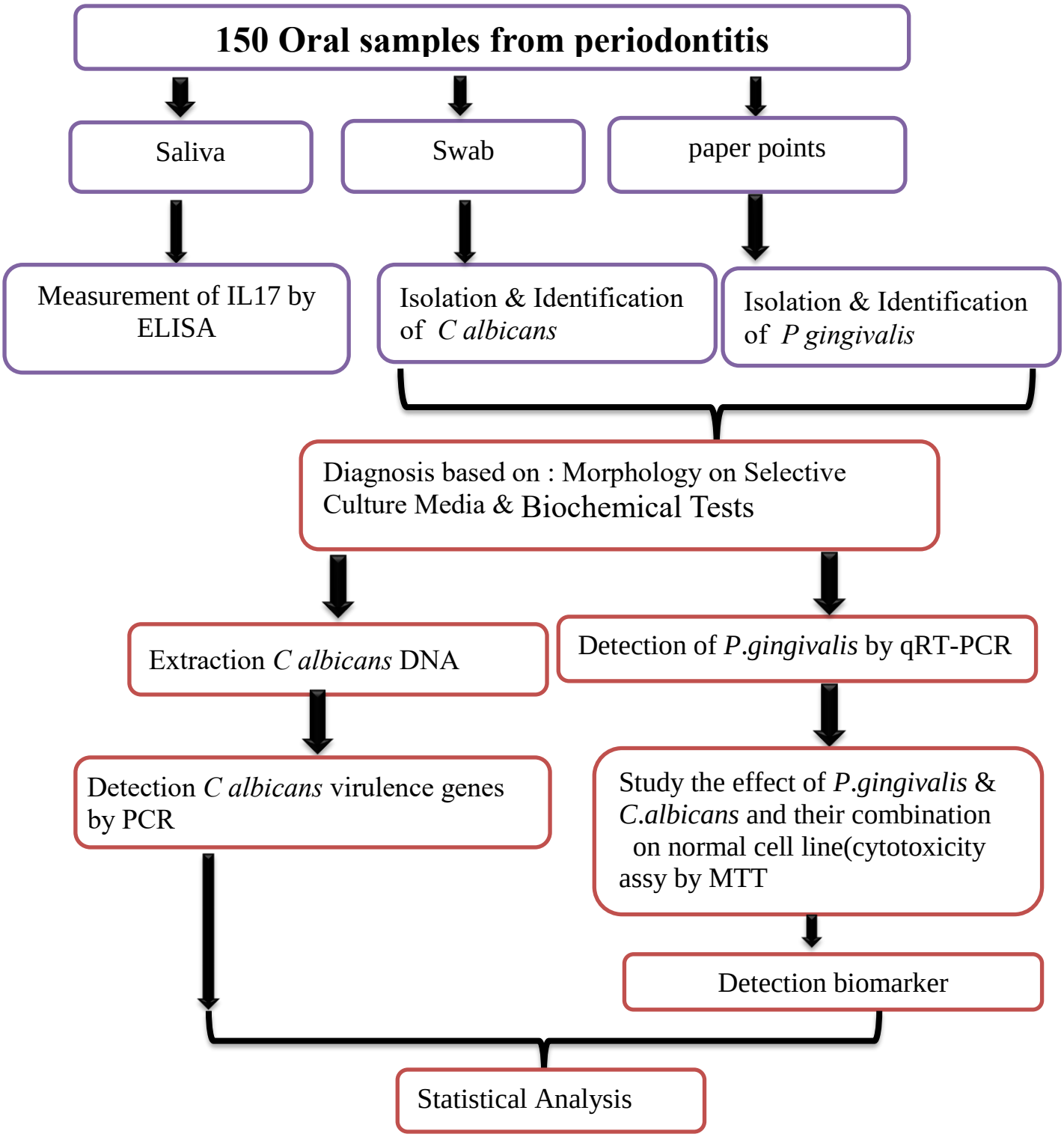


Figure (2-1): Demonstrating the Study analysis.

2.3.2. Preparation of Materials

2.3.2.1 Preparation of Stains ,Solutions and Reagents

2.3.2.1.1 Gram Stain

Ready to use stains and reagents: crystal violet, gram iodine, ethanol 70% and safranin stain, which were used for staining bacterial isolates for microscopic examination (Forbes *et al.*, 2007).

2.3.2.1.2 Catalase Reagent (3%)

This reagent was prepared by adding a 3ml of 6% H2O2 solution to 1 ml of D.W and stored in a dark container , to identify catalase producing bacteria (Forbes *et al.*, 2007).

2.3.2.1.3 Kovac's Reagent

It was prepared by dissolving 5g of p-dimethyl-l-aminobenzal-dehyde in 75 ml of isoamyle alcohols, then 25 ml of concentrated HCl was added carefully and gradually, and kept at 4°C until use. The reagent was used for detection the presences of indole (McFadden, 2000).

2.3.2.2. Preparation of Culture Media

The media were shown in Table (2-4) for isolating and diagnosing bacteria , fungi and yeasts according to the instructions of the manufacturer installed on the packages or according to the references of the scientific references and pH modification where necessary using the KOH and HCL the diluted dose were measured using pH strip. All the media were sterilized in a temperature of 121 C° , under pressure of 15 PSI for 15 minutes and the most important of these media is :

2.3.3.1.1. Blood agar medium

Blood agar medium performed according to manufacturer by dissolving 40 gm blood agar base in 1000 ml D.W. The medium was autoclaved at 121ºC for 15 minutes at15 pound/inch$_2$, cold to 45ºC and 5% of fresh human blood was added. It was used as enrichment medium for the bacterial isolates and to determine their ability to hemolysis RBCs (Wanger *et al.*, 2017).

2.3.3.1.2. Brain heart infusion broth(BHI)

This medium prepared by melting 37g in 1000 ml of Distille water (D.W.) into and heated to boiling to dissolve completely, as well adding chloramphenicol and streptomycin to make the fungal growth medium more competitive and preventing the growth of bacteria, sterilize at 121Ċ° for15min (Washington, 2012). This medium is useful for the grow for stored wide range of microorganisms.

2.3.3.1.3 Sabouraud Dextrose Agar medium (SDA)

It was prepared by suspending 65 g of SDA medium in 1000 ml of D.W and regulating the pH to 6.5 according to the instruction of the manufacturers 0.05 gm of Chloromenphenicol is added for each to prevent growth of bacteria. 0.5 gm of Cyclohexomide is added to prevent growth of saprophytic fungi. Then mixed and sterilized by autoclave. This medium is used for culturing and maintaining the pathogenic fungi and yeast isolates(Raines *et al*.,2013).

2.3.3.1.4 CHROM agar candida medium

The chrom agar is prepared by suspending 47.7 g of chrom agar in 1000 ml of D.W. and heated to the point of effervescence (for yeast cultivation), as instructed by the manufacturers. It was then poured into a plastic 9 cm petri dishes. The media is used for researching and diagnosing the Candida spp. Their appearance is based on color (Nadeem *et al.*, 2010).

2.3.3.1.5 Potato Dextrose Agar medium

This medium was prepared by following the instructions of the manufacturing by suspending 39 gm. dissolved in 500ml and complete the volume for 1000m and add 0.5 gm. of both antibacterial chloramphenicol , streptomycin and erythromycin then mixed and autoclaved at 121°C\15 minutes(MacFaddin, 2000).

2.3.3.1.6 Preparation of Sabouraud Dextrose Broth medium (SDB):

According to the manufactures instruction this medium is prepared by suspending 30 gm. of medium in 1000 ml of distilled water. Then 0.05 gm of Chloramphenicol is added and sterilized by autoclave (Odds, 1991).

2.3.3.1.6 Peptone water Medium

This medium was prepared by dissolving 8 gm peptone in 1000 ml of distilled water, and autoclaved at 121oC for 15 minutes at15 pound/inch2. It was used for the demonstration of the bacterial ability to decompose the amino acid tryptophan to indole (MacFaddin, 2000).

2.3.3.1.7 Selective media of *P.gingivalis*

The supragingival biofilms from clinically diagnosed chronic periodontitis patients were gently removed with sterile cotton pellets and (GCF) samples were collected using sterile paper points inserted in periodontal pockets during (30-60) seconds and (2-4) soaked paper points for each subject, were placed in 1.5 ml microcentrifuge Eppindorf tube containing 1 ml Sodium Thioglycolate Transport Fluid (STTF) .The periodontal samples were transferred to the microbiology laboratory during a period about four hours and subjected to *in vitro* cultivation on (P.GING) medium is locally prepared and it consists of Columbia Agar Base, supplemented with Sheep blood, Hemin, L-cystein, Vitamins K1,K3 and other selective agents for the isolation of *P. gingivalis* from other periodontal pathogenes in the periodontal samples as mentioned in the following table(2-6). and detection procedures Jousimies-Somer *et al*., (2002) and NCCLS, (2004).

After that , the periodontal sample tubes were incubated vertically at 37°C for 48 hours, then, 100 µl aliquot from each periodontal sample was streaked on *P. gingivalis* agar (P.GING) medium which is an enriched selective medium for the isolation and presumptive identification of *P. gingivalis* according to Jousimies-Somer *et al*., (2002) and NCCLS, (2004).

Table (2-6) Cultural requirements of *P. gingivalis* in (P.GING) selective medium according to Jousimies-Somer *et al*., (2002) and NCCLS, (2004).

Compositions	Dosage	Origin
Columbia Agar base	42.5 g /L.	Himedia , India .
L-Cystein	1mg /ml (1g /L.)	(BD BBLTM).
Hemin	5µg /ml (5 mg /L.)	Sigma Chemical Co.
Vitamin K_1	1µg /ml (1mg /L.)	Hoffman-LaRoche Ltd/ France
Vitamin K_3 (Menadione)	1µg /ml (1mg /L.)	Hoffman-LaRoche Ltd/ France
Agar Bacteriological powder	6.5 g./L.	(Oxoid) Basing stoke, U. K.
Bacitracin	10.0 mg/L.	Himedia Laboratories/ India
Colistin methane sulfonate	15.37 mg/L	Himedia Laboratories/ India
Nalidixic Acid	15.0 mg/L.	Himedia Laboratories/ India
Sheep Blood	50.0 ml/L.	Local sheep
Distilled Water	1000.0 ml	Local product
Blood agar base	39 gm/L.	Oxoid, no.2,Basing stoke, U. K.

Selective medium plates were incubated in a tightly packed anaerobic atmosphere jar using gas pack (OXOID Limited, Basingstoke, Hampshire, England) at 37°C for 7-14 days. In order to obtain pure culture, displayed colonies were recultivated on other selective blood agar plates (Oxoid, Basingstoke, United Kingdom) supplemented with all the additives in table (3-8) at the same circumstances.

2.3.3. Identification of Bacterial Isolates

2.3.3.1Cultural Characteristics

Each primary positive culture was identified depending on the morphological properties such as (Colony size, shape, color , translucency, edge and elevation of texture) (Wanger *et al.*, 2017).

2.3.3.2 Microscopic Examination

A single colony was selected by a loop from culture media and spread on a clean slide and fixed with heat to be attained using the gram stain according to. The morphology of bacterial cells was investigated by Gram- stain to observe the shape and arrangement of cells under oil immersion (100X magnification) microscopically (Brenner *et al*. 2005).

2.3.3.3 Cultivation on Blood Agar

All gram negative anaerobic bacilli inoculated on blood agar give black-pigmented colonies . All *P.gingivalis* gave catalase negative and Indole positive.

2.3.4 Biochemical Test

2.3.4.1Catalase test

A colony of organisms was transferred by sterile wooden stick to a clean, dry slide and mixed with few drops of 3% H_2O_2. A positive result indicated by the evolutions of bubbles as a result of the presence of catalase that hydrolyzes hydrogen peroxide to water and oxygen (Thille, 2016).

2.3.4.2 **Indole test**

The amino acid tryptophan is found in nearly all proteins, bacteria that contain tryptophanase can hydrolyze tryptophan to its metabolic products namely indol, pyruvic acid and ammonia to satisfy nutritional needs .The appearance of red ring on the surface medium was regarded as a positive result (MacFaddin, 2000).

2.3.4 Identification of Isolated Fungi

The identification tests, including cultural, morphological and biochemical characteristics were done for each isolate.

2.3.4.1 Cultural Characteristics

Each primary positive culture was identified depending on the morphological properties such as (Colony size, shape, color and natural of pigments, edge, texture) .

2.3.4.2 Microscopic Examination

Fungi isolates are examined microscopically, the fingerprint of the champignon in the colony is taken by adhesive tape, transparent adhesive tape is used, touches the surface of the fungal colonies and then pastes the tape on a glass slide containing a drop lacto phenol blue cotton. Slides examined under magnification 10X, 40X and 100X as described by (Pitt and Hocking, 2009; Rai, 2016; Samson and Pitt, 2000).

2.3.4.3. Culture Characters According to CHROM agar

This test is performed by inoculating the medium CHROM agar Candida, which was previously prepared from Candida isolates cultivated on SDA for 24 h. Single colonies of any yeast isolates are then picked and stretched on medium chrom agar , then incubated at 30 C for 24-48 hours (Sivakumar *et al*., 2009) Chrome agar test is used for presumptive identification of Candida species by different color production on this medium(*C. albicans*= green/ blue green, *C. dubliniensis* = dark green, *C. parapsilosis* = cream white, *C. krusei*=pink) (Sivakumar *et al*., 2009).

2.3.5 Preservation

2.3.5.1 Bacterial Isolates

1- Short Time Preservation

Single pure colony of bacterial isolate was inoculated on Brain heart infusion broth . Incubated at 37ºC for 24 hrs, sealed well and stored at 4ºC in the refrigerator one month for the plates and three months for the slants.

2- Long Time Preservation

The bacterial isolate was inoculated into the Brain heart infusion broth and incubated at 37°C for 24 hrs then the broth culture was preserved by adding glycerol to a final concentration of 20% and stored at -20ºC for 12-18 months (Forbes *et al*., 2007).

2.3.5.2 Preservation of *C albicans* Isolates

The isolates was inoculated into the Brain heart infusion broth and incubated at 37°C for 24 hrs then the broth culture was preserved by adding glycerol to a final concentration of 20% and stored at -20ºC for 12-18 months (Forbes *et al*., 2007).

2.3.6. Molecular Methods

2.3.6.1. Preparation of Molecular Materials

2.3.6.1.1. Preparation of 1X TBE Buffer

The preparation of 1X TBE buffer was performed by dilution of a concentrated 10X TBE buffer, this dilution was accomplished as 1:9 (v/v); 1 volume of 10X TBE: 9 volumes of distilled water. This solution was used to prepare agarose gel and as a transmission buffer in electrophoresis process. (Sambrook and Russel, 2001).

2.3.6.1.2. Preparation of Agarose Gel

The agarose gel was prepared according to the method of Sambrook and Rusell (2001) by adding 1-1.5gm agarose to 100ml of 1x TBE buffer. The solution was heated to boiling (using water bath) until all the gel particles dissolved. The solution was allowed to cool down within 50-60ºC, and mixed with 0.5µg/ml ethidium bromide (Sambrook and Russel, 2001).

2.3.6.1.3. Rehydration of Primers

Lyophilized primer pairs were rehydrated by DNA rehydration solution 1X (pH 8.0) Tris- EDTA buffer (TE-buffer). Initially, primer storage-stock tube prepared and then the working solution would prepared from primer stock tube. Consistent with the instructions of the producer (Bioneer/Korea), TE buffer was added to produce 100 picomole/microliter concentration of primer stock solution. The working solution prepared from stock as 1:9 (v/v) by dilution with TE buffer to get 10 picomole/microliter.

2.3.6.1.4 Ethedium Bromide

Prepared by dissolving 0.25 g from ethidium bromide in 50 ml D.W to get a final concentration of 0.5 mg/ ml (Sambrook and Russel ,2001).

2.3.7. Genomic Fungal DNA Extraction

Fungal genomic DNA was extracted from SDB. The extraction was done by using (Presto™ Mini gDNA Fungi Kit) according to company instructions as following steps:

1. One milliliter of (18 hours) incubated cultured fungal cells (up to 1 × 108) was transferred to 1.5 milliliters micro-centrifuge tube, then, was centrifuged at 10000 rpm for 2 minute then the supernatant was discarded.
2. Totally, 180 microliters GT buffer was added to the tube and the cell pellet suspended by vortex. After that, 20 microliters of Proteinase K was added, and the mixtures was incubated at 60ºC for 10 minutes. During incubation periods, the mixtures tubes were inverted every 3 minutes.
3. Totally, 200 microliters of GB buffer were added to each tube and mixed via vortex for 10 seconds. Then, the tubes were incubated at 60ºC for 10 minutes with inverted the tubes every 3 minutes throughout the incubation periods.

4- Totally, 200 microliters of absolute ethanol were added and immediately mixed via vortex. Then, precipitates if happen was broken by pipetting.

5- The GD column was placed in a 2 milliliters collection tube and the mixtures was transferred (including any precipitate) to the GD column. Then, the mixtures were centrifuged at 10000rpm for 1 minute. The 2 milliliters collection tubes that contained the flow-through were discarded and placed the GD column in a new 2 ml collection tube.

6- Totally, 400 microliters of W1 buffer were added to the GD column then centrifuged at 10000 rpm for 1 minute. The flow-through was discarded and placed the GD column back in the 2 milliliters collection tube.

7- Totally, 600 microliters Wash Buffer was added to the GD column. Then, it was centrifuged at 10000 rpm for 1 minute. The flow-through was discarded and

placed the GD column back in the 2 milliliters collection tube, after that, the tubes were centrifuged again for 2 minutes at 12000 rpm to dry the column matrix.

8- The dried GD column was transferred to a clean 1.5 milliliters micro-centrifuge tube and 100 microliters of pre-heated elution buffer was added to the center of the column matrix.

9- The tubes were let stand for at least 3 minutes to ensure that, the elution buffer was absorbed by the matrix. Then, it was centrifuged at 10000 rpm for 1 minutes to eluted the purified DNA

10- .All samples were stored at (- 10 to -20) ^{o}C .

2.3.8. Genomic Bacterial DNA Extraction

DNA extraction by using G-Spin^{TM} kit (iNtRON/ Korean) and performed as follows:

1- Transfer the appropriate number of Bacterial cell (up to 1×10^{9}) to a 1.5ml microcenterifuge tube and centrifuge at full speed.(14.000 rpm or 10.000× g) for 1 minute. Then Discard the supernatant.

2- CL Buffer (200µl) , lysozyme (100µl), RNase (5µl) and proteinase K (20µl) were applied to the tube and resuspended the cell pellet by vigorously shaking the vortex, after that incubated the tube at 25C for 10 minutes, and the tubes inverted each three minutes during periods of incubation.

3- BL Buffer (200µl) was applied to each tube and blended vigorously for 5 seconds. the tubes were Then incubated in 60^{o}C water bath for ten minute and inverted each three minutes during incubation period.

4- 200 µl Absolute ethanol has been applied to the clear lysate and immediately bended with vigorous shaking, after that precipitates have been broken up by pipetting.

5-The spin column was put in a collection tube(2ml)and all the mixture (including any precipitate) was transported to the spin column . After that the tube centrifuged at 15,000 rpm for two minute and the collection tube 2 ml containing the flow-through was discarded and put the spin column in a new collection tube 2 ml.

6- WA Buffer (700µl) was applied to spin column, after that the tube centrifuged at 15,000 rpm for thirty second. The flow through was removed and the spin column returned to the 2 ml collection tube.

7- 700µl of WB Buffer was applied to the spin column. After that centrifuged at 15,000 rpm for thirty second. The flow-through was removed and the spin column breturned to 2 ml collection tube. Then, the tubes were again centrifuged for three minute at 15,000 rpm to dryed the column matrix .

8- A dry spin column was transported to a clean 1.5 ml microcentrifuge tube and 100 ML of initial heating elution buffer were applied to the middle of the column matrix.

9- A tubes were left standing to at least three minute to ensure that elution buffer was absorbed by the matrix.
The extracted DNA was then centrifuged at 15.000 rpm for 30 seconds.

10- The purified DNA was preserved at -20°C.

2.3.9. Determination DNA Concentration and Purity

The extracted genomic DNA was checked by using Nanodrop spectrophotometer, which measured DNA concentration (ng/µL) and check the DNA purity by reading the absorbance at (260/280 nm) as following steps:

1. After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, DNA).

2. A dry paper-wipe was taken and clean the measurement pedestals several times. Then carefully pipet 2µl of ddH2O onto the surface of the lower measurement pedestals for blank the system.

3.The sampling arm was lowered and clicking OK to initialized the Nanodrop, then cleaning off the pedestals and 1µl of extracted DNA carefully pipet onto the surface of the lower measurement pedestals ,then check the concentration and purity of extracted DNA.

2.3.10 Detection of Virulence Gene of *C albicans* by PCR

DNA was used as a template for specific PCR to detect (ALS1, HWP1,EAP1) virulence gene . A pair of specific primer were used for the amplification of a fragment gene shown in table(2-7). A single reaction mixture contained 2.5ml of each primer (forward and reverse) , 5 ml of DNA extract , 5 ml of master mix and 5 ml of nuclease free water to obtain a total volume 20 ml . The amplicon were run in 1-1.5 a garose gel.

Table (2-7): Primer and condition for amplification *Candida albicans virulence genes*.

C albicans Genes	Sequence of Primer (5' -------- 3')		PCR amplicon size	Condition	References
ALS1	**F**	GACTAGTGAACCAACAAATACCAGA	318	**Initial Denaturation** (94Cº for 5 min No.of cycle 35) **Denaturation (**94Cº for 1 min) **Annealing (**55Cºfor 30sec) **Extension (**72Cºfor 1min) **Final extinction (**72Cºfor 5min)	**(Melek *et al*.,2013(**
	R	CCAGAAGAAACAGCAGGTGA			
HWP1	**F**	ATGACTCCAGCTGGTTC	503	**Initial Denaturation** (94Cº for 5 min No.of cycle 35) **Denaturation (**94Cº for 30 sec) **Annealing (**55Cºfor 40sec) **Extension (**72Cºfor 1min) **Final extinction (**72Cºfor 5min	**(Ardehali *et al*.,2019)**
	R	TAGATCAAGAATGCAGC			
	F	TGTGATGGCGGTTCTGTTC	66	**Initial Denaturation** (94Cº for 5 min No.of cycle 35) **Denaturation (**94Cº for 30 sec) **Annealing (**55Cºfor 30sec) **Extension (**72Cºfor 30Sec) **Final extinction (**72Cºfor 5min	
EAP1	**R**	GGTAGTGACGGTGATGATAGTGACA			

2.3.11 PCR Products Investigation

Successful PCR amplification was confirmed by agarose gel electrophoresis by visualization against UV light (Sambrook and Russell, 2001).

Agarose gel was prepared according to (2.3.3.3.2.). Then the comb was fixed at one end of the tray for making wells used for loading DNA sample. The agarose was poured gently into the tray, and allowed to solidify at room temperature for 30 min . The comb was then removed gently from the tray. The tray was fixed in an electrophoresis amplicon was transferred into each well of agarose gel, and in one well we put the 5µl DNA ladder.

The electric current was allowed to pass at 70 volts for 50min. UV trans-illuminator was used 280 nm for the observation of DNA bands, and the gel was photographed using digital camera.

2.3.12 Real Time -PCR for Detection *P.gingivalis* .

DNA was used as a template for RT-PCR to detect *P.gingivalis* . A pair of specific primer were used for the amplification of a fragment gene shown in table(2-8).

Table (2-8):The Sequence of Primer and probe that was Used in the Present Study for Detection *P.gingivalis*

Genes	Primer Sequence (5' ----- 3')		Probe (5' ----- 3')	Reference sequence
P. gingivalis	F	TGGTTTCATGCAGCTTCTTT	CGTACCTCATATCCCGAGG	PG1370b
WaaA	A	TCGGCACCTTCGTAATTCTT	GGCTG	

2.3.13 PCR Master Mix Preparation and Condition

PCR master mix *P . gingivalis* gene was prepared by using (Trans Script One Step **qRT-PCR Super Mix**) and this master mix done according to company instructions as following table(2-9):

Table (2-9): Contents of the qRT -PCR reaction mixture with their volumes

PCR master mix	Volume
DNA tamplate	4µL
forward primer (10µM)	1µL
reverse primers (10µM)	1 µL
One step qPCR SuperMix	9.5 µL
TransScript Greenone step RT/RI Enzyme Mix	0.5 µL
Rnase –free water	4
Total	**20µL**

Thermocycles condition

Rotagene Q (Qia gene , Germany)
95-5 min
95-20 Sec 40 cycle
60-30 Sec 40 cycle
Data collected at green channel.

2.3. 14. Atotal RNA extraction :

2.3.14. 1.A.totale RNA Mini Kit:

The RNA extraction from Cell line specimens were carried out according to the manual of manufacturer of Geneaid company **:**

The Genomic RNA extracted kit for tissue Component show in Table (2- 10).

Table (2-10) RNA extraction kit for tissue contents

Component	Volume Final
Lysis Buffer	10 ml
RB Buffer	2ml
DNase I Reaction Buffer	200 µl
W1 Buffer	2 ml
Wash Buffer (Added Ethanol)	1.5 ml (6 ml)
RNase-free Water	1 ml
RB Column	4
2 ml Collection Tube	8

Protocols:

1. A volume of 50µg of cell was added to a sterile 1.5 ml microcentrifuge tube. Mixed by inversion.
2. A volume of 1 ml of RBC Lysis Buffer was added to the tube and incubated on ice for 10 minutes (briefly vortex twice during incubation).
3. Centrifuge at 3,000 x g for 5 minutes then remove the supernatant completely.
4. A volume of 400 µl of RB Buffer was added, and the tube was incubated at room temperature for 5 minutes.
5. A volume of 400 µl of Wash Buffer was added to the RB Column. Ethanol was confirmed to be added to the RB Column and centrifuge at 14000 x g for 30 seconds. Flow- through was discarded and the RB Column was placed in the 2 ml collection tube.

6. DNase 1 solution was prepared in a 1.5 ml microcentrifuge tube (RNase-free) as follow:

Table (2-11) The DNase I prepared contents

Content	Volume
D Nase 1	5 µl (2 U/µl)
DNase 1 Reaction Buffer	45 µl
Total Volume	50 µl

7. By pipette DNase 1 solution was mixed carefully.
8. A volume of 50 µl DNase 1 solution was added into the RB column and Incubated for 15 minutes at room temperature (20-30ºC).
9. A volume of 400 µl of W1 Buffer was added into the RB Column, centrifuge at 14000 x g for 30 seconds. Flow-through was discarded then the RB Column was placed in the 2 ml collection Tube.
10. A volume of 600 µl of Wash Buffer was added into the RB Column. Centrifuge at 14000 x g for 30 seconds .
11. A volum of 600 µl of Wash Buffer was added into the RB Column, Centrifuge at 14000 x g for 30 seconds . The RB Column was placed back in the 2 ml collection tube and centrifuge at 14000 x g for 3 min to dry the column.
12. The dried RB Column was place in a clean 1.5 ml microcentrifuge tube.
13. A volume of 50 µl of RNase-free Water was added into the column. It was left for at least 1 minute to ensure that the RNase-free water was absorbed. Centrifuge at 14,000 x g for 1 minute to elute the purified RNA.
14. DNA digestion in Solution the DNase 1 reaction in a 1.5 ml microcentrifuge tube (RNase-free)was prepare as follows :

Table (2-12) The DNase I reaction contents

Content	Volume
RNA in RNase-free Water	1-40 µl
DNase I	0.5 µl/µg RNA
DNase I Reaction Buffer	5 µl
RNase-free Water	Added to final
Total Volume	50 µl

15.By pipette DNase 1 solution was mixed carefully.

16.The microcentrifuge tube was incubated at 37ºC for 15-30 minutes.

17.The tube containing RNA was kept at -20 °C.

2.3.14.2 qRT-PCR for Detection *H-RAS* gene Expression

RNA was used as a template for qRT-PCR to detect the expression ***H-ras gene***.

A pair of specific primer and primer for housekeeping gene were used for the amplification of a fragment gene shown in table(2-13).

Table (2-13):The Sequence of Primer that was Used in the Present Study for Detection *H-RAS gene Expression*

Genes	Primer Sequence (5' ----- 3')		size	Condition	Reference
Acti	F	'CGTGCGTGACATTAAG GAGAAG		Temperature 45 Time 5 min	**(Azab *et al*.,2012)**
	R	GAAGGAAGGCTGGAA GTG			
H-ras	F	TGAGGACATCCACCAG TACA	118	Temperature 45 Time 5 min	**(Krishna *et al*.,2018)**
	R	CGAGATTCCACAGTGC			

One step (TRANS super mix)

Rotagene Q (Qia gene , Germany)

Thermocycles condition (H-ras gene)

45c -30 Min cDNA synthesis

95-5 min

95-20 Sec 40 cycle

60-30 Sec 40 cycle

Data collected at green channel.

2.3.15 Cytology Study(Cell line and cell culture)

2.3.15.1 Chemicals and reagents

Table (2-14): The chemicals and reagents that are used in tissue culture of oral epithelial cel (HGF1)

No.	Items	Company	Country
1	Trypsin/EDTA	Capricorn	Germany
2	DMSO	Santacruz Biotechnology	USA
3	RPMI 1640	Capricorn	Germany
4	MTT stain	Bio-World	USA
5	Fetal bovine serum	Capricorn	Germany

2.3.15.2 Instruments

Table (2-15): The instruments that are used in tissue culture of HGF1

No.	Item	Company	Country
1	CO_2 incubator	Cypress Diagnostics	Belgium
2	Microtiter reader	Gennex Lab	USA
3	Laminar flow hood	K & K Scientific Supplier	Korea
4	Micropipette	Cypress Diagnostics	Belgium
5	Cell culture plates	Santa Cruz Biotechnology	USA

2.3.15.3 Description of HGF1 cell line

The HGF1-PI 1 cell line was derived from an explant culture of gingival biopsy taken from a normal 28-year-old Caucasian female in NCBI. Obtain from Institute Pasteur in Iran .

2.3.15.4 Handling Procedure for Frozen Cells

1- The vial was thawed by gently agitation in a 37C water bath, and to reduce the possibility of contamination the Oring and cap were keep out of water, the thawing was rapid approximately 2 minutes.

2- The vial then removed from the water bath as the contents are thawed and decontaminate by spraying with 70% ethanol and all of the operations carried out under strict aseptic conditions.

3- The contents of the vial transferred to a centrifuge tube containing 9.0ml complete culture media and then spine at approximately 125 xg for 5-7 minute and the supernatant was discarded.

4- The cell pellet re-suspend with RPMI 160 and dispense into a T25 cm culture flask (prior to addition of the vial contents, the culture vessel containing the complete growth media placed into the incubator for at least 15 minutes to allow the medium to reach its normal pH(7.0-7.6)).

5- The culture incubated at 37C in a suitable incubation with 5% CO2 .

2.3.15.5 Sub culturing procedure

1- The culture media aspirated from the culture vessel without disturbing the cell monolayer**.**washing with PBS **.**

2- Trypsin-EDTA added to the culture vessel, the culture vessel gently rocked to ensure complete coverage of the Trypsin-EDTA over the cells.

3- The cells observed under a microscope to confirm they are dissociating from each other and are rounding up. the culture vessel gently taped from several sides to promote cell detachment. Cells that are difficult to detach can be put in 37oC for several minutes to facilitate dispersal.

4- Equal volume of the complete media added into the culture vessel to neutralize the trypsin-EDTA, the culture suspension then swirled or pipetted to ensure the neutralization is complete.

5- The culture suspension transferred to a sterile centrifuge tube and the cell suspension Centrifuged at 1500 rpm for 3 minutes.

6- The supernatant aspirated after checking all cells are pulled down into the pellet. The cell pellet re-suspended in pre-warmed fresh complete media.

7- The newly seeded culture vessel was placed in a 37ºC, 5% CO2 incubator. Incubated for at least 24 –48 hours before processing the cells for downstream experiments.

8- The culture media was renewed every 2-3 days if the cells did not reached 80% confluence.

2.3.16. Cytotoxicity Determination Using Methyl Thiazolyl Tetrazolium (MTT) Assay

This assay done according to (Abdul-majeed,2000 ; Freshney,2001) Briefly, cells were seeded at 1×10^5 cells/mL in 96 well micro titer plates in RPMI medium. The cells were incubated overnight for attachment candida albicans(st1,st2,st3)and *P.gingivalis*(st1,st2, st3) and combination *C.albicans* and *P.gingivals* were added in triplicate and incubated for 72 hrs. Thereafter, the cells were treated with Methyl Thiazolyl Tetrazolium (MTT) at concentration 2mg/ml for 3hr.

After the incubation time, all the contents of well aspirated. DMSO was added to each well after incubation period and the absorbance was measured at 570 nm using microplate reader. For visualizing the shape of cells under inverted microscope, 120 µL of cell suspensions were seeded to suspensions in 96-well micro-titration plates at density $1x10^4$ cells mL^{-1} and incubated for 48 hrs. at 37ºC. Then the medium removed and added 50 µL *C.albicans*(st1,st2,st3) *P.gingivals*(st1,st2,st3)and componation After *C.albicans* and *P.gingivals* exposure periods, the plates were stained by 50 µL

with Crystal violet and incubated at 37ºC for 10-15 min, the stain was washed gently with tap water until the dye was removed. The cell observed under inverted microscope at 20 and 40x magnification microscope filed and photographed with digital camera.

2.3.17: Cytotoxicity assays:

2.3.17.1: MTT Assay:

2.3.17.2.: Principle:

The general purpose of the MTT assay is to measure viable cells in relatively high throughput (96-well plates) without the need for elaborate cell counting. Therefore the most common use is to determine the cytotoxicity of several drugs at different concentrations. The principle of the MTT assay is that for most viable cells mitochondrial activity is constant and thereby an increase or decrease in the number of viable cells is linearly related to the mitochondrial activity. The mitochondrial activity of the cells is reflected by the conversion of the pale yellow tetrazolium salt (MTT dye) into dark purple formazan crystals by NADH (Figure 2.2) which can be solubilised for homogenous measurement. Thus, any increase or decrease in viable cell number can be detected by measuring formazan concentration reflected in optical density (absorbance) using a plate reader at 570 nm. The darker the solution, the greater the number of viable and metabolically active cells (Meerloo *et al;* 2011).

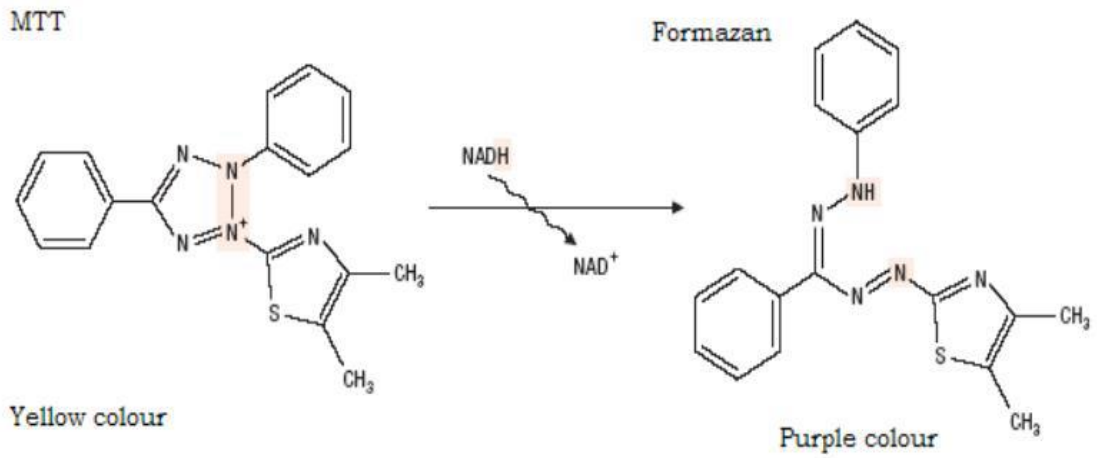


Figure 2.2: Principle of MTT Assay (Sukhramani et al; 2011)

2.3.17.3: Procedure (Meerloo *et al;* 2011):

1- At the end of the drug exposure period, the medium was removed from the wells and then the cells were washed with PBS. A blank control was carried to assess unspecific formazan conversion.

2- A volume of 1.2 ml of MTT solution (5 mg/ ml) was added to 10.8 ml medium to obtain final concentration of 0.5 mg/mL. Then, 200 µl of the resulting solution was added in each well.

3- The plate was incubated for 3 hours at 37°C until intracellular purple formazan crystals were visible under the inverted microscope.

4- The supernatant was removed and 100 µl DMSO was added in each well to dissolve the resultant formazan crystals.

5- The plate was incubated at room temperature for 30 minutes until the cells have lysed and purple crystals have dissolved.

6- Absorbance was measured by a microplate reader at 570 nm. The absorbance reading of the blank must be subtracted from all samples. Absorbance readings from test samples must then be divided by those of the control and multiplied by 100 to give percentage cell viability or proliferation. Absorbance values greater than the control indicate cell proliferation, while lower values suggest cell death or inhibition of proliferation. Percent of cell viability or percent of inhibition was calculated by the following formula:

% viability = (AT – AB) / (AC – AB) × 100%

Where, AT = Absorbance of treated cells (drug).

AB = Absorbance of blank (only medium).

AC = Absorbance of control (untreated).

% Inhibition = 100 – % viability

2.3.17.4 Apoptosis Effect Assay

The induced death of HGF-1cell was performed using Acridine orange – Ethidium bromide (AO/EtBr) dual staining method (Kuan Liu.,*et al.,* 2015). Briefly, the cells in 96- well plates were treated with C.albicans, P.gingivalis and combination and incubated for 16 hrs. The cells were detached and washed twice using PBS, and transferred to a clear 96-well plate. Dual fluorescent dyes (10 μL) were added into the cells at equal volumes. Finally, the cells were visualized under fluorescence microscopy.

2.3.17.5 : IC50 Calculate : To calculate IC50, you would need a series of dose-response data (e.g., drug concentrations x_1, x_2, ...,x_n and growth inhibition y_1, y_2, ...,y_n). The values of y are in the range of 0-1.(Cell Biology , online website)**.**

Linear Regression

The simplest estimate of IC50 is to plot x-y and fit the data with a straight line (linear regression). IC50 value is then estimated using the fitted line, i.e.,

$$Y = a * X + b,$$

$$IC50 = (0.5 - b)/a.$$

2.4. Detection The level of IL-17

2.4.1 Materials Used in ELISA Technique

Table 2-16 *: Cytokines (IL-17) kit contains:*

No	Items	Specifications
1	Standard solution (160ng/L)	0.5ml x1
2	Pre-coated ELISA plate	12 * 8 well strips x1
3	Standard diluent	3ml x1
4	Streptavidin-HRP	6ml x1

5	Stop solution	6ml x1
6	Substrate solution A	6ml x1
7	Substrate solution B	6ml x1
8	Wash buffer Concentrate (25x)	20ml x1
9	Biotinylated Human IL-17B antibody	1ml x1
10	Plate sealer	2 pics

3.4.2 Preparation of Solutions for ELISA Technique

Washing Buffer : Dilute 20ml of Wash Buffer Concentrate 25x into deionized or distilled water to yield 500 ml of 1x Wash Buffer. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

Standard : Reconstitute the 120ul of the standard (160ng/L) with 120ul of standard diluent to generate a 80ng/Lstandard stock solution. Allow the standard to sit for 15 mins with gentle agitation prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (80ng/L) 1:2 with standard diluent to produce 40ng/L, 20ng/L, 10ng/L and 5ng/L solutions. Any remaining solution should be frozen at -20°C and used within one month.

Dilution of standard solutions suggested are as follows:

320ng/L	Standard No.5	120ul Original standard + 120ul Standard diluent
160ng/L	Standard No.4	120ul Standard No.5 + 120ul Standard diluent
80ng/L	Standard No.3	120ul Standard No.4 + 120ul Standard diluent
40ng/L	Standard No.2	120ul Standard No.3 + 120ul Standard diluent
20 ng/L	Standard No.1	120ul Standard No.2 + 120ul Standard diluent

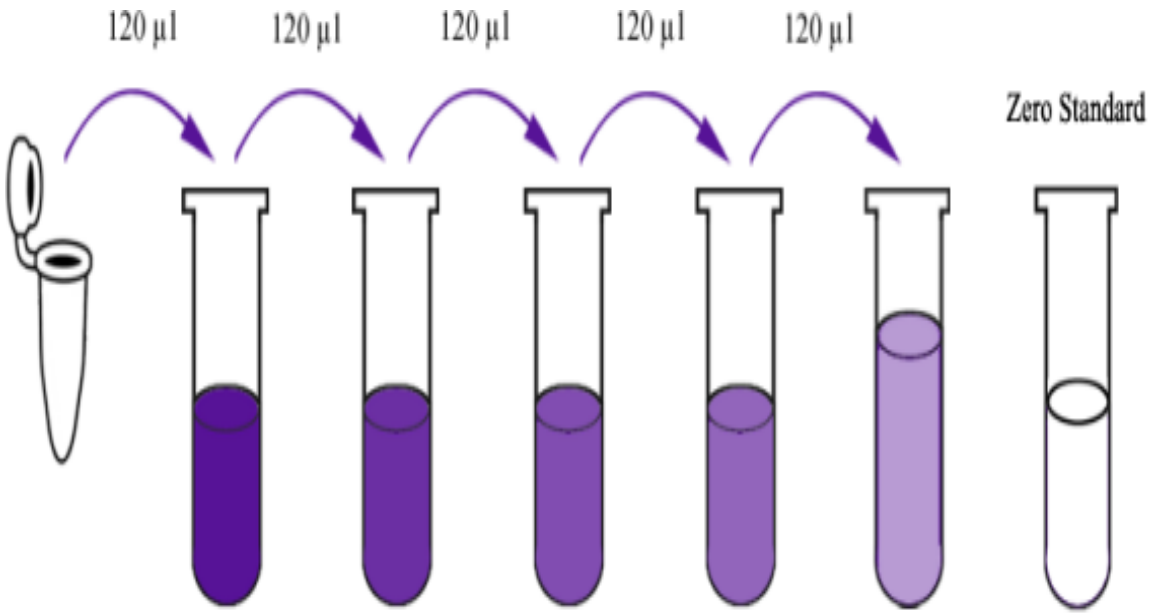


Figure (2.3) Dilution of standard

Standard concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
640ng/L	320 ng/L	160ng/L	80ng/L	40ng/L	20 ng/L

Biotinylated Human IL-17B antibody: Calculated the required amount before the experiment (100µL/well). In actual preparation, you should prepare 100~200µL more. Centrifuged the stock tube before use, diluted the concentrated Biotinylated Detection Ab to the working concentration using Biotinylated Detection Ab Diluent (1:100). **Streptavidin-HRP:** Calculated the required amount before the experiment (100µL/well). In actual preparation, you should prepare 100~200µL more. Diluted the Concentrated HRP Conjugate to the working concentration using Concentrated HRP Conjugate Diluent (1:100).

2.4.3 Detection of IL 17 Assay Procedure

Principle of test :

This ELISA kit uses Sandwich-ELISA as the method. The assay uses This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with Human IL-17B antibody. IL-17B present in the sample is added and binds to antibodies coated on the wells. And then biotinylated

Human IL-17B Antibody is added and binds to IL-17B in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated IL-17B antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of Human IL-17B. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

Procedure :

1. Prepare all reagents, standard solutions and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature.
2. Determine the number of strips required for the assay. Insert the strips in the frames for use. The unused strips should be stored at 2-8°C.
3. Add 50ul standard to standard well.
4. Add 40ul sample to sample wells and then add 10ul Human IL-17B antibody to sample wells, then add 50ul streptavidin-HRP to sample wells and standard wells (Not blank control well). Mix well. Cover the plate with a sealer. Incubate 60 minutes at 37°C.
5. Remove the sealer and wash the plate 5 times with wash buffer. Soak wells with 300ul wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirate or decant each well and wash 5 times with wash buffer. Blot the plate onto paper towels or other absorbent material.
6. Add 50ul substrate solution A to each well and then add 50ul substrate solution B to each well. Incubate plate covered with a new sealer for 10 minutes at 37°C in the dark.
7. Add 50ul Stop Solution to each well, the blue color will change into yellow immediately.
8. Determine the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

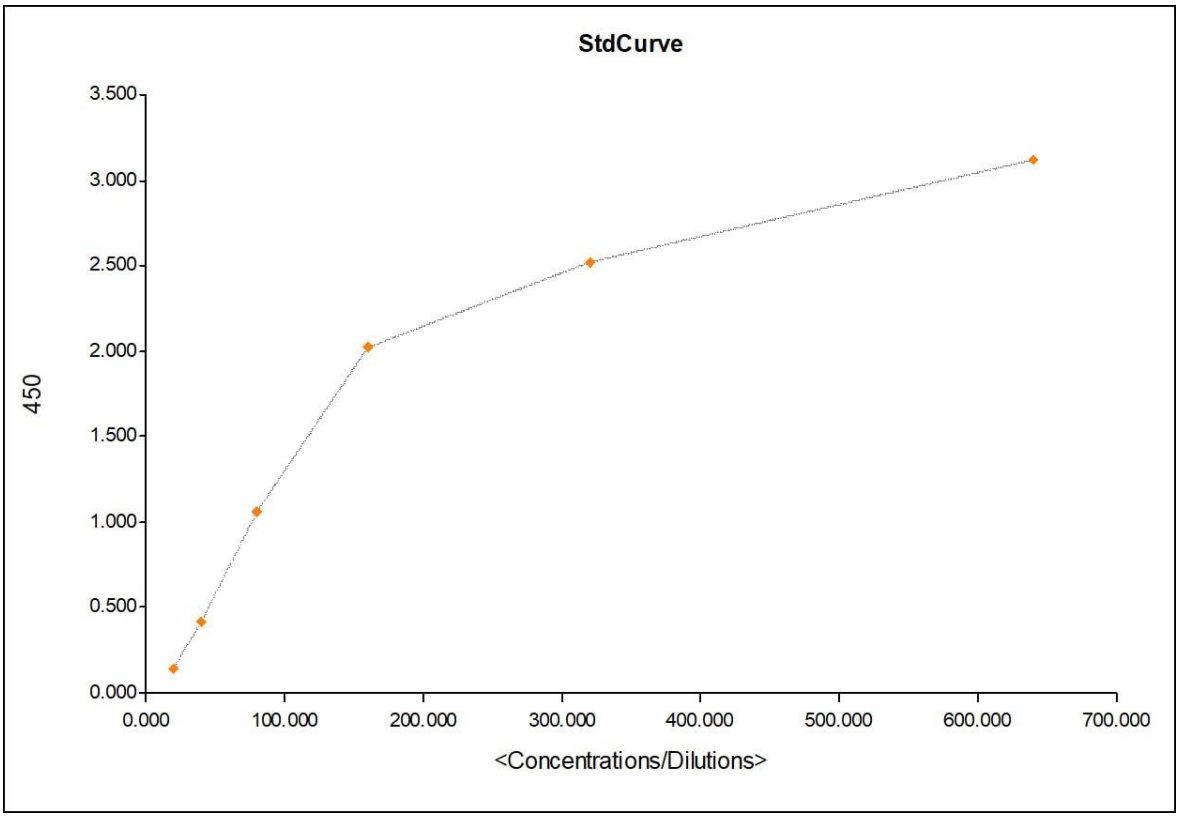


Figure (2-4) Standard curve of IL-17

2.5. : Statistics analysis

Statistical analysis was conducted by using (SPSS 23). Determining the statistical differences among different groups was made using the Pearson Chi-square test .Probability of ($P \leq 0.05$) was considered to be statistically significant (TEAM, 2010).

Also using two software programs, data were presented, summarized and analyzed. These programs were Microsoft Office Excel 2010 and the Graph Pad prism ,7 version using (t-student test) , One Way ANOVA test and (Tukey's Multiple Comparison Test) at the level of significant alpha ≤ 0.05.

3. Result and Discussion

3.1. Isolation and Identification of *Candida albicans*

From A total of 150 patients with oral infection swab , paper point and saliva were taken from the same patient . The result of culture showed 25(16.6%) samples identify as *candida albicans* and 125 (83.3%) samples negative for *C albicans* as shown in figure (3.1). The negative percentage may be due to other causes ,like aerobic bacterial and viruses that not detection in this study.

The result of the percent study was similar to study done by (Oka et al.,2022) who was detected *C. albicans* in 22 (25.6%) of the 86 patients . Also study done by (Khaleefa *et al.*,2020) showed the isolates of oral samples were 12 (24*%) C. albicans* and 18(36.0%) non *C. albicans.*

Previous study done by (Mohammed *et al*.,2017) shown that 22(47%) out of 47 *Candida* isolates from oral swabs were *C. albicans* and 25(53%) were non *albicans*.

The current result is also different to that was reported by AL-Ruaby and Kadhum (2019) who found that the isolation rate of *C. albicans* from oral swabs that were collected from human in Wasit province was 43.3%, and all these isolates were detect by PCR.

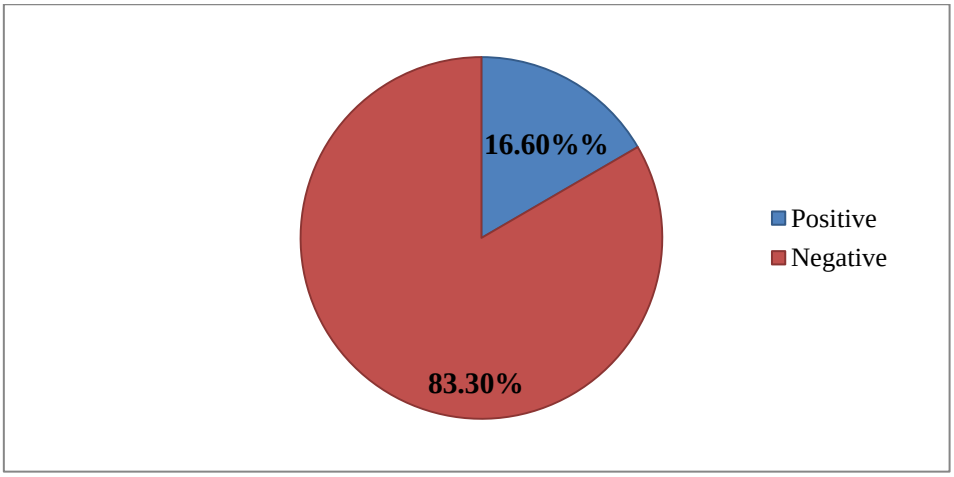


Figure (3.1): Percentage of *C albicans* isolates in oral infection samples.

All these samples were cultured on different media. The suspected plaque samples were transported to the Laboratory of microbiology department of medicine collage by using PBS. After the recording of patient information, the obtained samples were cultured on a sabouraud chloramphenicol medium and chroma agar media for the isolation of *Candida species* .

Then identification of *C.albicans* from other *Candid spp* depending on its morphological properties (colony form, size , color, borders, and texture)(Bhavan *et al.*,2010). *C albicans* colonies on the Sabouraud chloramphenicol medium are creamy whitish and smooth (Figure 3.2).

These results were agreed with (Bhavan *et al*., 2010). Especially, the colonies of *C. albicans* on SDA appeared white to creamy in color with round edges, soft and smooth associated with curved top, with yeast odor. The yeast growth reached to the typical form within three days.

The current result was consistent with that reported by (Singh and Chakrabarti, 2017) who detected cream-colored shiny and circular colonies of *C.albicans* in presence of suitable cultivation conditions.

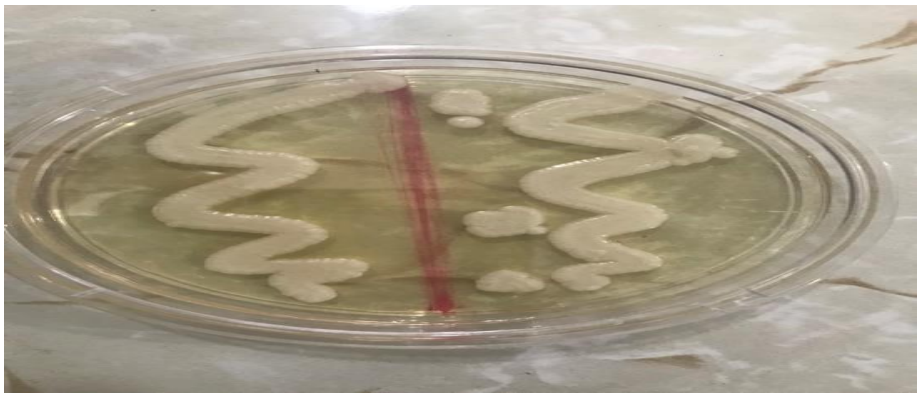


Fig. (3-2): Colonies color of *C albicans* on Sabouraud chloramphenicol medium at 30˚C for 24-48h.

C. albicans has a preponderant role among other species of it genus in periodontal disease , the presence of its hyphae has been demonstrated in the connective tissue of periodontal patients in associated with highly

invasive anaerobic bacteria such as *prophyromonas gingivalis* (Unniachan *et al*.,2020).

In a study conducted by DelaToree *et al,* chronic periodontitis patients were reported to have a higher Candida colonization rate than those without chronic periodontitis ,it was found a relationship between colonization of candida and the severity of periodontitis (DelaToree *et al*.,2018).

(Jabri *et al* .,2022) found that 20% of periodontitis patient were positive for *C albicans*. *C albicans* can grow either aerobically or anaerobically which may explain their presence in deep periodontal pockets (Colombo *et al*.,2016).

The role of *C. albicans* in periodontitis pathogenesis is yet unclear. Indeed, this yeast could alter the oral microbiome and, therefore, influence significantly bacteria colonization(Janus *et al*.,2017; Bartnicka *et al*.,2019). Coadherence between *Candida albicans* and some bacteria may help the formation of complex biofilms with mixed species affecting (Xu *et al*.,2014; Wu *et al*.,2015), it was suggested also that *C. albicans* promotes bacterial invasion of host cells by anaerobic bacteria such as *P. gingivalis* and, thus, induces infections by anaerobic bacterial diseases.

In addition, *C. albicans* can grow either aerobically or anaerobically (Dumitru *et al*.,2004), which may explain their presence in deep periodontal pockets (Wu *et al*.,2015, Colombo *et al*.,2016). Other studies reported that *C. albicans* is capable of adhering to epithelial cells and induce inflammation (Hube and Naglik ,2001).

3.1.1 Identification of *C. albicans* by CHROME agar medium

The result of this test of the colonies produce different color on chrome agar medium . The result in the present study show the ability of C albicans grow on chrome agar and *C. albicans* produce the light green color on this medium as shown in figure(3-3) the result are considered with (Nadeem *et al*., 2010; Vignesh Kanna *et al* 2017).

Candida chromogenic agar which was used according to the manufacturer's guidelines. Green colonies were identified as *C.albicans* (Ganguly *et al*.,2011).

CHROM agar Candida contains enzymatic chromogenic substrates, which combine with certain enzymes secreted by the types of *Candida* when they grow on this medium, which leads to different colors depending on the Candida species, this test is useful in the laboratory diagnosis of yeast (Murray *et al*., 2005).

It is a rapid diagnosis testing for *Candida* species, colonies growth is observed after 24-48 hours. Detection of *Candida species* and recognition of their growth; Compared to traditional methods, it has the advantage of being technically simple, fast and cost effective (Vijaya *et al*., 2011).

Figure (3-3): Colonies color of *C albicans* on CHROM agar medium .

3.1.2 Isolation and Identification of *P. gingivalis* according to Culture Characters

The results of isolation and identification of *P.gingivalis* indicated that 15 (10%) samples were as *P.gingivalis* as shown in table(3.1) .

Table (3.1) : Percentage of isolation *P gingivalis* in oral infection samples

Samples	Positive %	Negative%	Total
P.gingivalis	15(10%)	135(90%)	150(100%)
C albicans + *P.gingivalis*	4(2.66%)	146(97.33%)	150(100%)

The Identification of *P* gingivalis isolates depends mainly on the cultural , biochemical characteristics and microscopic examination. The result in Table (3.1) demonstrates that *P gingivalis* is an aerobic, Gram-negative small coccobacilli. The colonies on blood agar forms black spots, black pigmented colonies, due to it takes part in Iron transport, the way it does this is by using a hemin as a device to help it transport iron. When this builds up the black pigmentation appear as show in figure (3.4) (Ogrendik *et al*.,2005;Al-Kafagee *et al.*,2013).

P gingivalis is cultured on selective media (P.GING) it is enriched selective media for the isolation and presumptive identification of *P gingivalis* (Jousimies-Somer et al.,2002;NCCLS,2004).On these media the bacteria appears to be required Nalidixic acid ,Colistin and Bacitracin requirement ,these reqirment considered as a good enrichment agents , to provide the bacteria with the needed nutritional factors (Grenier and Dang,2011).

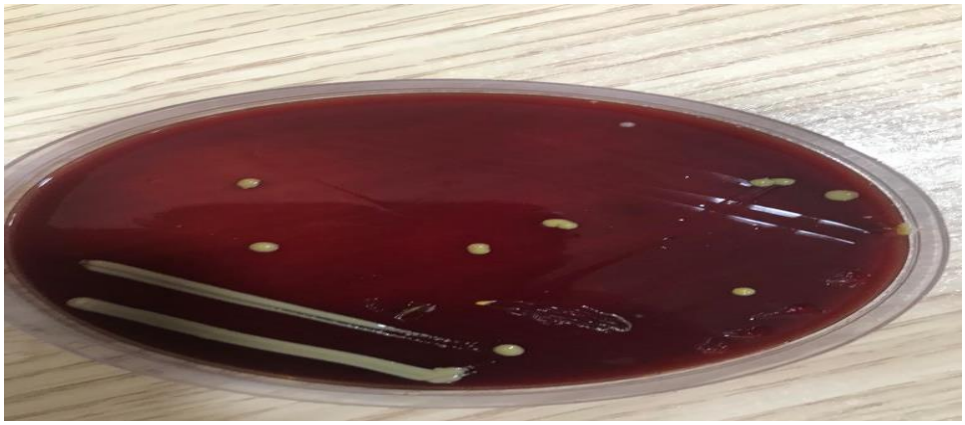


Figure (3.4): Colonies of *P gingivalis* on Blood agar

P gingivalis showed a positive reaction for Indole and negative reaction for catalase (MacFaddin,2000) .The diagnostic feature summarized on table (3.2). However, the identification isolates of *P gingivalis* confirmed by PCR.

Table (3.2):Biochemical Tests and the Microscopic Examination of *P.gingivalis* .

NO	Test	Result
1	Gram stain	G-Ve, Coccobacilli
2	Catalase	-Ve
3	Indole	-Ve
4	Grow on(P.GING)	+Ve
5	Grow on Blood agar	Black pigmented colonies

A previous study done in Iraq by (AL-Bdery and Al-Yasseen, 2018) found that out of 150 subgingival dental plaque samples only 78 isolates were belonged to *P. gingivalis*, which appeared as a small to large colonies convex, semi mucoid, translucent after 48 hr of incubation anaerobically and formation of black pigmented colonies after 7 days of incubation anaerobically on blood agar supplemented with 5% sheep blood, hemen and vitamin K and all isolates were negative to oxidase, catalase, methyl red and simmon citrate while it's

gave positive results to indole test and Alk/Alk without gas and H_2S production on TSI agar.

According to the culturing identification 15(10%) *P.gingivalis* were detected only this result is agrees with(Gomes *et al*.,2005) , they found that *P. gingivalis* was rarely isolated by culture methods (1%).

However , other studies have indicated that the prevalence rate of *P. gingivalis* in healthy subjects was 36.8% and in the periodontitis patients was 87.1%(Amano *et al*.,2000;Bostanci and Belibasaki,2012*)*.

Another study done by Hajishengallis,(2011)who found that *P.gingivalis* isolation rate from subgingival sample was(4%).

This anaerobic bacterium is a natural member of the oral microbiota ,yet it can become highly destructive and proliferate to high cell number in peroidental lesions(Clais *et al*.,2014).

Research by Bostanci and Belibasakis ,(2012) on *P*.gingivalis as a periodontal pathogen has provided a special attention should be paid to clinically relevant properties of *P.gingivalis*,such as pathogenicity and it possible relation systemic disease.

A previous study done by Atanasova and Yilmaz ,(2014) found that the rate of *P.gingivalis* in subgingival plaques reach to 60% of cases.

A synergistic interaction between *Candida albicans* and oral bacteria promoting the virulence of polymicrobial biofilms was reported (Koo *et al*.,2018; Montelongo-Jauregui and Lopez-Ribot ,2018). Indeed, regarding the specific case of periodontitis, the consumption of oxygen by *Candida albicans* seems to create an oxygen tension that helps *P. gingivalis* growth (Sztukowska *et al*.,2018) and support *P. gingivalis* ability to invade host cells (Tamai *et al*.,2011).

A study done by (Oka *et al.*,2022) found co infection *C. albicans*/*P. gingivalis* in 12 patients (14%) higher than the result in this study which was 4(2.66%).

Results of (Oka *et al.*,2022) suggest that co-infection of *C. albicans* and *P. gingivalis* rather than *C. albicans* infection alone contributes to active periodontitis. *C. albicans* may be involved in periodontitis in cooperation with periodontopathic bacteria.

The viability and hemagglutination activity of *P. gingivalis* was enhanced in the *P. gingivalis/C. albicans* mixed biofilm under low heme conditions, suggesting that *C. albicans* can enhance the virulence of *P. gingivalis* under the conditions of insufficient heme (Guo *et al*.,2020).

The capacity of *P. gingivalis* to invade gingival fibroblasts and epithelial cells was enhanced by mannans derived from *C. albicans* (Tamai *et al*.,2011). Therefore, *C. albicans* is thought to play a supportive role in the progression of periodontitis in the presence of *P. gingivalis*. Additionally, *C. albicans* may be associated with periodontopathic bacteria other than *P. gingivalis*

In the diagnosis of *P.gingivalis* infection ,currently rely on the traditional culture method requires the isolation ,culture and identification of the microorganism ,and has the disadvantages of being time –consuming insensitive , and cumbersome with the development of molecular biology techniques , rapid diagnostic studies of *P.gingivalis* have progressed rapidly (Ge *et al.*,2022) .

3.2 Molecular diagnosis of *P.gingivalis* by quantitive Real Time PCR

From 15(10%) positive culture to *P.gingivalis* only 10(6.66) isolates give positive result to *P.gingivalis* specific gene (waaA) by q RT-PCR as shown in figure(3.5).

Several diagnostic methods can be used to detect bacterial species that have been identified as periodontal pathogen including *P.gingivalis* . These methods include bacterial culture, enzyme assays , immunoassays nucleic acid probes, DNA-DNA hybridization and PCR(Kulkarni *et al*.,2018).

Study by (Kugaji, *et al*.,2019) detected *P. gingivalis* by qRT-PCR Chronic Periodentitis group in a rate 79.16%, whereas 29.17% samples were positive in the Healthy group.

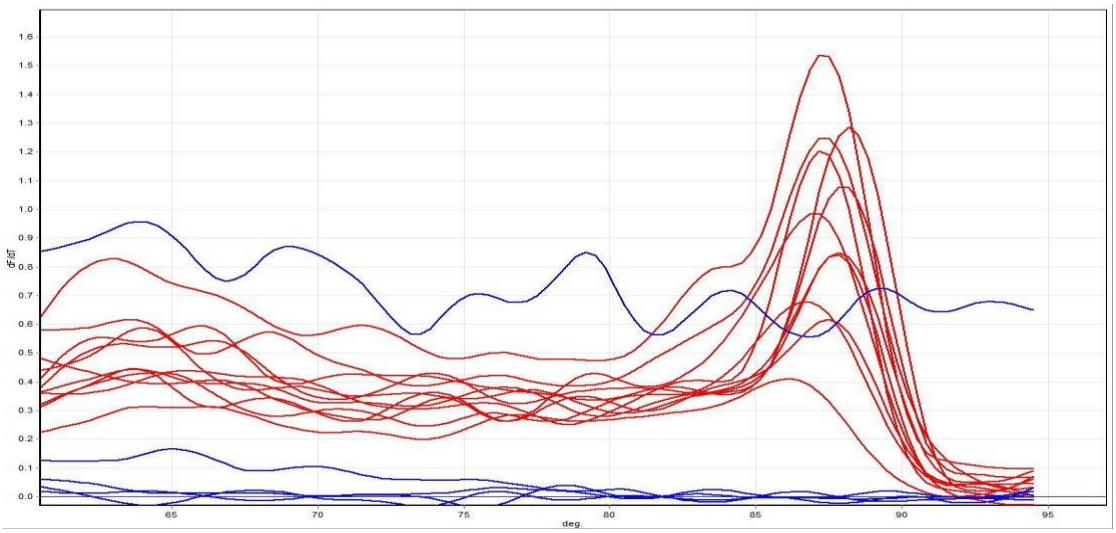


Figure (3-5) :Detection of *P. gingivalis* by real time PCR , amplification and melting curve , the red curve represent positive detection.

Also these result correlated with other result(ALwarid,2017)that indicate the percentage of *p.gingivalis* by PCR (17.7%);but this result is not agreement with (Gomes *et al*,2005) that indicate the isolate of *p. gingivalis* by PCR is (38%) . Also there is other study state that the percent of *P.gingivalis* is 3% (Ercan,2006).

Joshi (*et al*.,2016) found the percentage of prevalence of *P. gingivalis* was 66% in the chronic periodontitis (CP)group in a study which was carried out in Indian subjects. There are also researchers found that the prevalence of *P. gingivalis* more than 80% in the chronic periodontitis(CP)group (Chen *et al*.,2005; Chaudhary *et al*.,2013).

In few other studies prevalence of *P. gingivalis* in the chronic periodontitis (CP) group which was ranging from 45% to 53%(Boutaga *et al*.,2006; Boutaga *et al*.,2003).

The difference in the prevalence of bacteria in the CP group could be due to different geographical locations, inclusion criteria, technique used for detection, and variation in sample size between other previous studies.

The PCR examine gave more positive results than culture techniques when traditional culture methods are used, laboratories may need 7-14 days to identify anaerobic strict bacteria, followed by biochemical and other tests to identify the microorganism; the time required for identification can be even longer for slow-growing microorganisms or samples with low microbial counts, while PCR can offer information in only a few days and faster (Siqueirs and Rocas,2003).Previous studies found higher prevalence rate (49.1%) of *P. gingivalis* was reported by using Real-Time PCR (Avila-Campos 2003; Herrera *et al*., 2008).

On studying the microbiology of subgingival plaque samples from patients with severe chronic periodontitis, were reported a much lower detection rate (25.9%) of *P. gingivalis* yielded from anaerobic culturing (Boyanova *et al*.,2009).

However, all the mentioned references agreed on that the rate of detection is higher in disease than health. Presence of this bacterium in low number of healthy individuals (9/35) and in a significantly higher number of subjects in Chronic Periodontitis group indicates that it is an opportunistic pathogen. Healthy periodontium is maintained through a good oral hygiene of the individual. Opportunity for higher growth rate of *P. gingivalis* is usually generated through plaque accumulation in the sub-gingival area in which the growth of early plaque colonizers (gram positive cocci and rods) provide necessary growth factors such as attachment sites, substrate, reduced

oxygen tension and an area away from host's oral immunity(Nelson *et al*.,2003; Kawada *et al*.,2004).

Although microbiological culture technique is still considered as a gold standard for the detection of *P. gingivalis*, RT-PCR provided several advantages over the conventional methods. In addition to quantitative evaluation, RT-PCR offers the advantage of eliminating false positives which could otherwise play abysmal role in conventional detection techniques.

3.3 Distribution of Patients According to Age

As showed in figure (3-6), A total of 150 patients are diagnosed as periodontist the mean age of patients in the study group are ranging from (5-69) years old.

The highest percentage of infection was 43(28.66%) among patients in the age range (55-70) , 30(20%) among patients in the age (25-35) years, 26 (17.33%) among patients in the age (35-45)years , 20 (13.33 %) among patients in the age (5-15)years , 17 (11.33%) patients in the range of age (45-55), while the range of age (15-25) scored the lowest rate of infection 14 (9.33%).

The high infection rate at age group range from 55 to 70 years may be due to that the disease may come from constant neglect of not caring for the health of the teeth ,in addition to smoking and alcohol abuse this lead to lower immunity .Previous study by(Jabri *et al*.,2022) found that 16% of the studied population aged between 12 and 25 years old had periodontitis. All over the world, studies suggested that the prevalence of periodontitis is 15–30% in adults, and sometimes higher (Baelum *et al.*,2003; Holtfreter *et al*.,2009) among African adolescents, the prevalence of aggressive periodontitis was estimated as 3.4% to 6.5% (Albandar *et al*.,2002; Elamin *et al.*,2011). In a

Moroccan population (Kissa *et al*.,2016) found a prevalence of periodontitis was 11.3% (94/830) in students aged range from 12 to 25 .

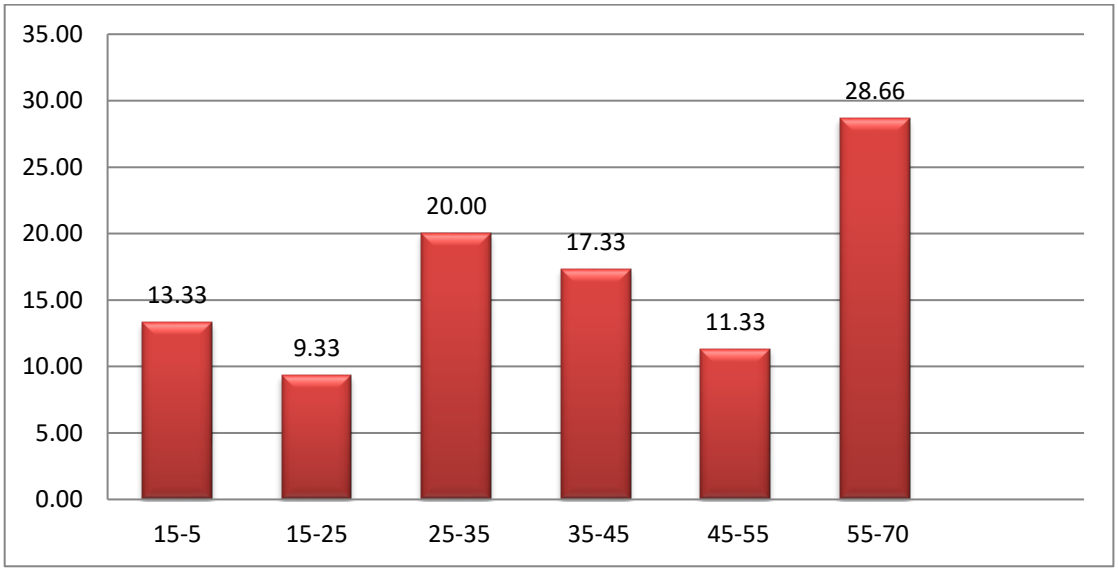


Figure (3-6): Frequency Distribution Of Periodontitis Patients According to Age group.

The higher age group (25-35) in which *C albicans* was isolated at a rate 10(40%), 7 (32%) in the age group (15-25) and 5(20%) *C albicans* isolated from age group (55-70) , 2(8%) also *C albicans* isolated in the age group (35-45) while in the age group (45-55) scored the lowest rate of *C. albicans* isolated 1(4%) as show in figure (3-7).

Previous study conducted by (Oka *et al*.,2022) found a higher *C. albicans*-positive rate (35.3%) in Patients in 80 years old compared with other participants.

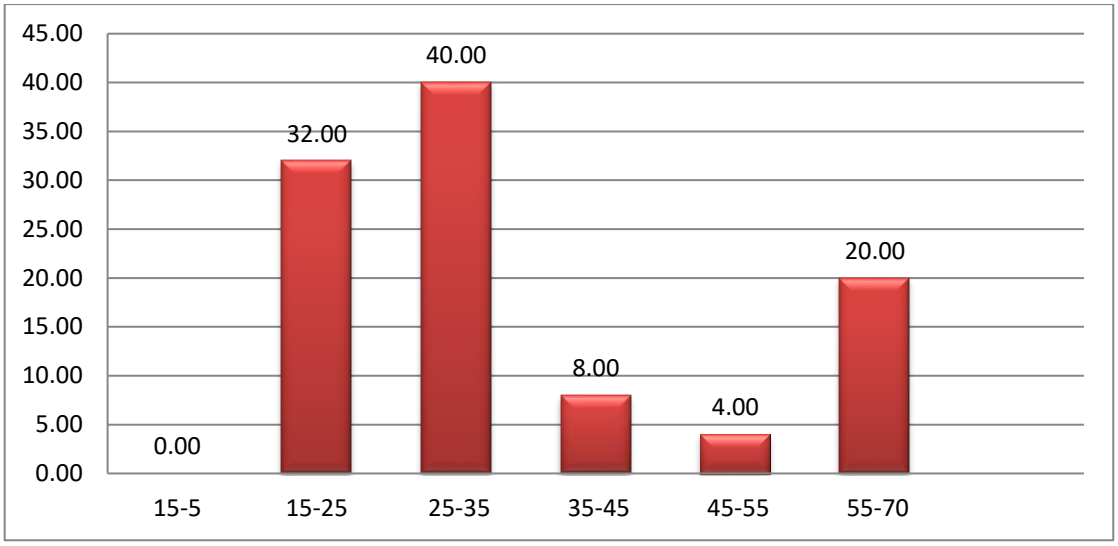


Figure (3-7): Frequency Distribution Of *C albicans* isolation By Age group.

P.gingivalis isolated in high rate 6(4%) from age group (25-35) and 4(2.66) in the age group (15-25) while only 2 (1.33%) isolate obtained from the age group (45-55) and 3 (2%) isolate from age group (55-70)as show in figure (3.8).

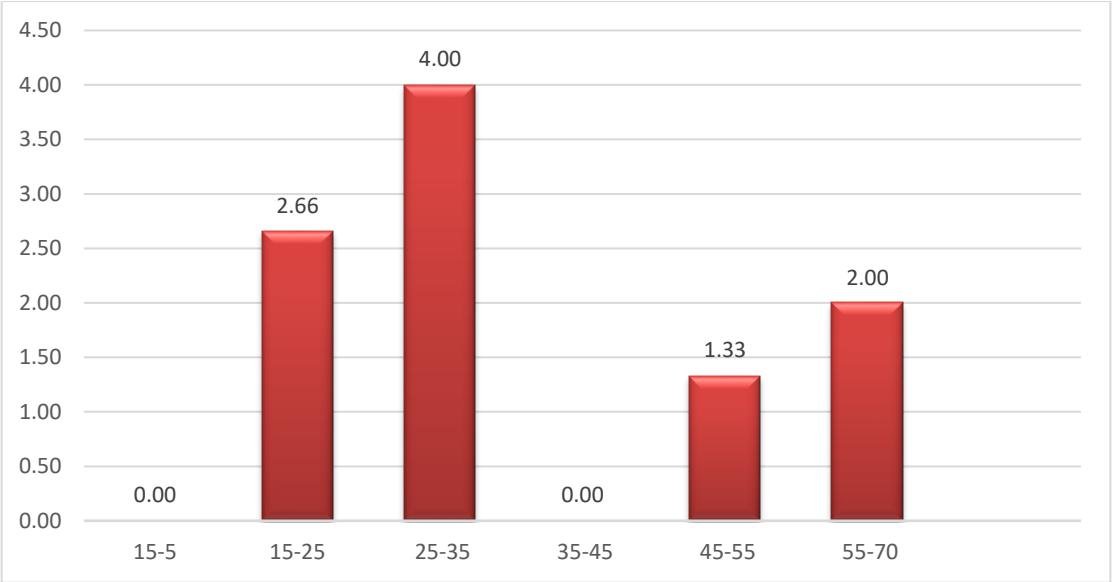


Figure (3-8): Frequency Distribution Of *P. gingivalis* isolation By Age group.

The present study agree with study done by (Al-Rawi.,2012) who revealed that there are effects of age and sex on isolation rate and the results

indicated that percentage of *P.gingivalis* was detected in 20-30 years old and males were more infected than females.

The current result similar to study conducted by (Kugaji, *et al*.,2019) who found a higher rate of detection of *P. gingivalis* In the older age groups. Also (Oka *et al.*,2022) found Patient in their 80 years old showed a higher rate of co-infection of *C. albicans and P. gingivalis* (23.5%) compared with other participants.

The older age groups were detected with more number of positive cases compared to younger age groups, which could mean the older age group is at higher risk to get infected with *P. gingivalis*. The pathogenicity of *P. gingivalis* is attributed to different virulent factors which directly or indirectly destabilize the immunogenic responses from host and help the bacterium to invade the host tissue.

3.4: Molecular study of *Candida albicans* Virulence Gene

The result show that the agglutinin-like sequence 1 (*ALS1*) gene detect in 13 (52%) isolates by using PCR with amplicon size 318 bp when compared with allelic ladder as shown in figure(3.9) and (table 3.3).The result in the current study Similar to a study conducted by (Ali,2014) who isolated the *ALS1* gene in a rate 12 from twenty five isolates.

Also the current study detected other *C. albicans* virulence gene *(HWP1)*and it was found 17 (68%) isolates out of twenty five isolates were positive to *HWP1* gene with amplicon size 503 bp as shown in(figure 3.10) and (table 3.3). Also the current study detected other *C. albicans* virulence gene ***(EAP1)*** which found that 19 (76%) isolates were positive out of twenty five isolates with amplicon size 66 bp as shown in(figure 3.11) and table 3.3).

Table 3.3: Percentage of *C. albicans virulence genes (ALS1 , HWP1 and EAP1)detected by PCR.*

Gene	Positive Number	Negative Number	Total
C. albicans ALS1	13(52%)	12(48%)	25(100%)
C. albicans HWP1	17(68%)	8(32%)	25(100%)
C. albicans ***EAP1***	19(76%)	6(24%)	25(100%)

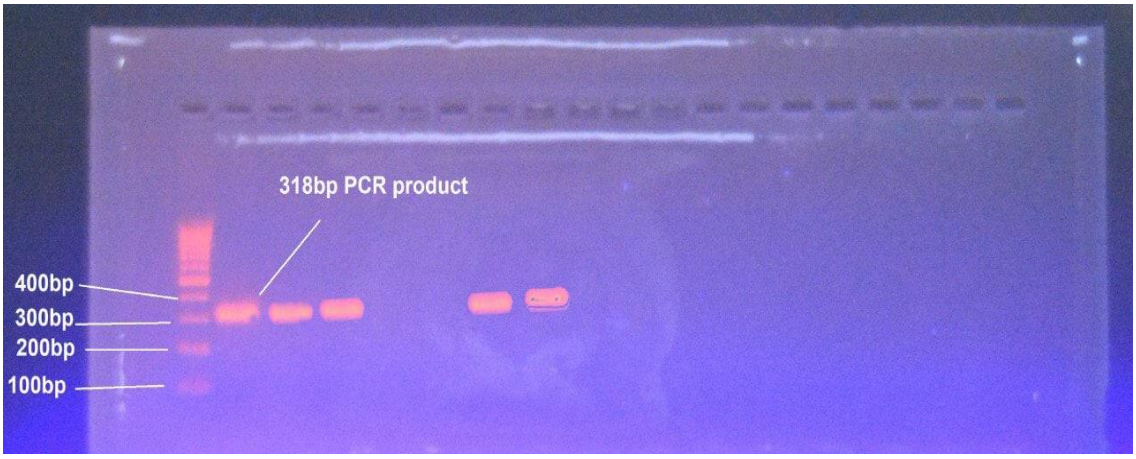


Figure (3-9): Gel electrophoresis of single PCR products of ALS1 genes **(318pb)** with in *Candida albicans* on 1% agarose gel at 70volt / cm for 45 minutes. Lane 1: 100bp DNA ladder,1,2,3,7and 8, positive for this gene and other show negative .

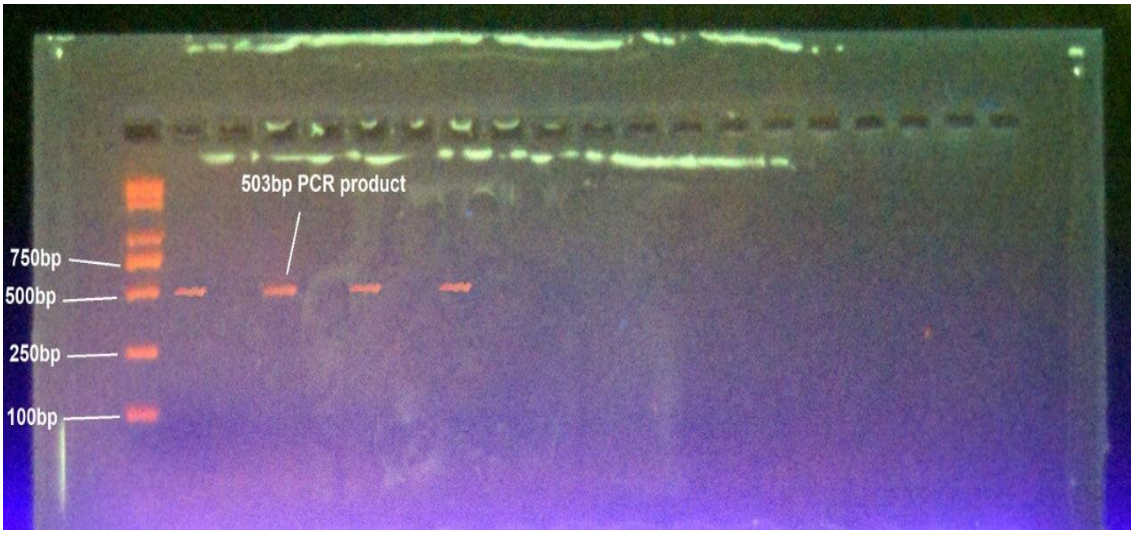


Figure (3-10): Gel electrophoresis of single PCR products of HWP1 genes (**503pb**) in *C albicans* on 1% agarose gel at 70volt / cm for 45 minutes. Lane 1: 100bp DNA ladder.1,3,5and 7 positive for this gene and other negative.

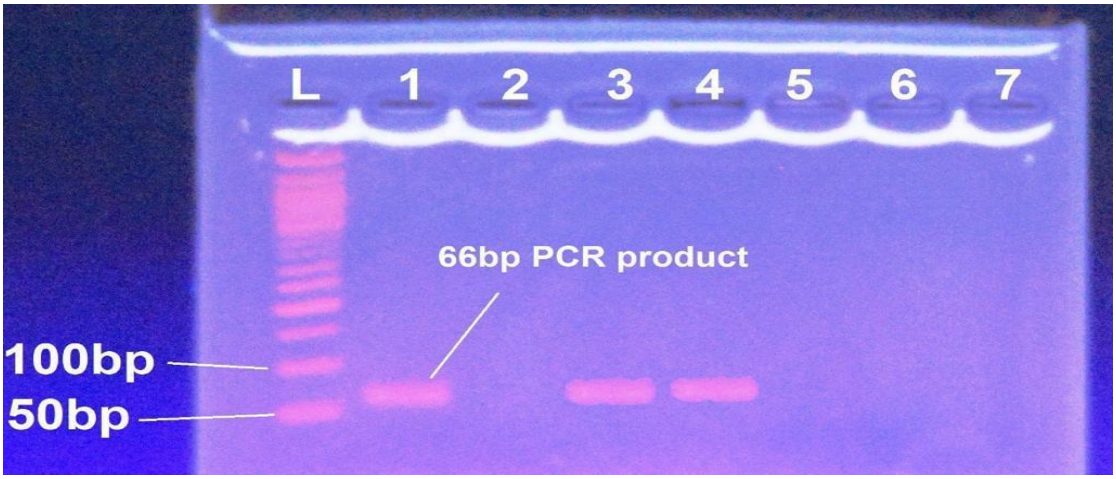


Figure (3-11): Gel electrophoresis of single PCR products of EaP1 genes (**66pb)** in *C albicans* on 1% agarose gel at 70volt / cm for 45 minutes. Lane 1: 100bp DNA ladder ,1,3 and 4 show positive result for this gene while other negative .

It was widely documented that *C. albicans* has a large number of virulence factors that causing disease, including phenotypic switching, filamentation, adherence and secreted hydrolyses. Some of these pathogeneses are associated with gene families, particularly, *ALS1*, secreted aspartyl proteinase, and lipase families (Gropp *el al*., 2009; Sun *et al*., 2010).

In both commensal and pathogenic lifestyles, *C. albicans* utilizes a set of proteins called adhesins to prime adherence between *C. albicans* and host cells or with inanimate surfaces **(**Maras *et al*., 2021).*C. albicans* isolation , which was encodes a cell surface protein that mediates adherence of *C.alblcans* to endothelial cell such as mentioned in the study of (Nadeem *et al*.,2010).

The most important *C. albicans* adhesins virulence genes are ALS proteins (ALS1-7 and ALS9)(Hoyer *et al*.,2008). ALS1 was reported as the most commonly expressed genes of the ALS gene family(Nas *et al*.,2008).

The result of present study disagree with study conducted in Iraq by (Mohammed *et al*.,2017) were found that 100% of *C. albicans* isolates

from oral and vaginal infection are ALS1 positive. This variations might be due to associated with the number of samples studied or with the virulence of the strains analyzed and with the methodology employed or the microorganism may be contain other gene responsible for the same virulence factors .

study of (Monroy -Pérez *et al*.,2016) show that ALS1gene was detected in 39/39 (100%) of *C. albicans* isolates and HWP1was detected in 35/39 (89.7%) and all strains were positive for HWP1 35/35 (100%) expressed this gene during infection.

The current detection results were much higher than other Iraqi thesis by (Ali ,2014)who found that the gene detection of each of ALS1 and HWP1was done by PCR methods. Out of 25 *C. albicans* twelve isolates were positive for ALS1 gene and only nine samples positive for HWP1 gene, eight isolates were positive for both genes by multiplex PCR method.

The study of (Inci *et al*., 2013) found that the presence of the ALS1 gene was detected in 53.9% of all strains, while the HWP1 gene was present in 5.3% which are much lower than present findings. The high frequency of detected ALS1and HWP1in this study may be related to the high pathogenicity of *C. albicans* that isolated from patients in ruled in this study.

The different in the results in this study with others studies could be related to differences in *Candida* strains, Candida pathogenicity, sampling numbers, sites and degree of infection.

The ALS proteins that were coded by Als1-Als9 genes are essential extracellular components to adhesion and colonization (Murciano *et al*.,2012, Moeckli ,2014). Moreover, SAP4 and HWP1 are other virulence factors that were shown has a significant role in hyphae Production, host tissue damage

and biofilm development, respectively (Khodavandi *et al*.,2011, Inci *et al*.,2013).

HWP1 protein is revealed that contributed in *C. albicans* covalently binding to epithelial cells and data of published studies have shown that HWP1 surface protein and ALS1/3 association is necessary to initiation and development of biofilm formation. A complementary role was proposed for ALS1/3 and HWP1 genes in biofilm formation (Sundstrom .,2002).

Also, the finding of many studies suggested that several genes included HWP1and HWP2 have the main role in biofilm formation, but between them, HWP1 was the essential factor(Inci *et al*.,2013, Nobile *et al*.,2008).The *HWP1* is a main adhesin protein, commonly expressed on the germ tube and hyphal surface of Candida species as a substrate attach covalently to host cells transglutaminases and cross-links of this genus to epithelial cells of mucosa (Chaffin, 2008; Romeo and Criseo, 2008).

HWP1 is proposed as an essential substrate that could contribute to covalent attachment of *C. albicans* to host cells (Sundstrom ,2002). This result supports the hypothesis that HWP1 gene has a significant role in the initiation and development of candida infections at the various tissue site. (Peters *et al*., 2012 , Schlecht *et al.*,2015).

Moreover, in vivo model of biofilm formation, it has been proposed that *HWP1* adhesin retains Candida in the biofilm (Mirhendi *et al*., 2011). Additionally, detecting the presence of the *HWP1* gene in *C. albicans* isolates that was recovered from clinical specimens will help to ascertain the role of this gene in colonization, which is a vital stage in infection process. Therefore, it has been found that *HWP1* gene was an excellent marker for

the identification of *Candida species*, as well as, the phylogenetic analysis of the most clinically significant *Candida* species(Abastabar *et al*., 2016).

The result in this study is inconsistent with NAS *et al.* (2008) who found that *HWP1* gene was detected in 73% of the isolates .However, the current result is in concordant with the result of a molecular study that was carried out by Mohamed *et al*. (2017) in Baghdad who reported that the detection rate of *HWP1* gene was 100% of isolates.

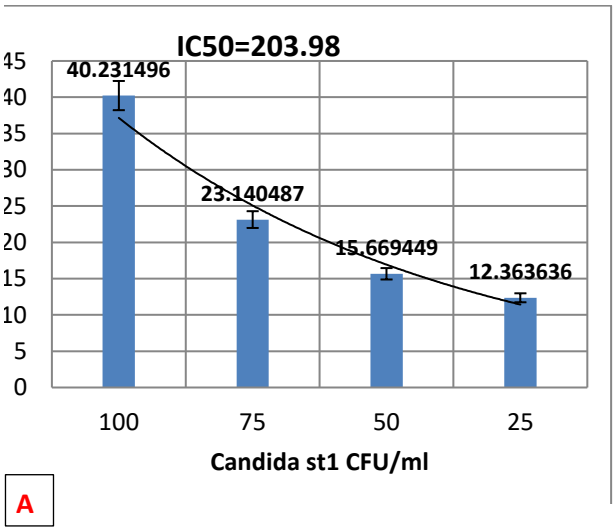
Study done by (li *et al.*, 2007) describe the role of the *C. albicans EAP1* gene, which encodes a glycosylphosphatidylinositol-anchored, glucan-cross-linked cell wall protein, in adhesion and biofilm formation in vitro and in vivo. Deleting *EAP1* reduced cell adhesion to polystyrene and epithelial cells in a gene dosage-dependent manner. Furthermore, *EAP1* expression was required for *C.albicans* biofilm formation in an in vitro parallel plate flow chamber model and in an in vivo rat central venous catheter model. *EAP1* expression was upregulated in biofilm-associated cells in vitro and in vivo. Our results illustrate an association between Eap1p-mediated adhesion and biofilm formation in vitro and in vivo.

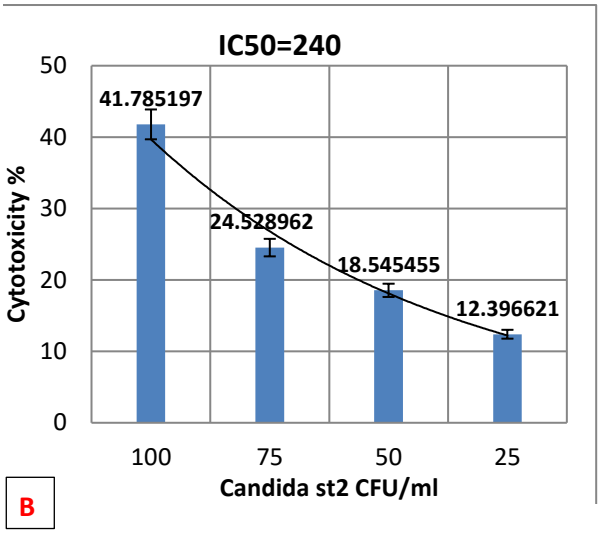
3.5 Cytology Study

3.5.1Determine the Cytotoxicity activity of *Candida spp.*

I. Determine the cell cytotoxicity by using MTT assay

The cytotoxicity potentials of *Candida* with 3 isolates were evaluated by MTT assay against HGF-1 cell line culture after 48 hrs., which appear that Candida spp exhibited selective cytotoxicity against HGF-1 cell line with inhibitory concentration (IC50) 203.98 µg/ml, 240 µg/ml as showed in figure (3.12-a, b)respectively for strain-1 and strain-2 and, while the IC50 of the Candida strain-3 HGF-1 cell line was 54.45 µg/ml as illustrated in figure (3.12.C)





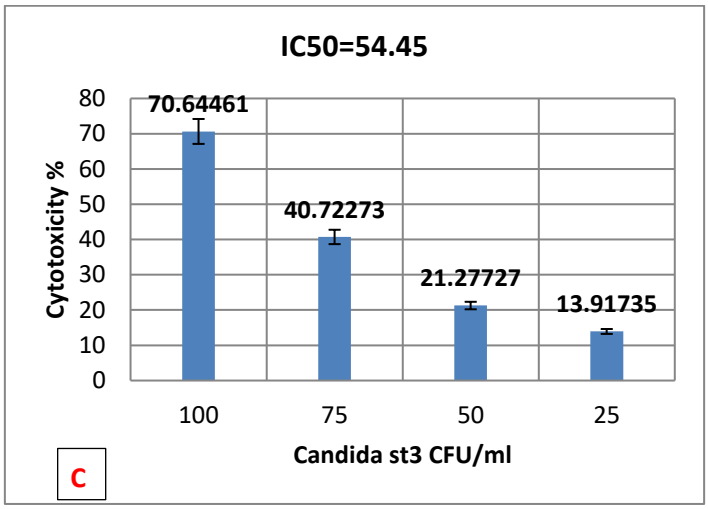


Figure (3.12) The cytotoxic effect of Candida spp (against HGF-1 cell line **after 48 hrs of exposure.**

A. The IC50 of the Candida spp strain-1 against HGF-1 cell line B. The IC50 of the Candida spp strain-2 against HGF-1 cell line. C. The IC50 of the Candida spp strain-3against HGF-1 cell line. Both strain1 and 2 shows IC50 more than the concentration used the experiment, while the strain3 was more impact on the cells than strain1 and 2

Candidalysin is the first and currently only ,peptide toxin identified in human fungal pathogen , causes cell damage in mucosal and systemic infection molds(Ito *et al.*,2020).*C albicans* candidalysin damages human oral epithelial cell in mannar is independent of apoptotic caspases (Blagojevic *et al.*2021).

Also candidalysin activates the NLRP3 inflammasome and induces cytolysisin both murine and human mononuclear phagocytes,cell death(Kasper *et al*.,2018).

The role of *Candida albicans* in the process of carcinogenesis tends to be complex, such as the role of virulence factors, the host genome, influence on the immune response, and oral dysbiosis, as noted in a review conducted by (Di Cosola *et al*., 2021)

.Increased colonization of *Candida albicans* is one of the strong associations with oral epithelial dysplasia and neoplastic transformation leading to the Oral Squamous Cell Carcinoma (OSCC)process (McCullough *et al*.,2000). The number of colonies and excessive density of *Candida albicans* can damage host cells and promote the development of carcinogenesis (Gallè *et al.*,2013).

Study condected by (Ayuningtyas *et al*.,2022) found the presence of *Candida albicans* in the form of colonies and biofilm formation found in the healthy mucosa group compared to moderate, severe dysplasia and OSCC showed high statistical significance.

Candida albicans also have the potential to induce OSCC by producing carcinogenic compounds. Certain strains of *Candida albicans* and other yeasts play an essential role in developing oral cancer by creating endogenous nitrosamines. *Candida albicans* can convert both nitrite and/or nitrate into nitrosamines and other substances to produce acetaldehyde (Gayathri *et al.*,2015). Acetaldehyde can induce tumor development in various ways. This carcinogen binds to proteins and DNA, changes its structure and function, and the reduction in the antioxidant activity of glutathione increases the content of reactive oxygen species (ROS) in the cells. These changes can lead to genomic instability, inhibiting the apoptotic system and tumor development (Ramirez-Garcia *et al*.,2014).

C albicans has the ability to convert alcohol to acetaldehyde,which has carcinogenic role in oral cavity. This conversion is facilitated by *Candida albicans* Alcohol dehydrogenase 1 (CaADH1) mRNA gene (Alnuaimi *et al*.,2015).

Chronic contact with microorganisms and their products such as endotoxins, enzymes are toxic for host cells, which can either trigger mutations or alter the signaling pathways to influence on cell proliferation or the survival of the epithelial cells (Kurago *et al*.,2008).

Candida albicans can produce carcinogens such as acetaldehyde, nitrosamines, and enzymes (proteases, lipases, esterases, and phospholipases) that can promote cancer formation (Alnuaimi *et al*.,2016, Ramirez-Garcia *et al*.,2014; Kurago *et al*.,2008).

One of the proteins identified in the mannoprotein infraction of *Candida albicans*, which increases tumor adhesion by triggering inflammation in endothelial cells, is alcohol hydrogenase (ADH1), which is associated with a cancer-stimulating mechanism by acetaldehyde production. *Candida albicans* use the enzyme alcohol hydrogenase (ADH1) to convert alcohol and other substances, such as carbohydrates into carcinogenic acetaldehyde (Ramirez-Garcia *et al*.,2014).

Nitrosamines produced by *Candida albicans* individually or in combination with other carcinogenic compounds can activate specific proto-oncogenes that can cause the development of cancer lesions that lead to changes in dysplastic conditions in oral epithelium and cancer (Sanjaya *et al*.,20211; Ramirez-Garcia *et al*.,2014).

3.5.2 The Effect of *P gingivalis* on HGF1 Cell line .

The cytotoxicity potentials of *P gingivalis* with 3 strains were evaluated by MTT assay against HGF-1 cell line culture after 48 hrs., which appear that *Gingivalis spp* exhibited selective cytotoxicity against HGF-1 cell line with

inhibitory concentration (IC50) 192 µg/ml 72.46band 63.73, as for strain 1,2 and 3 respectively showed in figure (3.13).

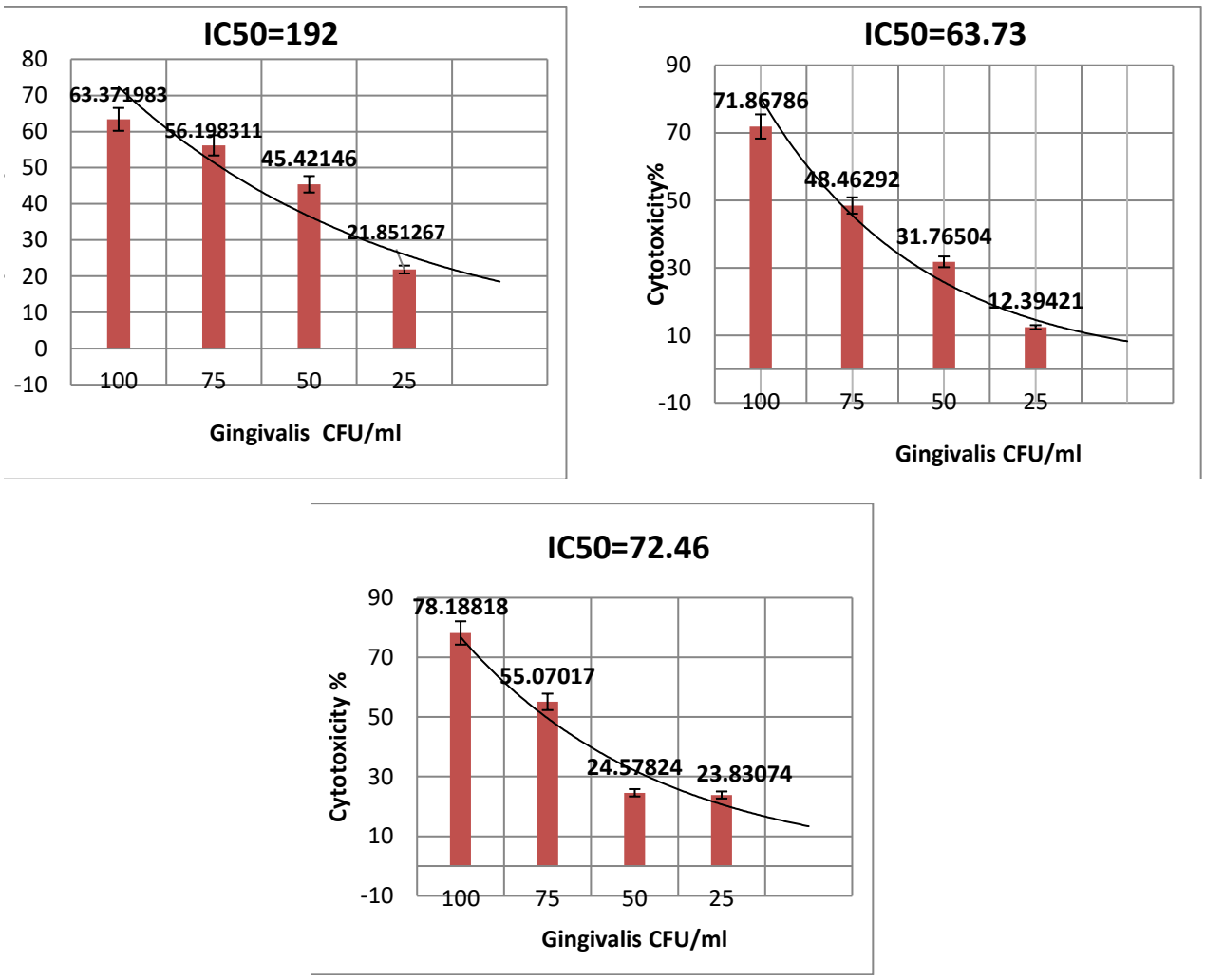


Figure (3.13) The cytotoxic effect of *Gingivalis spp* (against HGF-1 cell line **after 48 hrs of exposure.**

A. The IC50 of the *Gingivalis spp* strain-1 against HGF-1 cell line B. The IC50 of the *Gingivalis spp* strain-2 against HGF-1 cell line. C. The IC50 of the *Gingivalis spp* strain-3 against HGF-1 cell line. sStrain1 IC50 more than the concentration used the experiment, while the strains 2 and strain3 wrer more impact on the cells than strain1.

Porphyromonas gingivalis has been recovered in abundance from oral squamous cell carcinoma (OSCC). Recently established tumorigenesis models have indicated a direct relationship between *P. gingivalis* and carcinogenesis. The bacterium upregulates specific

receptors on OSCC cells and keratinocytes, induces epithelial-to-mesenchymal (EMT) transition of normal oral epithelial cells and activates metalloproteinase-9 and interleukin-8 in cultures of the carcinoma cells. In addition, *P. gingivalis* accelerates cell cycling and suppresses apoptosis in cultures of primary oral epithelial cells. In oral cancer cells, the cell cycle is arrested and there is no effect on apoptosis, but macro autophagy is increased. *Porphyromonas gingivalis* promotes distant metastasis and chemoresistance to anti-cancer agents and accelerates proliferation of oral tumor cells by affecting gene expression of defensins, by peptidyl-arginine deiminase and noncanonical activation of β-catenin.

The pathogen also converts ethanol to the carcinogenic intermediate acetaldehyde. Although coinfection with other bacteria, viruses, and fungi occurs in periodontitis, *P. gingivalis* relates to cancer even in absence of periodontitis. Thus, there may be a direct relationship between *P. gingivalis* and orodigestive cancers (Olsen and Yilmaz,2019)

P. gingivalis secretes a variety of metabolic end products as a result of its asaccharolytic metabolism; however, study of the carcinogenic potential of these is scant. Volatile sulfur compounds, such as hydrogen sulfide, methyl mercaptan, dimethyl sulfide, and dimethyl disulfide, are cytotoxic, and hydrogen sulfide in particular may also be genotoxic and stimulate cell proliferation(Nguyen *et al*.,2020).

Short-chain fatty acids, such as butyrate and propionate, are produced in abundance by *P. gingivalis* and influence the physiology of epithelial and immune cells through serving as energy sources(Blacher *et al.*,2017; Meijer *et al.*,2010) .

Hence, an imbalance in the levels of short-chain fatty acids in the tumor microenvironment has the potential to impact cell proliferation and differentiation; however, the matter requires experimental investigation. Butyric acid produced by *P. gingivalis* can contribute to activation of the

Epstein-Barr virus lytic cycle. Butyric acid inhibits histone deacetylases, thus increasing histone acetylation and the transcriptional activity of the Epstein-Barr virus *BZLF1* gene, which encodes ZEBRA, a master regulator of the transition from latency to the lytic replication cycle(Imai *et al*.,2012).Another study done by (Johansson *et al*.,1996)to analyze the cytotoxicity of some bacterial species associated with periodontal diseases. The specificity of cytotoxicity was estimated against cells of various origin and from different individuals.

3.5.3 Determine the Cytotoxicity activity of the combination of *P.gingivalis and Candida spp*

The cytotoxicity potentials of combination of *P .gingivalis* and *Candida spp* were evaluated by MTT assay against HGF-1 cell line culture after 48 hrs., which appear that combination exhibited selective cytotoxicity against HGF-1 cell line with inhibitory concentration (IC50) 80.3699 µg/ml, as showed in figure(3.14).

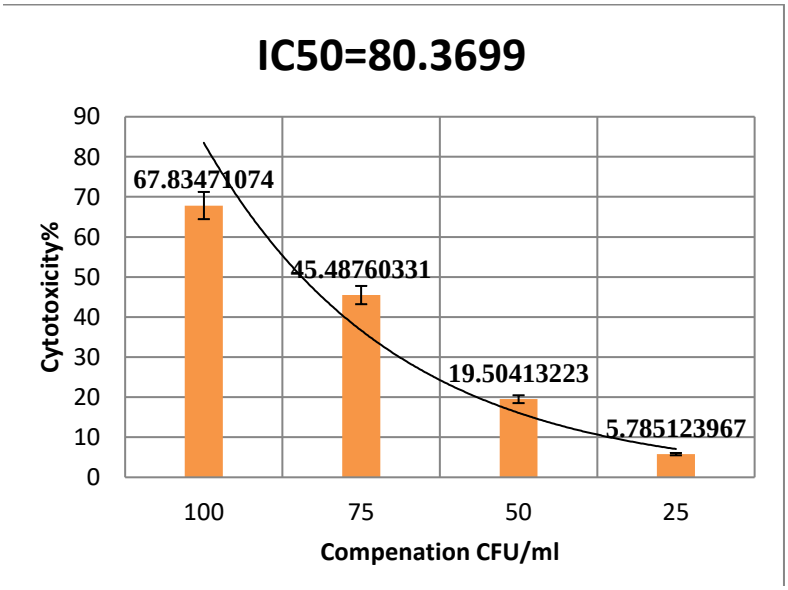


Figure (3.14) The cytotoxic effect of combination of *Gingivalis spp* and *Candida spp* (against HGF-1 cell line after 48 hrs of exposure.

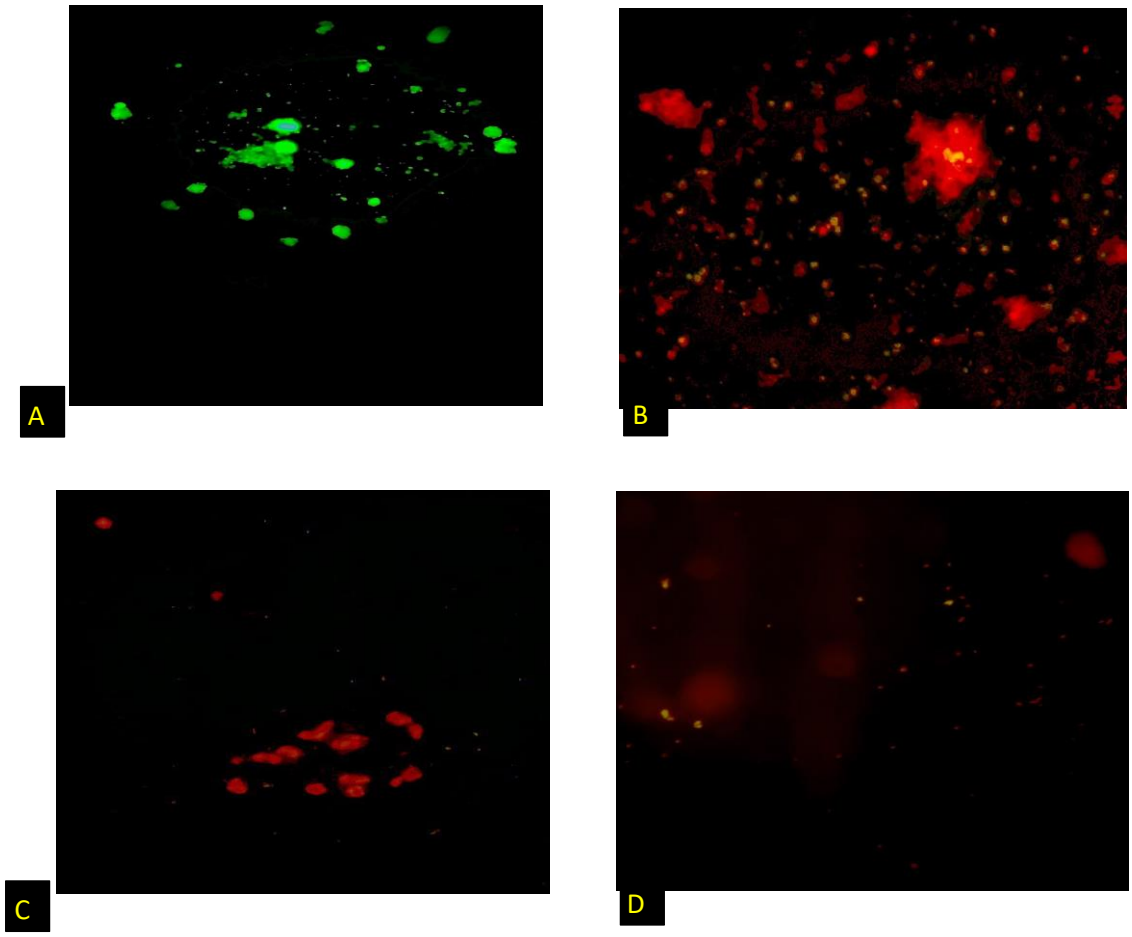


Figure (3.15): The fluorescence microscope image to HGF-1 of healthy human oral cavity cells stained with AO / EtBr and represent the apoptosis method. (A)Untreated HGF-1 cells (100x) . (B) HGF-1 cells treated with Gingivalis strian3 with 100ug/ml after 48 h (100 x) .(C) HGF-1 cells treated with Candida strain3 with 100ug/ml after 48 hr (40 x).(D) HGF-1 cells treated with combination of *Gingivalis spp* and *Candida spp* with 100ug/ml after 48 hrs. (100 x).

The effect of a mixed infection of *Candida spp* and *P.gingivalis* on the inhibition of epithelial cell migration was also studied. The inhibition of cell migration challenged with a mixed infection was stronger than the inhibition caused by one of both microorganisms separately. The inhibiting effect might partly be attributable to the oxygen-reducing effect of both *Candida spp.* Within a biofilm, bacterium–fungus interactions influence the overall survival and proliferation of the respective species (Martin *et al.*,2011).

C. albicans promotes growth and biofilm formation of anaerobic bacteria under aerobic conditions (Janus *et al*.,2016, van Leeuwen *et al*.,2016). An explanation for this might be that Candida creates a pro *P. Gingivalis* anaerobic microenvironment by using oxygen for its own metabolic processes(metabolic interaction).

Epithelial cell death was not excluded as a mechanism of inhibition of epithelial cell migration in a study done by (Haverman *et al*.,2017). However, during all experiments, the epithelial cells were strongly attached to the surface, and the cells looked morphologically viable. Moreover, in a previous study, using the same model, epithelial cell viability was confirmed (Laheij *et al*.,2013).

Therefore, epithelial cell death would not appear to be the mechanism that is responsible for the inhibition of cell migration by *Candida spp*. That was observed in this study.

The study done by (Haverman *et al*.,2017)found that the presence rather than the concentration *of P. gingivalis* was important for the additional inhibitory effect on cell migration when both *Candida spp.* were present. First, it is possible that the inhibitory effect on epithelial cell migration of *Candida* and *P. gingivalis* is at its maximum within the model that was used. Another explanation might be that one Candida cell can only interact with a certain amount of *P. gingivalis,* which means that after a certain threshold, extra *P. gingivalis* does not result in an additional effect.

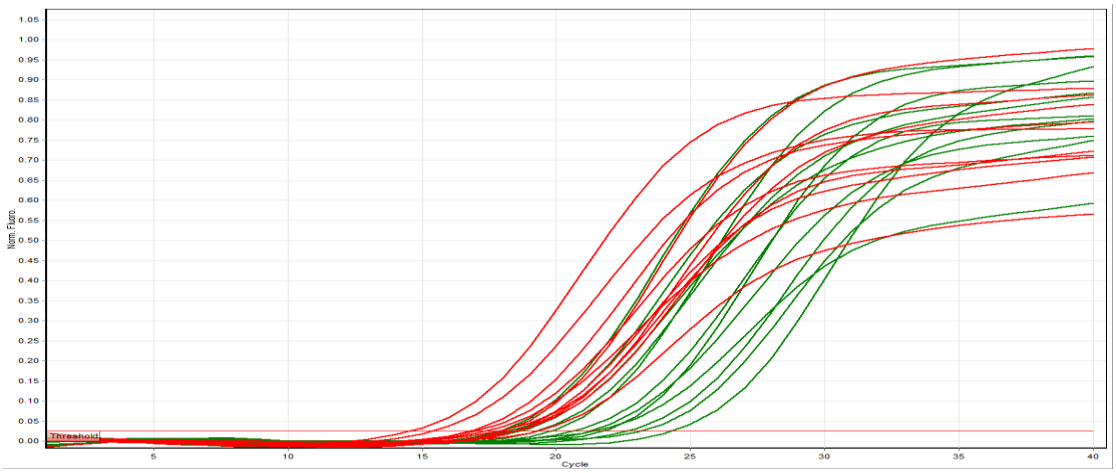
3.6 Determine the gene Expression of Harvey -Ras Gene by RT-qPCR

Gene expression of the *Ras* gene was determined to assess its role in the carcinogenic effect. Quantitative-Real Time-polymerase chain reaction (qRT-PCR) was used to determine the level of *Ras* RNA

transcripts,.Results in (Fig. 3-16) showed that the PCR cycle was proportional to the amount of PCR products after measuring the fluorescence during each cycle. Gene expression estimated by qRT-PCR red trace *Ras* gene , Green house keeping gene .

A total from 4 tissue cell culture infected with candida albicans with different concentration(25%,50%, 75%,100%) , 4 tissue cell culture infected with *P.gingivalis* and 2 tissue cell culture infected with *C albicans and P.gingivalis* ,and 2 sample as control group.

RNA was extracted to study the gene expression by of H-Ras gene using real time PCR(Relative gene expression) (2ddcT) methods ,in this method the level of expression H-ras gene in test samples as well as control samples normalize with house keeping gene for test sample as show in figure 3.16).



(Figer 3-16) : H- Ras gene Expression level .This is the first run for samples (red target gene) , green house keeping gene (GAPDH).

The present study found that the expression of H-Ras gene significant decrease in the patient group (both patients who infected with candida or infected with p.gingivalis or infected with both types)when compared with control group(cell line not infected with any

microorganism) so the gene expression is decrease more than (4.5)fold in compared with normal tissue line as show in figure(3-17).

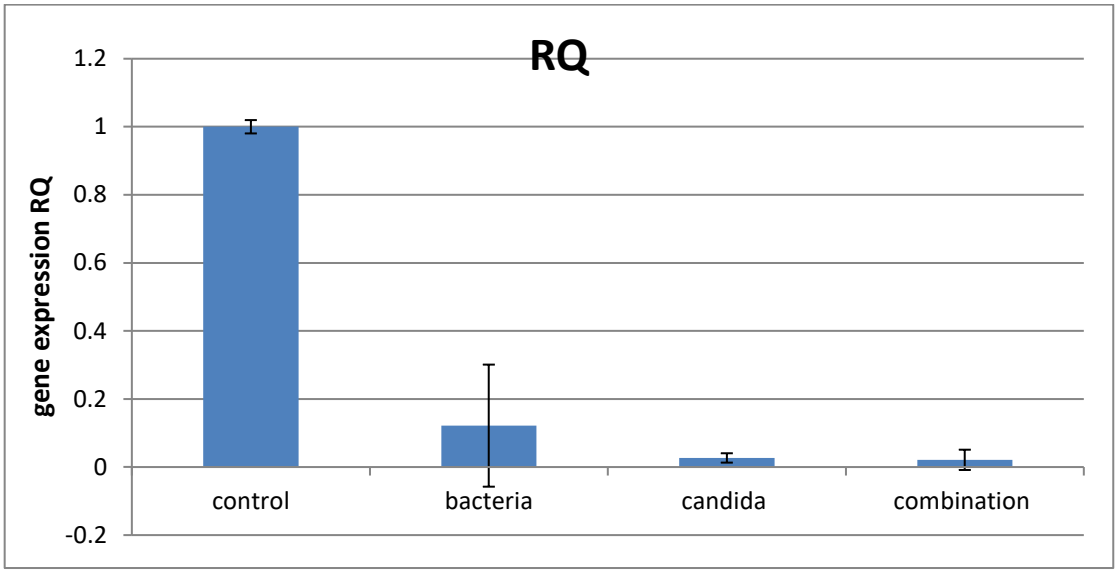


Figure (3-17) :Ras gene expression among samples versus the reference gene (GAPDH).

Also the results in this study show that the CT in normal tissue line less than the value in tissue infected with candida or with p.gingivalis as show in table (3.4).

Table 3.4: ΔΔCt for isolates

	sample	gene Ct	house Ct	ΔCt	ΔΔCt	RQ
Bacteria	1	20.45	27.4	-6.95	2.35	0.196146
Bacteria	2	18.46	23.2	-4.74	4.56	0.042394
Bacteria	3	19.47	28.1	-8.63	0.67	0.628507
Bacteria	4	17.94	21.1	-3.16	6.14	0.01418
Bacteria	5	19.93	25.27	-5.34	3.96	0.064257
Bacteria	6	17.42	21.27	-3.85	5.45	0.022876
Bacteria	7	21.71	27.2	-5.49	3.81	0.071298
Bacteria	8	22.46	28.22	-5.76	3.54	0.085971
Bacteria	9	14.6	18.67	-4.07	5.23	0.026645
Bacteria	10	18.07	20.66	-2.59	6.71	0.009552
Bacteria	11	18.95	22.57	-3.62	5.68	0.019505
Bacteria	12	18.81	26.27	-7.46	1.84	0.279322
Candida	13	18.69	22.27	-3.58	5.72	0.018972

Candida	14	16.8	21.77	-4.97	4.33	0.049721
Candida	15	21.41	25.87	-4.46	4.84	0.034915
Candida	16	21.7	26.17	-4.47	4.83	0.035158
Candida	17	21.55	24.78	-3.23	6.07	0.014885
Candida	18	20.53	25.51	-4.98	4.32	0.050067
Candida	19	22.68	25.28	-2.6	6.7	0.009618
Candida	20	22.67	25.43	-2.76	6.54	0.010746
Candida	21	23.49	27.71	-4.22	5.08	0.029564
Candida	22	22.13	25.59	-3.46	5.84	0.017458
Candida	23	21.43	25.61	-4.18	5.12	0.028756
Candida	24	24.11	27.88	-3.77	5.53	0.021642
Combination	25	18.49	24.34	-5.85	3.45	0.091505
Combination	26	26.1	28.95	-2.85	6.45	0.011438
Combination	27	18.49	22.67	-4.18	5.12	0.028756
Combination	28	25.39	27.77	-2.38	6.92	0.008258
Combination	29	26.5	27.31	-0.81	8.49	0.002781
Combination	30	19.33	18.28	1.05	10.35	0.000766
Combination	31	17.87	21.42	-3.55	5.75	0.018581
Combination	32	17	19.3	-2.3	7	0.007813
Control	33	18.19	27.51	-9.32	-0.02	1.013959
Control	34	20.72	30	-9.28	0.02	0.986233

The Ras pathways is one of the most prevalent oncogenic alterations in both human and animal in vivo oncogenic mutation have been shown to occur at exon 12 ,13 and 61 resulting of possible point mutation for each RAS is forms (miller and miller,2012).

The main member of the RAS gene family-KRAS,HRAS and NRAS-encode proteins that have a pivotal cytoplasmic role in cell signaling ,when RAS gene are mutated ,cell grow uncontrollably and evade death signals ,also RAS mutations also make cell resistant to some available cancer therapies (simanshu *et al* 2017).

RAS proteins are important for normal development active RAS drives the growth , proliferation and migration of cell ,in normal cell RAS receives signal those signals rapidly switch between the active GTP form inactive GDP form stste(prior *et al*.2020).

Mutated RAS is stuck in the active state and drives cells to become cancerous, there are distinctive patterns in the mutation frequencies associated with each RAS gene and cancer type (prior *et al*. 2020)

The result in this study , show decrease in H-RAs gene expression this may be due to the gene have a mutant so it detected in low value and this results make patient more susceptible to cancer type due to which infected with candida or *P.gingivalis* .

The significant differences in the RAS signaling capacity can result depending on the tissue isoform mutation combination (pershing *et al*.,2015).

Results in (Fig. 3-17) indicated that there is a significant differences in *RAS* gene expression between bacteria and *Candida albicans* with control. when the isolates is treated with cell line the results indicated inhibition gene expression with candida albicans higher than the *P .gingivalis* and control.

The Current result showed that high significant difference (P value 0.05%) in concentration (75%) with the third isolated of bacterial (0.043) while the four isolates of bacteria show significant difference (0. 54%) in a concentration (100%) .

Also the present study showed significant difference(0.043) in second concentration (50%) isolates of *C albicans* while the combination between *C albicans* and *P gingivalis* show no significant with all concentration as show in table (3.5) and (3.6).

Table (3.5): Concentration of isolates and combination

Descriptives								
RQ								
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1.00	2	1.0001	.01961	.01386	.8239	1.1762	.99	1.01
2.00	12	.1217	.17940	.05179	.0077	.2357	.01	.63
3.00	12	.0268	.01376	.00397	.0180	.0355	.01	.05
4.00	8	.0212	.02977	.01053	-.0037	.0461	.00	.09
Total	34	.1162	.25188	.04320	.0284	.2041	.00	1.01

Table (3.6). Concentration of isolates and combination:

Multiple Comparisons						
Dependent Variable: RQ						
LSD						
(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	.87838*	.08398	.000	.7069	1.0499
	3.00	.97330*	.08398	.000	.8018	1.1448
	4.00	.97886*	.08693	.000	.8013	1.1564
2.00	1.00	-.87838*	.08398	.000	-1.0499	-.7069
	3.00	.09493*	.04489	.043	.0033	.1866
	4.00	.10048	.05019	.054	-.0020	.2030
3.00	1.00	-.97330*	.08398	.000	-1.1448	-.8018
	2.00	-.09493*	.04489	.043	-.1866	-.0033
	4.00	.00555	.05019	.913	-.0969	.1081
4.00	1.00	-.97886*	.08693	.000	-1.1564	-.8013
	2.00	-.10048	.05019	.054	-.2030	.0020
	3.00	-.00555	.05019	.913	-.1081	.0969

*. The mean difference is significant at the 0.05 level

*1.00 mean control , 2.00 Bacteria , 3.00 Candida and 4 the combination .

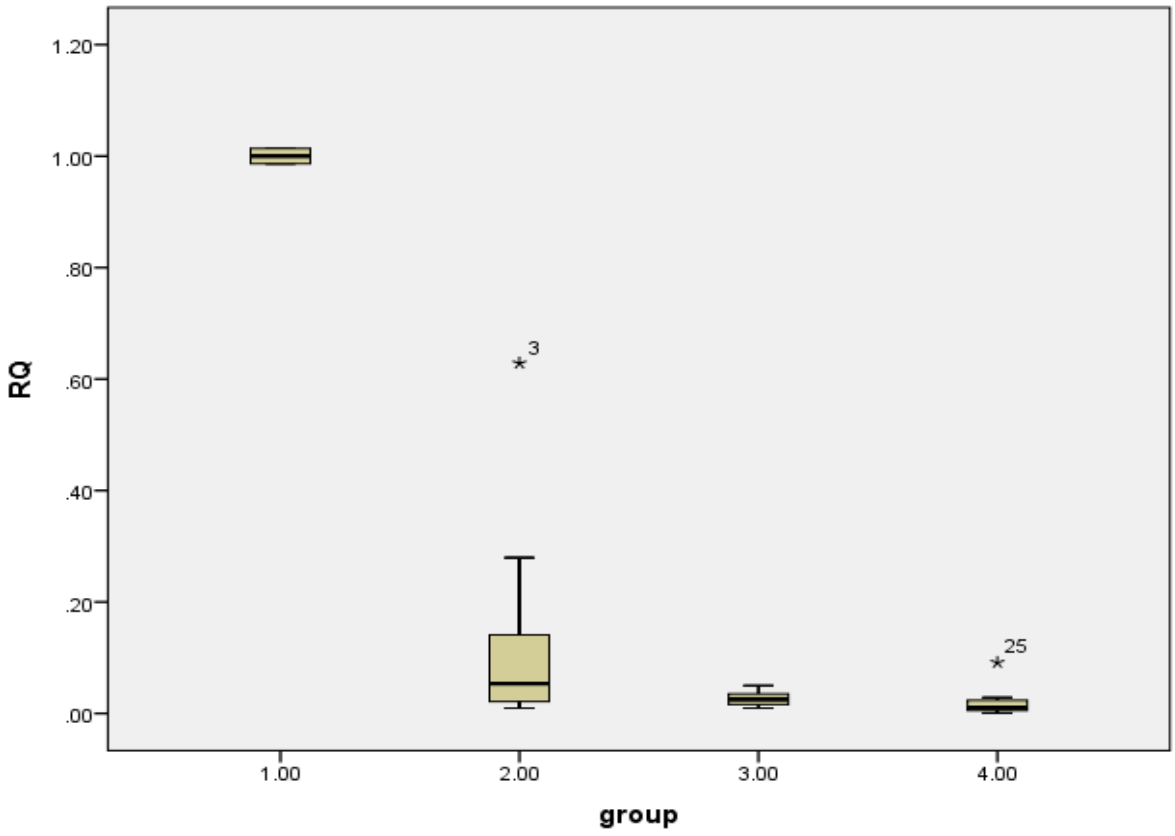


Fig 3-18: B ox plot Represent the value gene expressions of Ras gen different group at four 1=control ,2=bacteria ,3=candida ,4=compensation

A study proposed by (Krishna *et al.*,2018) found that the H-Ras protein was significantly overexpressed in the oral carcinoma group compared to the normal group (P = 0.03) . H-Ras positivity increased in cases affected with buccal mucosa site and higher grade of carcinoma. Relative mRNA level of H-Ras was significantly elevated in oral carcinoma as compared with the control group ($P \leq 0.001$). Protein and mRNA levels of H-Ras in case group was poorly correlated .

Also (Krishna *et al.*,2018) concluded that H-Ras oncogene expression was markedly higher in oral carcinoma, and it can be a prognostic marker and target for an effective molecular therapy.

Mutations in any one of three members of the Ras family are common events in human tumorigenesis. Several studies reported that most

oncogenic mutations predominantly affect the K-Ras locus and express altered protein in oral carcinoma and some other cancer.(Vitale-Cross *et al.*,2004; Camps *et al.*,2005; Vaughn *et al.*,2011) .

The exact interpretation of this finding remains unclear, but previous Indian studies based on etiological factors and mutational status of Ras oncogenes indicate that rare mutations or transcriptional splicing in H-Ras gene might be related to overexpression or mutant type H-Ras protein in tumoral tissues. More studies are required on the functional and mutational gene expression of H-Ras in oral carcinogenesis.

3.7 Interleukine 17 (IL-17B) Assessment

The results of this study showed there was no significant p value (0.398) of IL-17 in saliva of periodontitis patient compared with control group as shown in Table(3.7) and figure (3.20) . .

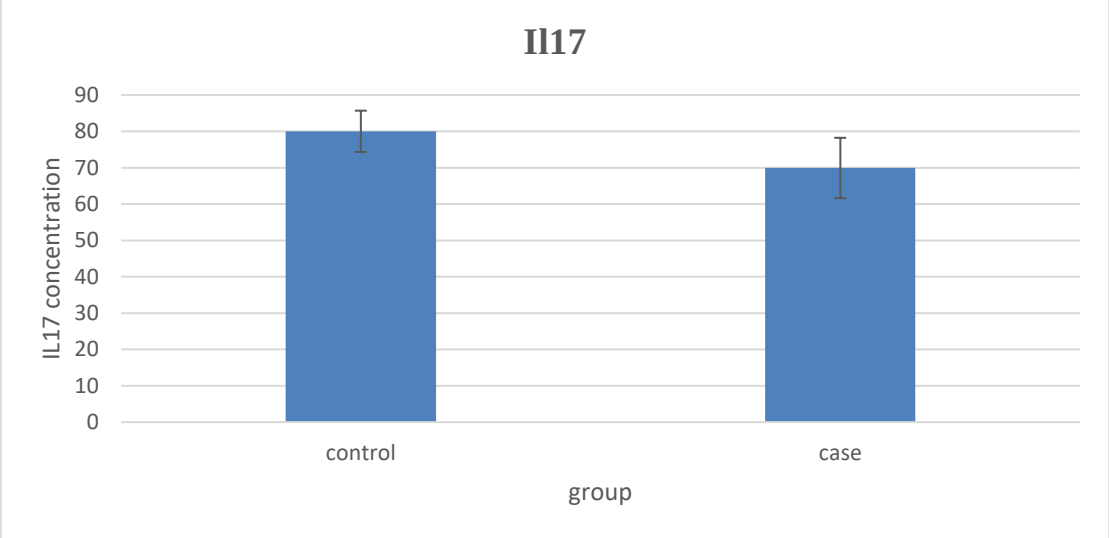


Figure (3.20): The level of salivary IL-17 in case and control groups

Table (3.7): The level of Salivary IL-17 in case and control group.

Group Statistics					
	group	**Mean**	**Std. Deviation**	**Std. Error Mean**	**P value**
Il17	control	80.0288	17.96528	5.68112	0.398
	case	69.9388	34.24481	8.30559	

*Non significant

Interlukin-17 stimulates the release of cytokines which particularly attract neutrophils to the inflammatory site. However, AgP known to have divergent PMN function shows defect in neutrophils chemotactic response. It might possible that despite stimulation of IL-17, inflammation may remain unnoticed in AgP due to lack of chemotactic response. Study conducted in a Vermin model reveal the significance of IL-17 in mobilization of neutrophil in the control of the any bacterial infection(Ye *et al*.,2001).

A previous study done by (Azman *et al*.,2014) demonstrated that Serum, saliva and gingival crevicular fluid (GCF) IL-17A levels were higher in periodontitis patients and correlated positively with clinical parameters of attachment loss, pocket depth and bleeding on probing and In vitro, IL-17E inhibited *Porphyromonas gingivalis* and IL-17A induced expression of chemokines by reducing phosphorylation of the NF-κB p65 subunit.Also concluded that IL-17E may have opposing roles to IL-17A in periodontitis pathogenesis. IL-17E can negatively regulate IL-17A and periodontal pathogen induced expression of chemokines by oral keratinocytes.

While the results showed there was a significant increase in IL-17 concentration incomperation to treatment with *C albicans* alone and

combination with *P .gingivalis* at P value (0.001) as show in table (3.8) and figure(3.21) .

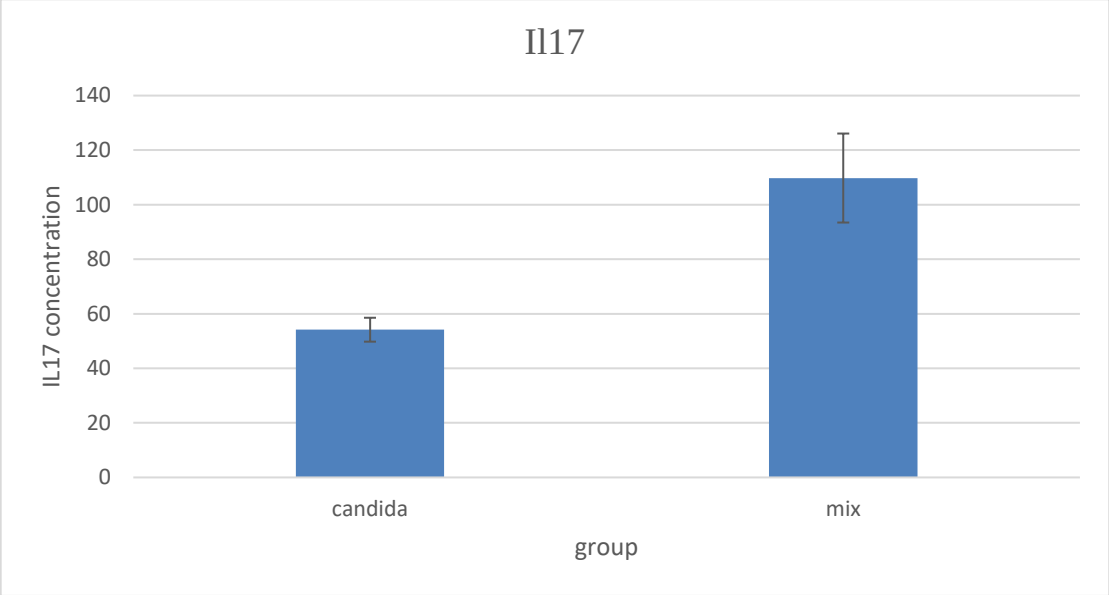


Figure (3.21): IL17 Salivary concentration among candida and mixed infection

Table (3.8): IL17 Salivary concentration among candida and mixed infection

Group Statistics					
	Group	Mean	Std. Deviation	Std. Error Mean	P value
	Candida	54.1665	14.53969	4.38388	0.001
	Combination	109.7686	36.47849	16.31368	

IL-17A plays a protective and destructive role in the progression of chronic periodontitis. When periodontal infection occurs, neutrophils can quickly move out of the blood vessel and are the first effector cells that reach the infected site. IL-17A can regulate neutrophils to leave the bone marrow and enter the blood circulation, and recruit neutrophils to the infected site of periodontal tissues (Hajishengallis,2015;, Hajishengallis,2020).

Studies have mentioned that many defense mechanisms were included in response to candidiasis, and IL17 is the most strongly recommended in such immune response, It was reported that IL-17 and INF-γ play an important roles in protection against fungal infections, when they effectively enhanced neutrophils and macrophages in killing of fungi during the innate responses of the host (Fidel.,2011) .

Result of (Cardoso *et al.*,2009) demonstrated elevated levels of IL-17, TGF-β, IL-1β, IL-6, and IL-23 messenger RNA and protein in diseased tissues as well as the presence of Th17 cells in gingiva from patients with periodontitis.

Another study by (Chen *et al*.,2015) found that IL-35 and IL-17 were significantly higher in GCF from patients with periodontitis than healthy participants (P <0.01, P <0.05, respectively).

CP represents long lasting inflammation of periodontal tissues where microbes are a major etiological factor and the hallmark of periodontal diseases is bone loss. Virulence factors produced by *P. gingivalis* such as Arg- and Lysgingipain proteinases are key factors for host tissue invasion which leads to activation of immune-infammatory processes. Subsequently, various molecules (proteases, MMPs, cytokines, etc.) are activated leading to destruction of connective tissue attachment and alveolar bone loss (Chen *et al*.,2015).

In the study of (Batool *et al*.,2018) was high in CP compared to healthy controls and it gradually increased with the severity of disease. Corroborating our data, previous studies have documented increased level of IL-17A in saliva, serum, and gingival cerevicular fluid (GCF) in periodontitis compared to healthy subjects and also reported important role

of IL-17 in gingival infammation and bone loss (Prakasam and Srinivasan, 2014; Azman *et al*.,2014; Cheng *et al*.,2014).

(Batool *et al*.,2018) Concluded Salivary levels of IL-6 and IL-17 were signifcantly higher in patients with calculus associated CP compared to healthy subjects. These cytokines increased as the disease progressed from mild to moderate and severe form. Therefore, can conclude that the salivary level of IL-6 and IL-17 may help in the subcategorization of periodontitis.

According to (Rohaninasab *et al*.,2013; Abusleme and Moutsopoulos2017; Zenobia and Hajishengallis, 2015). IL-17 levels in the GCF of patients with periodontitis were higher compared to healthy control patients and IL-17 played a role in the pathogenesis of chronic periodontitis. This can occur because the role of IL-17 affects the immunity and contributes to making clinical disease, and also the identification and characteristics of other molecules play a role.1-3 The presence of concentrations of IL-17 is a consequence of the early stages of gingival inflammation, but it does not cause periodontitis lesions.

Neutrophils phagocytize bacteria and sterilize through oxygen dependent and independent mechanisms to achieve immune activation and protection. However, the main bactericidal substances of neutrophils are superoxide ions and lysosomal enzymes, whose excessive release will damage the surrounding cells and tissues and aggravate the inflammatory response. At the same time, the inflammatory cytokines produced and released by neutrophils in the process of phagocytosis of bacteria will also aggravate inflammation and promote the local inflammatory response of periodontal tissues, leading to damage and destruction of the periodontal tissues.

(Yu et al.,2007) have shown that IL-17A plays a major protective role in bone loss in *Porphyromonas gingivalis* induced periodontitis, although a large number of researches have shown that IL-17A is closely related to bone erosion in rheumatoid arthritis.

In addition, IL-17A can also act synergistically with IL-1 and TNF-α to induce gingival fibroblasts to produce MMP-1 and MMP-3, which plays an important role in the tissue destruction in periodontitis (Beklen et al.,2007).

IL-17A can promote keratinocytes to secrete antimicrobial peptides and play a defensive role, but whether it plays a role in the progression of periodontitis has not been confirmed. In the mouse model of ectopic tracheal transplantation, IL-17A is involved in the pathogenesis of obliterative bronchiolitis by regulating M1 macrophage polarization (Meng *et al*.,2017), but whether IL-17A mediates periodontal tissues destruction by promoting M1 macrophage polarization and secreting inflammatory factors remains to be studied.

IL-17 is found in high amounts in periodontal disease. IL-17 aggravates periodontal disease by activating gingival fibroblasts to produce inflammatory cytokines. There is abundant documentation that suggests major tissue destruction in periodontitis which results from the recruitment of host cells through the activation of monocytes/macrophages, lymphocytes, and fibroblasts cell(Cardoso *et al*.,2009) .

IL-17 is more frequently detected in periodontitis patients than in gingivitis patients. Takahashi *et al*. have suggested that IL-17 is produced in periodontitis, which may be involved in Th1 modulation and which

increase inflammatory reactions through gingival fibroblast-derived mediators and thus, IL-17 has a potential role in the pathogenesis of the periodontal disease(Oda *et al.*,2003; Takahashi *et al*.,2005).

IL-17 has an action on alveolar bone cells. It has been documented that T-cells can be directly involved in bone metabolism through T-cell-derived cytokines which includes IL-17(Takayanagi *et al.*,2000).

IL-17 has the ability to stimulate osteoclast cells and activate receptor activator of nuclear factor kappa-B ligand production by osteoblasts(Sato *et al.*,2006).

Aggressive periodontitis (AgP) is generally seen in teenagers and young adult. It is the most severe form of periodontitis which can lead to significant periodontal inflammation and premature tooth loss in maximum number of cases at early age. There is abundant literature suggesting aberrant polymorphonuclear leukocytes (PMN) function as a key pathogenic mechanism in AgP exhibiting defective *in vitro* PMN chemotactic response and enhanced oxidative metabolic responses(Baeten et al.,2013).

Evidence suggest that the rate of bone destruction is about the three to four times faster than in chronic periodontitis. Early age of onset is one of the main characteristics features of AgP; however, patients with AgP are clinically healthy(Andrukhov *et al*.,2011).

IL-17 may play a significant role in AgP because of the functional impairment of PMN and because of the association of IL-17 pathways with the recruitment of neutrophils which results in enhanced inflammation and bone resorption(Ay *et al*.,2011).

It has been hypothesized that IL-17 has a key role in regulating neutrophils *in vivo* and neutrophils play a crucial role in controlling periodontal infection. Neutrophils are considered as the first line of defense against a broad range of periodontal pathogens. Functionally intact neutrophils are necessary for defense in any inflammatory condition. Patients suffering from defects in neutrophils function suffer from recurrent and severe infection including AgP. Neutrophils are also potentially harmful if turned against host tissue(Linden *et al.*,2005).

The constant phases of neutrophil mobilization are key components of innate immunity contributing to host defense(Ibbotson et al.,2001; Shiohara et al.,2004).

IL-17 is important for neutrophil homeostasis and therefore for periodontal health. Any alteration from normal neutrophil activity (in terms of numbers or activation status) can potentially cause periodontitis(Darveau,2010; Hajishengallis, E., and Hajishengallis, G. (2014).

The functional pathways of the IL-17 cells in periodontitis are still not sufficiently understood, and thus, more research is required. There is abundance literature available with IL-17 levels in chronic periodontitis but miniscule information with IL-17 in relation to AgP cases. More studies in all ethnic groups are necessary which can be used in the development of individualized diagnostic and treatment plans of periodontitis, especially in case of AgP.

4.1 Conclusions

According to the results of the present study, the following conclusions could be elucidated:

- Detection of *C.albicans* virulence gene (ALS1, EAP1 HWP1) in some isolates by PCR techniques.
- Detection cytotoxicity potentials effect cell line study to3 isolates of *C albicans* were evaluated by MTT assay against HGF-1 cell line culture after 48 hrs., which appear that C albicans exhibited high toxicity (IC50 of the Candida strain-3 HGF-1 cell line was 54.45 μg/ml.).
- The cytotoxicity potentials Detectby cell line study effect to *P .gingivalis* in 3 isolates which found that they were evaluated by MTT assay against HGF-1 cell line culture after 48 hrs., which appear that *P .gingivalis* exhibited selective cytotoxicity against HGF-1 cell line with inhibitory concentration (IC50) 192 μg/ml.
- Cytotoxicity potentials detected for combination of *P .gingivalis* and *Candida albicans* by cell line study were evaluated by MTT assay against HGF-1 cell line culture after 48 hrs., which appear that combination exhibited selective cytotoxicity against HGF-1 cell line with inhibitory concentration (IC50) 80.3699 μg/ml.
- Gene expression of the H-Ras gene indicated that there was a significant differences in RAS gene expression between bacteria and candida albicans with control.
- IL-17 detected by ELISA technique indicated high concentration in patients infected with candida and *P. gingivalis.*

4.2 Recommendations

Depending on the finding of this study, the following recommendations are drawn:

- ❖ further research should be undertaken in this field to establish the exact pathogenic mechanism of this opportunistic fungus in periodontal diseases and also confirm the results using a long-term follow-up by evaluating the effect of periodontal treatment on this opportunistic fungus.
- ❖ Further studies will be necessary to identify the molecular pathways that promote the alteration in epithelial cells during *C. albicans* and *P .gingivalis* oral infection
- ❖ Further study relationship between existence of specific type of bacteria and its oncogenic effects
- ❖ More studies are required on the functional and mutational gene expression of H-Ras in oral carcinogenesis.
- ❖ Study the role of others' specific pro and anti-inflammatory cytokines levels and their gene polymorphism, which may have impact on the disease management.
- ❖ The application of new typing methods like PFGE type or sequencing typing methods may result in a better discrimination of isolates

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الخلاصة

دراسة جزيئيه وسميه ل *Candida albicans spp* and *Porphyromonas gingivalis* المعزولة من المرضى المصابين بالتهاب اللثة المزمن

تم اخذ (150) مريض مصاب بالتهاب اللثه , حيث تم اخذ مسحة فموية ,لعاب paper point , من كل مريض ، الذين حضروا إلى المركز التخصصي للأسنان والعيادات الخارجية لطب الأسنان وخلال الفترة من (نيسان 2022 إلى أيلول 2022) و تتراوح أعمارهم بين (5 - 70) سنة.

شخصت الدراسة الجزيئية جينات الضراوة (ALS1و HWP1 و EAP1) في *C albicans* عن طريق تفاعل pcr باستخدام برايمرات محدده ذات حجم جزيئي (318 bp ، 572 bp ، 66 bp) على التوالي. أظهرت النتائج أن جين ALS1 اكتشف بمعدل 15 (22.05٪) بينما جين HWP1 بمعدل 19 (27.94٪) و جين EAP1 بمعدل 19 (76٪).

في هذه الدراسه تم استخدام ثلاث عزلات من كل نوع من المايكروبات المستخدمه في الدراسه وبتراكيز متعدده في الخط الخلوي (,normal oral epithelial tissue)حيث

أظهرت الدراسة النسيجية أن تأثير السمية الخلوية على 3 عزلات من *Candida albicans* تم تحديدها بواسطة MTT assy ضد الخط الخلية HGF-1 بعد 48 ساعة ،

أن C albicans أظهرت سمية خلوية انتقائية ضد خط الخلايا HGF-1 بتركيز مثبط 203.98 (IC50) ميكروغرام / مل ، 240 ميكروغرام / مل بينما كان IC50 لخط خلايا Candida-HGF-1 3 سلالة المبيضات 54.45 ميكروغرام / مل.

كشفت الدراسة الحالية أيضًا عن إمكانات السمية الخلوية لـ P.gingivalis.

كذالك تُظهر الدراسة الحالية أن إمكانات السمية الخلوية لمزيج من *P .ingivalis* و *Candida albicans* قد تم اجرائها بواسطة MTT assy ضد الخط الخلوي HGF-1 بعد 48 ساعة ، والذي يبدو أن المجموعة أظهرت سمية خلوية انتقائية ضد خط الخلايا HGF-1 بتراكيز مختلفه (تركيز 80.3699 (IC50) ميكروغرام / مل.

كذلك بعد الزرع الخلوي النسيجي وتحديد السميه وكذاك ال apoptosis

تم تحديد التعبير الجيني لجين H-Ras لتحديد دور وامكانية التأثير السرطاني لهذه الكائنات على الخلايا المستخدمه في الدراسه

باستخدام (real time-PCR) ، وأظهرت النتيجة أن هناك اختلافات كبيرة في التعبير الجيني لل RAS جين بين البكتيريا والمبيضات مقارنة مع control عندما عوملت العزلات بخط الخلية أظهرت النتائج التعبير الجيني المثبط مع المبيضات البيضاء أعلى من *P .ingivalis* و control. أظهرت النتائج الحالية وجود high significant (قيمة P 0.05٪) في بعض التراكيز والعزلات بينما لم يظهر الخليط بين *C albicans* و *P gingivalis* فرقا معنويا مع كل التراكيز.

كذلك استخدمت تقنية الاليزا للكشف عن مستوى IL-17 من لعاب مرضى التهاب اللثة ومقارنتها مع الأشخاص الاصحاء حيث اظهرت النتائج ليس هناك فرق معنوي 0.398) p value بينما اظهرت النتائج هنالك فرق معنوي في تركيز الانترلوكين بين الاشخاص المصابين في *C albicans* و *P gingivalis*. P value (0.001).

وزارة التعليم العالي والبحث العلمي
جامعــة بابـــل
كليــة الطـــب

دراسه جزيئيه وسميه للمبيضات البيضاء وبكتريا Porphyromonus gingivalis المعزوله من المرضى المصابين باللتهاب اللثه

دراسة مقدّمة إلى
مجلس كلية الطب / جامعة بابل
وهي جزء من متطلبات نيل درجة الدكتوراه فلسفه في
العلوم / الأحياء المجهرية الطبيه

من قِبل

علي عـــــــــواد عبيـــــس عبد
بكالوريوس تحــليلات مرضيـــــة \2012
ماجستير احياء مجهريه طبيه/2019

إشراف

الأستاذ الدكتور

ميساء صالح مهدي **عبير فوزي مراد**

1444هـ **2023 م**

Chapter One

Introduction and Literature Review

Chapter Two

Materials and Methods

Chapter three

Results and Discussion

References

Conclusions and Recommendations

Supervisor certification

We certify that this thesis (Molecular and cytotoxic study of *Candida albicans* and *Porphyromonas gingivalis* isolated from patients with periodontitis **)** was prepared under our supervisor at the department of microbiology , college of medicine , university of Babylon in partial fulfillment of the requirement for the degree of doctor of philosophy in medical microbiology .

Supervised by

Professor Dr.Maysaa S.M. Al-shukri **Professor Dr. Abeer Fauzi M. Al-Rubaye**

In view of the available recommendation , I forward this thesis for debate by the examining committee

Professor

Dr. Hayam Khalis AL-Masoudi

Head of department of microbiology

College of medicine

University of Babylon

1. Introduction and Literatures Review

1.1. Introduction

Periodontitis is a chronic inflammatory disease characterized by the destruction of the supporting structures of the teeth. Its high prevalence and negative effects on quality of life make it one of the current problems in dentistry. *Porphyromonas gingivalis (P. gingivalis)* is the predominant periodontal pathogen that expresses a number of virulence factors involved in the pathogenesis of periodontitis : fimbriae, gingipains, haemagglutinin, and lipopolysaccharide (Oleinik and Goncharenko,2022).

Fimbriae that are encoded by fimA gene have been considered the main and the first virulence factor of this bacterium involved in adhesion, colonization, invasion, establishment and persistence within the host (Almaali, 2014). Invasion of *P. gingivalis* to host cell was relies on the ability of bacteria to produce gingipains (a trypsin – like cysteine proteinases) which support biofilm formation and regulate host defense response ((Ren *et al*., 2017).

The expression of several cytokines gingipains was modulate in multi cell kinds involved: gingival fibroblasts, endothelial cells, monocytes and T cells (Palm *et al*., 2015; Kariu *et al*., 2017).

Amongst the virulence factors of *P. gingivalis*, gingipains are the most important virulence factors which are responsible for damage of periodontal tissues inactivate and degrade a number of host defense and structural proteins, also it plays an essential role for *P. gingivalis* nutrient acquisition, colonization, immune subversion and signaling (Mahato *et al*., 2016). Initiation of periodontitis is relies mainly on embedding of microbes in subgingival dental plaque (biofilm) and interactions between microbes and host (Hajishengallis, 2015).

In addition to bacteria, fungi may also be related to oral mucositis. In immunocompromised individuals, *Candida albicans* frequently overgrows the microbial flora and causes infections and epithelial damage. *Candida* spp.,

particularly *C. albicans*, are associated with oral mucositis in patients with hematological malignancies(Sun *et al*., 2020).

C. albicans can induce carcinogenesis through its proinflammatory action, through induction of Th17 response, or through the production of direct carcinogene. *C. albicans* is capable of forming nitrosamines that act as carcinogens both alone and when combined with other chemical compounds. Their production leads to the activation of specific proto-oncogenes that can further support the formation of a carcinogenic lesion (Chang *et al*.,2019).

Interactions between *P. gingivalis* and *C. albicans* that can allow the cooperation of both microorganisms for mutual biofilm development and host invasion. For example, it was observed that *P. gingivalis* influences *C. albicans* morphology, enhancing germ tube formation (Guo *et al*.,2020). These findings were supported by the observed increased expression of genes encoding the *C. albicans* main adhesins, Als3 and Hwp1, and a secreted aspartic protease 6 (Sap6) that correlated with hyphal morphology (Bartnicka *et al*.,2019). However, some opposing effects have also been observed (Cavalcanti *et al*.,2016).

The mutual contact of both microorganisms was found to be based on direct interactions between the fungal adhesin *ALS3* and the adhesive domain of gingipain *rgpA* (Guo *et al*.,2020),. Another conductive interaction was also determined for the adhesion among both pathogens that induced the type 9 secretion system of *P. gingivalis* and increased the pathogenicity of the community (Hajishengallis *et al*.,2016).

On the other hand, the importance of a bacterial extracellular enzyme peptidylarginine deiminase (PPAD) for the mutual contact of both pathogens has been proposed (Karkowska-Kuleta *et al.*,2018). This enzyme converts protein arginine residues to citrullines, and this modification of selected surface-exposed *C. albicans* proteins was identified during the formation of

mixed biofilms by both microorganisms under hypoxic and normoxic conditions.

Aim of study:

This Case-control study aims to investigates effect of interaction of *Candida albicans* and *Porphyromonas gingivalis* in periodontitis at molecular level through following objectives:

1-Isolation and identification of *Candida albicans* and *Porphyromonas gingivalis* by using different media ,then identification of *P.gingivalis* by RT-q PCR.

2- Analysis of associated genes with important virulence factors include attachment and invasion gene by specific primers to *Candida albicans* .

3- Study the effect of *C.albicans* and *p.gingivals* alone and their combination on normal cell line (in vitro).

4-Study the effect of *Candida albicans* and *P*.gingivalis in tissue culture by detect specific biomarker by molecular method .

5-Detection of IL-17 level by ELIZA technique .

1.2. Literature Review

1.2.1 . Periodontitis

Periodontitis is a multifactorial chronic disease of the oral tissue, which is characterized by gingival inflammation and bone loss. More than 20 years ago, epidemiological studies reported that poor oral hygiene and tooth loss were significantly associated with oral squamous cell carcinoma OSCC, providing the first indication that oral bacteria might play a role in oral cancer development (Healy and Moran ,2019).

Clinically, periodontitis involves attachment loss around teeth, forming periodontal pockets, and bone destruction (Srivastava and Rana, 2022). Several studies have raised the heterogeneity of the microflora associated with periodontal disease. Indeed, various microorganism species, including Gram negative anaerobic bacteria organized in a complex biofilm, have been associated with the initiation and progression of periodontitis (Hussain *et al*.,2018).

Tezal *et al*.,(2009) reported that poor dental hygiene such as in the case of gingivitis and periodontitis are important risk factors for cancer inadequate poor oral hygiene is the primary cause behind periodontitis where accumulation of plaque and tartar stimulates production of inflammatory cytokines and prostaglandins to fight the infection.

Colonization of pathological bacteria is strongly correlated with inflammation and cancer progression. The immune system recognizes bacterial and viral invasion in periodontal pockets as foreign organisms damaging epithelial cells and attacks infected cells to remove the infection. Periodontitis is mainly caused by gram-negative anaerobic bacteria in dental biofilm which are responsible for secreting endotoxin substances destroying well-being of healthy epithelial cells (Peres *et al*.,2019).

In periodontitis, cellular migration and proliferation increases together with increased production of inflammatory cytokines, growth factors, enzymes

and some prostaglandins that are reported to be closely associated with cancer initiation and progression (Peres *et al*.,2019). Evidence indicates that chronic infection and inflammation are strongly associated with cancer (Peres *et al*.,2019).

A number of case-control studies describes the role of oral health and head and neck cancer and some cohort studies have explored the relationship between periodontitis and other types of systemic cancer reporting a significant positive association Tezal *et al*.(2007) .

A case-control study conducted in Beijing by Meyer *et al*.(2008)assessing the relationship between dentition and risk of oral cancer. This study recorded any missing tooth, gingivitis and periodontitis as a part of the oral exam. This study suggests that males and females suffering from periodontitis are more prone to develop oral cancerwhere males had a two to three-fold and females five to eight-fold increase in risk .

Sadighi Shamami *et al*. (2011) in a review of epidemiological research articles defines a clear association between tooth loss, periodontitis and carcinogenesis. Nine out of ten-case control studies reported significant increase in the risk of oral cancer in patients with periodontitis thereby indicating that there is a possible link between cancer and periodontal disease when modifying factors such smoking and drinking were controlled.

1.2.2. General Characteristic of *Candida* spp

Species of the genus *Candida* are found in their natural hosts, including humans. The genus *Candida* is distinguished by its oblong or oval shape, and it reproduces by bipolar bud (Alobaid *et al*., 2021). *Candida* grow on living tissue or culture media in the form of yeast or oval spherical cells and emerging yeast cells are cream-white in color range from 3 to 6 microns in length and may form what is known as pseudohyphae (Kadosh *et al* .,2020).

sometimes, this is called the case in the process of budding, identification methods have been developed for the purpose of diagnosing *Candida* species, such as the growth test on Chromagar *Candida* medium, where species grow on this medium in a range of different colors after an incubation period of 24 hours at 37°C (Rosiana *et al* .,2020), in addition to several differential tests Such as the formation of spores (Chlamydospores), which are characterized as large, thick-walled spores that contain a high content of proteins and fats, and the function of these spores remains opaque, which may represent a dormant stage of yeast, where these spores are formed in special conditions that differ from the normal state of growth such as Deficiency of Heat, Light, Nutrients and Oxygen, as well as *C. albicans* yeast is the main cause of fungal infection in humans, whether it is mucosal (Mucosal) or systemic (systemic) fungal infections (Villa *et al*.,2020).

The most common cause of superficial injuries to the nails and skin, a hot or humid environment of the body is important in the overgrowth of these yeasts because they stimulate their growth like skin folds in people with obesity, such as between the toes hand perineal and genitocrural (Webb *et al*., 2018).

The species of the genus *Candida* can be divided into two groups, *albicans* and non-*albicans*, which have the potential to cause infection, even partially in patients with somatic and immunocompromised (Webb *et al*., 2018).

Candida albicans is the main human pathogen among *Candida* yeast species. It is considered one of the yeasts that coexist in healthy people and is widespread in most environments as well as other species belonging to the genus *Candida* namely *C. krusei*, *C. glabrata, C. paraphsilosis, C. dubliniensis and C. tropicalis* (Thomaz *et al*., 2020).

Like the ability to Adhesion to cell walls (addition), which is the first step in the events of infection, and adhesion begins when a fibrous layer is

formed) of sugars on the surface of yeast cells, called cell adhesion, which helps to link yeast cells with carbohydrates and proteins. In the membranes of the host (Mourer *et al*.,2021).

This yeast is also characterized by its ability to form a germ tube, to which the mechanical strength is attributed to the resistance of immune cells or phagocytes in the host in addition to secreting enzymes that disintegrate the cells and tissues of the host and thus help the yeast to spread in the body host or extend between pus or chronic granulomas, or cause local infections suchas inflammation of the nails, skin, scalp, vagina, vulvitis and oral mucous membranes (Vila *et al*.,2020).

Among *Candida* species, *Candida albicans* is the most widespread yeast associated with healthy and pathologic oral conditions (d'Enfert *et al*.,2021). Indeed, this opportunist microorganism belongs to commensal microflora in the healthy human digestive tract, but can become pathogenic under the influence of general or local favorable factors. Periodontitis is a worldwide oral disease with a very complex etiopathogenesis, including dysbiosis and host immune responses that comaintain conditions for the occurrence of periodontal disease in susceptible individuals (Hajishengallis *et al*.,2017; , Lamont *et al*.,2018).

1.2.2.1.*Candida albicans*

It is classified as one of the most important types of opportunistic yeasts. These fungi form part of the normal flora of man. It turns into pathogens depending on the human immune status as a result of reduced cellular immunity and inhibition of the natural flora after antibiotic treatment that leads to the elimination of natural flora such as *lactobacilli* bacteria and exacerbation of *Candida* (Bohner *et al*., 2022).

This type of yeast is characterized by the presence of two dimorphic forms. The first form is single-celled yeast, and according to the environmental conditions in which it can be found, it grows in the form of yeast in a solid and

acidic medium that contains nitrogenous organic materials and sugar. As a carbon source, and another multicellular filamentous form (Multicellular filamentous form) (Rosiana *et al.*, 2020).

This species is characterized by the form of hyphae growing in acid culture medium that are neutralized with pH = 6.5 or more, media containing substances potato, dextrose, Or it may be pseudohyphae or the true hyphae, the transition from one form to another is due to intrinsic and extrinsic signals (Rane *et al*., 2019).

This process is of great importance in the pathogenesis of yeast, and this yeast can grow in different culture media such as blood agar, meat extract medium and Sabouraud dextrose cells, where the cells of this yeast appear under the microscope in an oval shape and may be irregular or elongated with a diameter ranging from It is between (10-12µ), and its soft and convex colonies may appear in a creamy white color and have a small yeast odor with a diameter of up to 0.5 mm in the case of growth for 18 hours, and when they remain for up to about a week with a diameter of 2 mm and may turn yellow, where It seems to have larger extensions and size, coarse and somewhat positive when using gram stain which is one of the most important diagnostic properties of white yeast, and the formation of the germination tube in the agricultural medium and under special environmental conditions, where the formation of this tube is an important diagnostic characteristic of these yeasts, as their ability to form squamous spores is another diagnostic feature of these yeasts (Lamoth *et al*., 2018).

1.2.2.2.Virulence Factors of *C. albicans*

A number of virulence factors support *C. albicans* to infect such complex host niches **(**Henriques, and Silva, 2021**)**. Virulence factors are found in many attributes like adhesive and invasive expression on the cell surface, biofilm production, secretion of hydrolytic enzymes and phenotypical switching (Staniszewska *et al*., 2020). Furthermore, *C. albicans* supports

virulence factors, colonize and avoid host defense acts through oral tissue adherence *C. albicans* (Fan *et al*.,2022).

Candida albicans must first react to environmental changes and transfer from the ordinary unicellular to invasive, multicellular filamentous forms to infect the host (Mundodi *et al*.,2021). Epithelial cell surfacing, germs and hydrolytic enzymes and hydrolytic materials are some of the essential factors , some of the hydrolytic enzymes are phospholipase, protease and lipase **(**Galocha *et al*., 2019).

Candida albicans produces a whole range of hydrolytic enzymes, which facilitates the adhesion of the pathogen to the cells host and is associated with the colonization of mucous membranes. This enables tissue penetration and digestion immune cells and antibodies (Yang *et al*.,2020; Dunker *et al*., 2021).

There are many reports in the literature that testify on the role of aspartyl protease (*sap*) in infections of *C. albicans* while examining the virulence characteristics of *C. albicans*, proved that that *sap* expression is associated with the formation of true hyphae, adhesion and phenotypic variability **(**Veni *et al*., 2022).

Hydrolytic enzymes (*sap*) coded by a family of ten *sap* genes catalyze hydrolysis peptide bonds (CO-NH) in proteins. *C. albicans* proteases digest cell membranes as well as immune system and antibodies it causes avoiding the host's defensive response in the process infections .*sap* proteins are key virulence factors having, participation in adhesion of *C. albicans* to host tissues (Permata *et al*., 2018).

Candida albicans adhesion to epithelial cells the mouth, esophagus, intestines and vagina is crucial stage of candidiasis development .Adhesion begins the process of *C. albicans* cell invasion into depth host tissues and this process follows that mannan is a component of the wall complex cellular containing highly glycosylated proteins, whose aspartic acid and serine/

threonine residues are joined sequentially by glycosidic bonds (N- and O- type) with sugar chains of different length (Mba and Nweze, 2020).

Mannan is detected on the outer surface of the cell wall *C. albicans*, and mannosebindings are responsible for the serospecificity of the strains ((Mba and Nweze, 2020). In adhesion to host cell surface are involved in *C. albicans* fimbrie , It has been shown that the main the structural subunit of fimbriae. According (Cavalcanti *et al*., 2017) . Mannoprotein sugar chains of *C. albicans* are involved in the adhesion process to cells the host. Cell surface hydrophobicity (CSH) is a virulence factor facilitating adhesion of *C. albicans* cells to tissues the host (Goswami, 2017). It has been proven that the inhibition of the process mannosylation increases the hydrophobicity cells and increased adhesion **(**Pérez-García *et al*, 2016).

Numerous authors have shown increased mannosylation external fimbriae proteins reduce adhesion *C. albicans* cells into epithelial tissues like also plastic medical tools (representing gateway to the development of disseminated candidiasis (Raška *et al*., 2007).

Hyphae forms have also been proven to adhere more strongly to the oral mucosa than the cells budding (blastoconidia) (Anderson *et al*., 2021).

The adaptability of *Candida* spp. is an important virulence determinant, as the fungus has to reproduce, respond to stress factors, and acquire nutrients in an efficient way to survive. Although *C. albicans* has been mostly considered an asexual fungus, thishas been denied by the discovery of a parasexual stage in its reproductive cycle. *C. albicans* can exist as sterile “white cells” and mating-competent “opaque cells” (Scaduto *et al*., 2014).

The two types of cells have the same genome, but they express different genes, and they have different metabolic preferences and different susceptibilities to antifungal drugs (Ciurea *et al*.,2020).

Sexual reproduction is inhibited by an acid pH and it is regulated by transcription factors encoded atthe mating-type loci (MAT) (Sun *et al*., 2015).

Mating competent/opaque cells communicate with mating incompetent white cells, enhancing the white cells' adherence and ability to form biofilms. This form of communication is conducted via pheromones (Dadar *et al*.,2018). *C. albicans*' response to stress is a factor of virulence, as it permits the yeast cells to survive and adapt. The response to stress is mediated by "heat shock proteins" (Hsps) (Jabra-Rizk *et al*., 2016).

The synthesis of Hsps-type proteins allows the survival of microorganisms at high temperatures, in environments with insufficient nutrients or under high oxidative stress conditions (Jabra-Rizk *et al*., 2016).

Heat-shock proteins (Hsps) 90, for example, is involved in the cellular dispersion inside biofilms, in *C. albicans* susceptibility to antifungals drugs, as well as in its temperature-dependent morphogenetic transition (de Aguiar Cordeiro *et al*., 2016).

The genetic exchange in *C. albicans* biofilms involves cell fusion and mating (Kowalski *et al* ., 2019). Inside a biofilm, sessile cells of *C. albicans* are less susceptible to antifungal drugs as compared with planktonic cells (free-floating cells). While forming biofilms in vivo¸ *Candida* spp. might use host components such as neutrophils, epithelial cells, proteins involved in inflammation (e.g., myeloperoxidase, alarmin S100-A9, Creactive protein) or site-specific proteins (Chong *et al*., 2018).

The interaction between *C. albicans* cells, for example, and the epithelium of the oral cavity is influenced by the presence of saliva. Some authors consider the possibility that proteins that are normally found in the saliva could act as bridging molecules between the hyphae and the epithelial cells and thus, they can facilitate the endocytosis of the yeast cells, but Sui *et al*.(2017) disproved this hypothesis. *In vitro* studies showed that even dead hyphae can adhere to

and are endocytosed by the epithelial cells found in the oral cavity, similar to live hyphae (Park *et al*., 2013).

Candida albicans exhibits a wide range of morphological phenotypes due to phenotypic switching and bud to hypha transition (Figure 1.1), (Gow and Yadav, 2017).

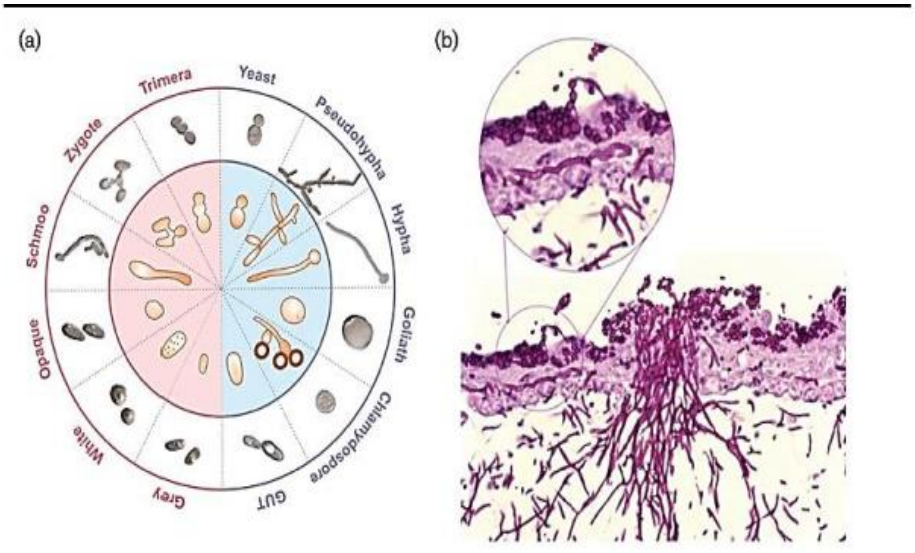


Figure (2.2): Morphology of *C. albicans*, showed (a): Morphotypes of *C. albicans*; blue sector represents the vegetative forms, whilepink sector represents the forms related to mating or changes in ploidy. (b): Superficial yeast colonization and invasion of chicken chorioallantoic membraneby *C. albicans* hyphal cells (Gow and Yadav, 2017).

Figure (1.1) :Morphology of *C albicans*(Gow and Yadav,2017).

The yeast-to-hyphae transition (filamentation) is a rapid process and induced by environmental factors, phenotypic switching is spontaneous, happens at lower rates, and in certain strains, up to seven different phenotypes are known **(**Kadosh *et al*., 2019).

Switching in *C.albicans* is often, but not always, influenced by environmental conditions such as the level of CO2, anaerobic conditions, medium used and temperature (Noble *et al*.,2017; Huang *et al*., 2019). In its yeast, form *C. albicans* ranges from 10 to 12 microns. Spores can form on the pseudohyphae called chlamydospores which survive when put in unfavorable conditions such as dry or hot seasons (Staib and Morschhäuser, 2007).

Unlike those of *Candida* species, *C. albicans* do not have a life cycle, but are either ovoid shaped budding yeast or ellipsoidally extended cells with restrictions of the sepsoidal tip, generally referred to as the pseudohyphae and as parallel wall-coated hyphae *C. albicans* have a period of life (Roselletti, *et al***,** 2019; Wijnants *et al*., 2021). Most pathogens are dimorphic, which helps them to exchange morphological states between the yeast and the germ tube **(**Kim *et al*., 2018). The fungal growth or invasion inside the host is often related to this exchange (Gow *et al*., 2017).

1.2.2.3.Pathogenesis

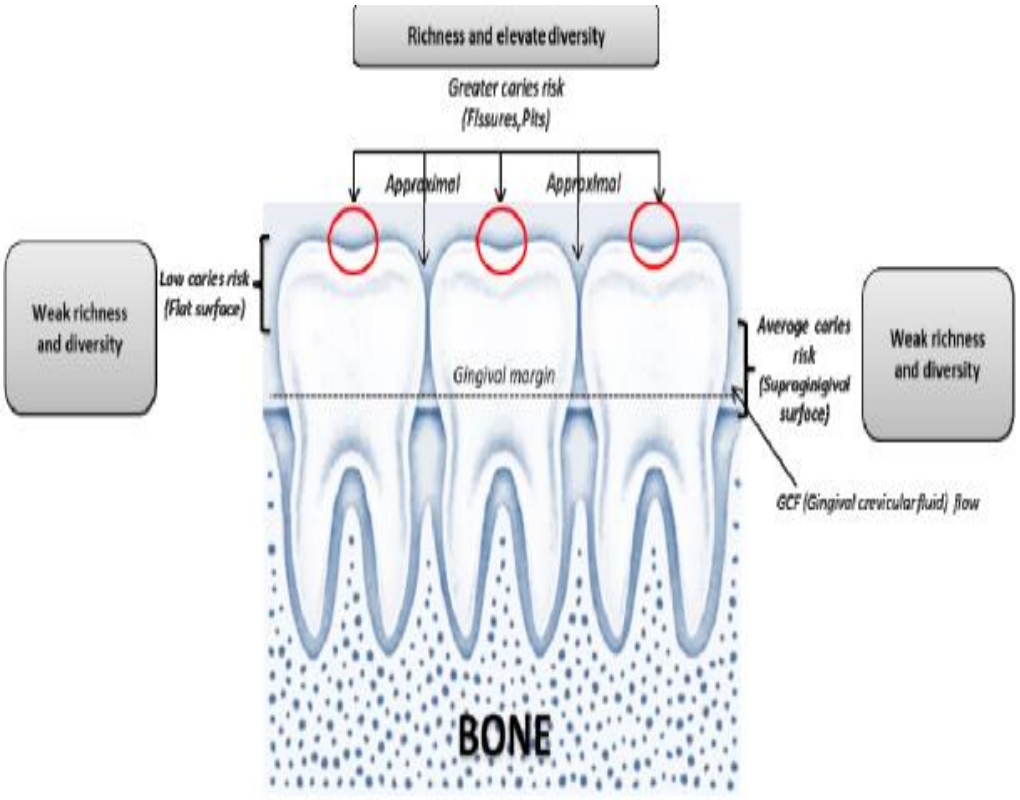
The formation of an oral candidiasis lesion is usually caused by the establishment of a complex biofilm containing *C. albicans* as well as other microorganisms (Contaldo *et al*.,2022). The biofilm adheres to oral surfaces, such as teeth, mucosa and dentures, and triggers an immune response heavy in neutrophils (McCall, *et al*.,2019). The biofilm is the perfect environment for the fungal cells to thrive, as the neutrophils cannot reach outside the body's own borders (Lalla *et al*.,2013).

C. albicans pathogenicity is linked to it phenotypic switching, between the commensal yeast form, and the invasive hyphal form (Gharaghani *et al* .,2021). Hyphae are elongations of the fungal cells, a tube without constrictions that can aid the pathogen in invading its host (Gharaghani *et al*., 2021).

C. albicans can invade the superficial layers of the oral epithelium, and cause proteolytic breakdown of E-cadherin (Lalla *et al*.,2013). E-cadherin is an important structural protein in the oral cavity, responsible for keeping the epithelium continuous, and a barrier against harmful substances. When E-cadherin is broken down, the tissue weakens and the protective barrier is compromised (Rouabhia *et al*.,2012). *C. albicans* uses this to migrate deeper into the tissue.

Interactions have been observed between *C. albicans* and *Porphyromonas gingivalis*, which have shown that cohesion caused by specific

proteins causes significant changes in gene expression by *P. gingivalis*, which could be used to increase infectivity. This means that interactions between different groups of microorganisms can trigger specific oral diseases such as the precancerous conditions. All the above show that the etiological flora of periodontal disease is not yet revealed and that several fungi can be a parameter responsible for the onset and progression of periodontal infection (Figure 1.2)(Sztukowska *et al* .,2018; Bottalico *et al*.,2016).



Figure(1.2). The risk associated with the areas that may be affected by caries and periodontal disease. Surfaces and locations with the highest variety and richness of oral microbial communities are more sensitive to caries and in the genital areas of periodontal disease. When tooth decay or periodontitis develops, the acidic environment reduces the variety and richness of local microbes.

1.2.2.4.The Biomolecular Mechanisms of *C. albicans*-Induced Oncogenesis

There are several hypothetical molecular mechanisms, that discuss the role of *Candida* cause dysplasia and malignant neoformations in the oral epithelium and can be mainly from (Patil *et al*.,2015; Sztukowska *et al.*,2018).

a. The production and release (via hyphal invasion) of nitrosamines, such as *N*-nitrosobenzyl-methylamine (e.g., caused by dysbiosis of the oral microbiota), which can lead to a tumor condition in mouse models (such as the Sprague-Dawley rats) (Sankari *et al.*,2015 ; Isacco *et al*.,2021).

b. An over-expression of P53, *Ki-67 labeling index*, and *Prostaglandin-endoperoxide synthase* 2 (COX-2) are some of the additional mechanisms by which *Candida* can affect malignant transformation into oral leukoplakia. P53 and Ki-67, which are markers of cell proliferation, have overexpression that is well established in malignant lesions, and COX-2, which is markedly increased in inflammation states and is associated with the release of prostaglandins, thus influencing cell proliferation, cell death, and tumor invasion (Warnakulasuriya, and Ariyawardana, ,2016 ; Alnuaimi, *et al.*,2016 ; Kiyoura and Tamai, 2015).

c. *Acid aspartyl proteinase* appears to be more present in oral lesions and therefore also in those with leukoplakia than in healthy subjects (Warnakulasuriya, and Ariyawardana, A ,2016).

The production of acid aspartylproteinase are putative virulence factors in candidiasis, and are why an acidic pH exists, thus degrading the sub endothelial extracellular matrix, as well as laminin 332 and E-cadherin. This induces dysplastic alterations and thus begins the *C. albicans* dissemination in the systemic circulation and therefore in the organs (Lim *et al.*,2017, Kiyoura and Tamai, 2015).

On the other hand, in a model of hyphal invasion (localized or uniform) of *Candida*, there is no difference between oral potentially precancerous disorders and oral squamous cell carcinoma. These biomolecular mechanisms

highlight the ability of *Candida* to influence malignant and cellular changes in oral leukoplakia (Kiyoura and Tamai, 2015).

d. Oral *Candida* infection is a cause of *up-regulation in proinflammatory cytokines* (interleukin (IL)-1α, IL-1β, IL-6, IL-8, IL-18, tumor necrosis factor (TNF)-α, IFN-γ, and GM-CSF), that influences the metabolic pathways and induces directly an endothelial dysfunction, playing a role in immune-related mechanisms with cancer development (Lim *et al*.,2017, Delaloye, and Calandra,2014; Jayachandran *et al*.,2016).

e. *C. albicans* can produce acetaldehyde (carcinogen due to mutagenic qualities in DNA) from precursors found in the oral cavity (metabolizing ethanol and glucose in high quantities, especially when associated with smoking and alcohol consumption)(Warnakulasuriya, and Ariyawardana,2016,Bombeccaria *et al*.,2016, Alnuaimi, *et al*.,2016). Thus, *Candida* can produce large quantities of acetaldehyde and acetyl-CoA synthetase (more in smokers) in cases of potentially malignant disorders and in oral carcinomas (concentrations of acetaldehyde and acetyl-CoA synthetase increase) compared to healthy individuals and those with ectodermal dystrophy and autoimmune polyendocrinopathy (with candidiasis) (Bombeccaria *et al.*,2016, Marttila *et al.*,2013).

However, the increase in the mutagenic amounts of acetaldehyde is more marked even in occupationally exposed workers to carcinogen and people with poor oral hygiene, than in healthy subjects, via the oral microbiota (*Streptococcus viridans* and resident fungi such as *Candida*) that can convert ethanol into acetaldehyde (possess the enzyme alcohol-dehydrogenase) (Gainza-Cirauqui *et al.*,2013). Indeed, the levels of acetaldehyde produced by *Candida* increase in proportion to the increase in alcohol consumption (Gainza-Cirauqui *et al*.,2013, O'Grady *et al.*,2020).

f. In oral squamous cell carcinoma, the reduction of β-defensins favors *Candida* superinfections. In chronic hyperplastic candidiasis, C. *albicans* is the

predominant species and is associated with high concentrations of alcohol dehydrogenase enzyme and P53 that suggests a dysplastic potential factor (Correia, *et al.*,2019). In fact, there is evidence that *Candida's* epithelial invasion can cause hyperplastic conditions (Figure 2.3) (Bombeccaria *et al.*,2016, Gupta, and Johnson, 2014).

g. The candidalysin (or 31-amino acid α-helical amphipathic peptide) is a cytolytic toxin of *C. albicans*. It is encoded by the ECE1 gene initially associated with fungal filamentation ability (release the toxin from the hypha) and host cell adhesion. Initially, ECE1 encodes 271 amino acid pre-proproteins that are cleaved by Kex8p enzyme into eight smaller peptides (Ece1-I to Ece1-VIII). Ece1-III6-93 is an epithelial immune activator and collaborates with the cytolytic activity of *C. albicans* (Moyes *et al.*,2016). Likewise, mmune activator and collaborates with the cytolytic activity of *C. albicans* (Moyes *et al.*,2016).

Likewise, candidalysin is an inducer for NF-κB and MAPK pathways. Candidalysin has been reported to excite granulocyte macrophage colony-stimulating factor GM-CSF, an essential molecule in carcinogenesis. After the macrophage death, the *C. albicans* can escape, survive, and outgrow other macrophages. On the other hand, it induces epithelial damage and elicits host inflammatory processes because it is a trigger for NLR family pyrin domain containing protein 3 (NLRP3) (Engku Nasrullah Satiman *et al.*,2020).

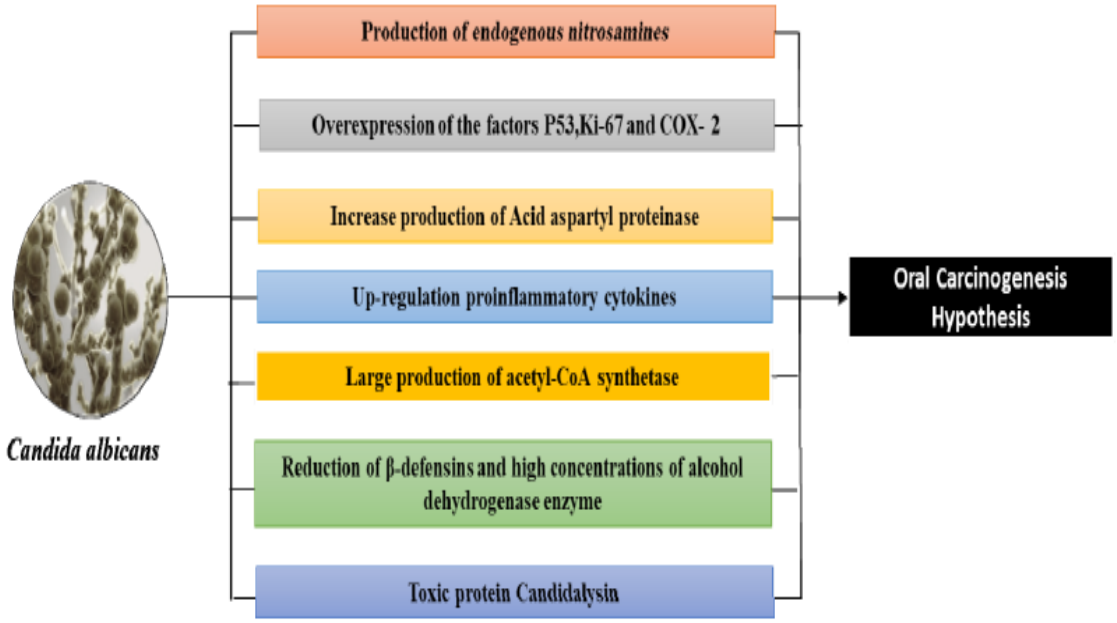


Figure 1. 3: The main hypothetical molecular mechanisms by which *C. albicans* can cause precancerous and malignant neoformations in the oral cavity.

1.3.1 Porphyromonas gingivalis

P. gingivalis is classified in the genus *Porphyromonas*, family *Porphyromonadaceae*, order Bacteroidales, class Bacteroides, phylum Bacteroidetes (Krieg *et al*.,2010). The bacterium is non-motile, Gram negative, rod-shaped, anaerobic, asaccharolytic and highly proteolytic. *P. gingivalis*, which is often found in deep periodontal pockets of humans, produces a broad array of potential virulence factors involved in tissue colonization and destruction as well as host defense perturbation (Silva and Cascales, 2021).

The major habitat of *P. gingivalis* is the subgingival sulcus of the human oral cavity. It relies on the fermentation of amino acids for energy production, a property required for its survival in deep periodontal pocket, where sugar availability is low (Bostanci and Belibasakis, 2012). Being an obligate anaerobe, *P. gingivalis* serves as the secondary colonizer of dental plaque, often adhering to primary colonizers such as Streptococcus gordonii and P. intermedia.

In the past few decades, *P. gingivalis* strains have been classified into invasive and non-invasive strains based on their ability to form abscesses in a mouse model. It has been demonstrated that the invasive strain of *P. gingivalis* possesses more pathogenic activities than the non-invasive strain both in vitro and in vivo (Dorn *et al*., 2000; Baek *et al*., 2015).

it was a member of the red complex (a group of three species including *P.gingivalis, Trepomema denticola* and *Tannerella forsythia*, which was strongly associated with each other and with periodontal disease site) and because it was the easiest of the three to grow and genetically manipulated, it became the most widely studied periodontal bacterium (Hajishengallis and Lamont,2012).

P. gingivalis can locally invade the periodontal tissues and evade the host defense system by utilizing a panel of virulence factors that cause disruption in the immune and inflammatory reactions. The potential virulence factors of *P. gingivalis* have been extensively described in several reviews (Mysak *et al*.,2014).

Porphyromonas gingivalis is strongly correlated with chronic periodontitis. Its chronic persistence in the periodontium depends on its ability to evade host immunity without inhibiting the overall inflammatory response, which is actually beneficial for this and other periodontal bacteria. Indeed, the inflammatory exudate (gingival crevicular fluid) is a source of essential nutrients, such as peptides and hemin-derived iron (Hajishengallis,2011).

Important features of *P. gingivalis*-mediated chronic periodontitis include the ability of the bacterium to adhere to and invade host cells, disseminate through host cells and tissues, and subvert host immunological surveillance and defense mechanisms(Carvalho-Filho *et al*.,2016).

Porphyromonas gingivalis rapidly adheres to the host cell surface followed by internalization via lipid rafts and incorporation of the bacterium

into early phagosomes. *Porphyromonas gingivalis* activates cellular autophagy to provide a replicative niche while suppressing apoptosis. The replicating vacuole contains host proteins delivered by autophagy that are used by this asaccharolytic pathogen to survive and replicate within the host cell. When autophagy is suppressed by 3-methyladenine or wortmannin, internalized *Porphyromonas gingivalis* transits to the phagolysosome where it is destroyed and degraded. For that reason, the survival of *Porphyromonas gingivalis* depends upon the activation of autophagy and survival of the endothelial host cell, but the mechanism by which *Porphyromonas gingivalis* accomplishes this remains unclear (Bélanger *et al*.,2006).

The harsh inflammatory condition of the periodontal pocket suggests that this organism has properties that will facilitate its ability to respond and adapt to oxidative stress. Because the stress response in the pathogen is a major determinant of its virulence, a comprehensive understanding of its oxidative stress resistance strategy is vital (Henry *et al*.,2012).

The ability of *Porphyromonas gingivalis* to cause adult periodontitis is determined by its arsenal of virulence factors. Biofilm formation and bacterial dipeptidyl peptidase IV (DPPIV) activity contribute to the pathogenic potential of *Porphyromonas gingivalis*. Furthermore, biofilm formation may enhance *Porphyromonas gingivalis* virulence through increased DPPIV activity. Because of their importance for bacterial colonization and growth, biofilm formation and DPPIV activity could present interesting therapeutic targets to tackle periodontitis (Clais *et al.*,2014).

1.3. 2.Porphyrmonas gingivalis Virulence factors

P. gingivalis expresses four major virulence factors: fimbriae, capsule, gingipains and lipopolysaccharides. The main function of fimbriae is to mediate adhesion and invasion into host epithelial cells. *P. gingivalis* fimbriae modulate proinflammatory cytokine production and also induce T cell

activation in mice . There are two main types of fimbriae that can be expressed by this pathogen, the major fimbria (FimA) and the minor fimbria (Mfa) (Liang *et al.*, 2020; Hasegawa and Nagano, 2021.).

The capsule of *P. gingivalis* aids in immune evasion, promoting survival of the bacterium within host cells and increasing virulence (Jia *et al.*, 2019). Along with immune evasion, encapsulation also reduces the host immune response in a variety of ways. Phagocytosis is reduced, survival increases in the presence of host cells, dendritic cell maturation induced by *P. gingivalis* is reduced, and virulence of *P. gingivalis* is enhanced compared to non-encapsulated *P. gingivalis* strains (Jia *et al.*, 2019).

P. gingivalis gingipains are proteases that function to degrade proteins into peptides as a source of nutrients. *P. gingivalis* utilizes hemin, iron-containing Protoporphyrin IX, as the primary form of iron , *P. gingivalis* acquires hemin from hemoglobin via the enzymic activity of gingipains (Priyadarshini *et al.*, 2013).

Gingipains also contribute to evasion of phagocytosis by degrading serum opsonins and host tissues (Hočevar *et al*., 2018). There have been three cysteine proteases purified from *P. gingivalis* with site-specific hydrolysis.Two of the proteases hydrolyze peptide bonds after Arginine residues and one hydrolyzes peptide bonds after Lysine residues. The proteases have recently been referred to as RGP and KGP or "gingipain R" and "gingipain K" (Cherian *et al*.,2019). Gingipain R aids in intracellular invasion and evasion of the host immune response by mediating vascular permeability through bradykinin release, enhancing binding of fimbriae to fibroblasts, and destroying the proteins of the complement system (Hočevar *et al*., 2018). Gingipain K mediates similar activities and is currently described as the most potent fibrinogenase (Lee, 2022).

The LPS of *P. gingivalis* has unique properties. Most gram-negative bacteria interact with TLR4 as the main transmembrane receptor for

lipopolysaccharides, while TLR2 is the main receptor to yeasts and gram-positive bacteria. *P. gingivalis* is an exception in that it can interact with TLR2 (Ingram *et al.*,2019; Hsieh e*t al*., 2020). Surface components of *P. gingivalis* including the LPS, lipoproteins, and fimbriae interact with TLR2 expressed on the surface of host cells , TLR2 mediates the expression of genes responsible for inflammation (De Andrade *et al.*,2019). The activation of TLR2 by *P. gingivalis* LPS may allow the pathogen to be able to regulate the class of the immune response *in vivo*, favoring a humoral response and enhancing its survival (Bourgeois, *et al*., 2019).

1.3.3. Pathogenesis of *Porphyromonas gingivalis*

The pathogenicity of *P. gingivalis* has been widely studied, including its ability to colonize surfaces of oral tissues, interact with other oral bacteria, induce a destructive immune response, and invade host cells (Ho *et al.*,2016).

Fimbriae were found to increase the tissue invasiveness and pro-inflammatory ability of *P. gingivalis* (Olsen *et al*.,2016).

Cell invasion by *P. gingivalis* occurs in oral epithelial cells, gingival fibroblasts, aortic and heart endothelial cells, and vascular smooth muscle cells (Chaudhuri *et al*.,2014; Ho *et al*.,2016).

P. gingivalis invasion is believed to protect the bacteria against environmental challenges including innate immune surveillance systems and antibiotic treatment (Damgaard *et al*.,2015). This likely plays a pivotal role in chronic bacterial infection (Park *et al*.,2016).

Previous work has shown that binding of *P. gingivalis* to red blood cells (RBCs) restricts phagocytosis of the bacterium by monocytes and neutrophils. This has led to the hypothesis that RBCs may also affect *P. gingivalis*-stimulated release of pro-inflammatory cytokines and production of intracellular reactive oxygen species (ROS) by neutrophils (Damgaard *et al*.,2017).

The perturbation of epithelial cells by bacteria is the first stage in the initiation of inflammatory and immune processes which eventually cause destruction of the tissues surrounding and supporting the teeth which ultimately result in tooth loss (Kinane *et al.*,2008).

Porphyromonas gingivalis can locally invade periodontal tissues and evade the host defense mechanisms. In doing so, it utilizes a panel of virulence factors that cause deregulation of the innate immune and inflammatory responses (Bostanci *et al.*,2012).

Porphyromonas gingivalis rapidly adheres to the host cell surface followed by internalization via lipid rafts and incorporation of the bacterium into early phagosomes. *Porphyromonas gingivalis* activates cellular autophagy to provide a replicative niche while suppressing apoptosis. The replicating vacuole contains host proteins delivered by autophagy that are used by this asaccharolytic pathogen to survive and replicate within the host cell. When autophagy is suppressed by 3-methyladenine or wortmannin, internalized *Porphyromonas gingivalis* transits to the phagolysosome where it is destroyed and degraded. For that reason, the survival of *Porphyromonas gingivalis* depends upon the activation of autophagy and survival of the endothelial host cell, but the mechanism by which *Porphyromonas gingivalis* accomplishes this remains unclear (Bélanger *et al.*,2006).

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Porphyromonas gingivalis contributes to the pathogenesis of aggressive periodontitis by inducing high levels of proinflammatory cytokines, such as IL-1β and IL-6 by peripheral $CD4^+$ T helper cells (Gonzales *et al*.,2014).

Porphyromonas gingivalis serotypes K1 and K2 but not others are associated with an increased production of the osteoclastogenesis-related factor RANKL. This important information suggests that these serotypes could elicit a greater bone resorption in vivo and have a significant role in the periodontitis pathogenesis. Destructive periodontitis is associated with a Th1-Th17-immune response and activation of RANKL-induced osteoclasts. In addition, *Porphyromonas gingivalis* K1 and K2 serotypes induce a strong Th1-Th17-response. These *Porphyromonas gingivalis* serotypes induce higher osteoclasts activation, by increased Th17-associated RANKL production and an antigen-specific memory T lymphocyte response (Vernal *et al*.,2014).

Chronic *Porphyromonas gingivalis* oral infection prior to arthritis induction increases the immune system activation favoring Th17 cell responses

which ultimately accelerate arthritis development. These results suggest that chronic oral infection may influence rheumatoid arthritis development mainly through activation of Th17-related pathways (Marchesan *et al*.,2013).

Salivary concentrations of metalloproteinase MMP-8, interleukin IL-1*β*, and *Porphyromonas gingivalis* are associated with various clinical and radiographic measures of periodontitis. The CRS index, combining the three salivary biomarkers, is associated with periodontitis. High salivary concentrations of metalloproteinase MMP-8, interleukin IL-1*β*, and *Porphyromonas gingivalis* have been associated with deepened periodontal pockets and alveolar bone loss and MMP-8 and IL-1*β* with bleeding on probing (Salminen *et al*.,2014).

The bacterium utilizes amino acids as energy and carbon sources and incorporates them mainly as dipeptides. Therefore, a wide variety of dipeptide production processes mediated by dipeptidyl peptidases (DPPs) could be beneficial for the organism (Ohara-Nemoto *et al*.,2014).

1.4.Role of interleukin-17 in Periodontal Disease

Periodontal disease is a multifactorial disease affecting the supporting tissues of the teeth and many etiologic factors are implicated in etiopathogenesis of periodontal disease. Although pathogenic bacteria have been the main causative factor in periodontal disease, host response accounts for the majority of periodontal destruction by releasing various inflammatory mediators, which has a negative impact on the periodontium. The host immune responses are regulated by various classes of T-cell subsets. Initially, periodontal disease can be explained with the T-helper 1/T-helper 2 paradigm. However, the discrepancies associated with it have led to the discovery of T-helper 17, which is responsible for the secretion of the cytokine interleukin-17 (IL-17) (Preeja, and Sivadas,2021).

IL-17 is a proinflammatory cytokine released from activated Th17 cells. Besides IL-17, TNF-α and IL-6 are released from Th17(Harrington *et al*.,2005).

IL-17 plays a major role in immune response because it stimulates the secretion of various chemokines resulting in recruitment of neutrophils and macrophages causing the subsequent clearance of pathogens, IL-17 mediates the actions of adaptive and innate immune systems resulting in proper regulation of immune response. Immune responses if not properly orchestrated can adversely affect the host which is manifested as bone destruction in periodontal disease. Evidence from various studies(Zwicky *et al*.,2020) substantiated that the destruction of bone in periodontal disease is mainly by immune response of the host rather than due to infectious microorganisms.

The interplay between osteoblasts and osteoclasts in bone remodeling has a crucial role in maintaining bone homeostasis. The differentiation of osteoclasts is under the control specific factors released from B and T lymphocytes (Jolink *et al*.,2017).

The occurrence and development of periodontitis involves a series of immune and inflammatory reactions. Periodontal tissues damage caused by periodontitis is mainly attributing to the host's immune response to infected microorganisms and their toxic products, not just directly caused by the infected microorganisms. The innate and adaptive immune defense and inflammatory defense that occur when the body prevents microbial invasion and diffusion will damage the local periodontal tissues. Therefore, the protective and destructive mechanism of the host immunity is an important link in the progression of periodontitis. When periodontal tissue was infected by pathogens such as *Tannerella forsythia*, *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans* and *Prevotella intermedia*, neutrophils and macrophages phagocytize and kill pathogens, which not only

play an immune activation and regulatory role, but also promotes the local inflammatory response of periodontal tissue (Nussbaum and Shapira, 2011; Kinane *et al*.,2017).

Activated B cells secrete antibodies while plasma cells secrete TNF-α, IL-6, IL-10, TGF-β and MMPs (Berglundh *et al*.,2007). Local infiltrating plasma cells during periodontal inflammation may be an important reason for the imbalance between MMPs and its blocker TIMPs. This lone-term chronic inflammatory response results in the absorption of alveolar bone by osteoclasts and the degradation of periodontal membrane fibers by MMPs. The ratio of Th17/Treg cells in gingival tissue and peripheral blood of patients with chronic periodontitis was significantly higher than that of healthy people (Okui *et al*.,2012), and the number of γδT cells in gingival tissue was also higher than that of healthy people (Barel *et al*.,2022).

As what mentioned before, both Th17 cells and γδ T cells are the cell sources of IL-17A, suggesting that the expression level of IL-17A in periodontal region of patients with chronic periodontitis is significantly higher than that of healthy people, which has been confirmed by many studies in recent years (Zenobia and Hajishengallis,2015 ; Kang *et al.*,2021). Furthermore, it has been reported that the expression level of IL-17A mRNA in gingival tissue of patients with chronic periodontitis was higher than that of patients with gingivitis (Thorbert-Mros *et al.*,2019).

Although IL-17 has a major role in periodontal pathogenesis whether they have a protective or destructive role in periodontal disease is still a matter of controversy (Xiong *et al.*,2019)

A previous study done by Yu *et al*(2007) found that the IL-17 has a bone protective effect in periodontal disease due to its crucial role in neutrophil

regulation. This can be explained due to the fact that neutrophils form the first line of defense and they play an active role in controlling periodontal infection; hence, impairment of neutrophil function can lead to increased microbial load. In sterile inflammatory conditions such as rheumatoid arthritis or other autoimmune disorders, IL-17 can cause tissue destructive effects.

However, in infectious inflammatory conditions such as periodontal disease, IL17 is having tissue protective actions due to its key role in the recruitment of neutrophils and other immune cells, thereby limiting the spread of periodontal infection.,Thus, Th17 cells play a key role against protection from bacteria and fungi which are not adequately dealt with Th1-mediated immunity.(Liu *et al*.,2021). However, there are various studies reporting elevated levels of IL-17 in the destructive phase of periodontal disease(Kumar *et al*.,2021).

Another previous study implicated a destructive role of Th17 cells due to the fact that Th17 acts as an osteoclastogenic lymphocyte that links T-cell activation to bone resorption , Therefore, IL-17 is a proinflammatory cytokine with dual action in various inflammatory disorders and further studies should be done in this field to evaluate the predominant role played by IL-17 in periodontal disease (Sato *et al*.,2006).

outer membrane protein of Porphyromonas gingivalis can induce the production of IL-17 in patients with periodontitis, and after its stimulation, it is detectable in patients with periodontitis than in those with gingivitis((Kumar *et al*.,2021) .

Another study investigated for the presence of IL-17 in periodontal lesions and to determine the effect of IL-17 on IL-6 production in human gingival fibroblasts. They found that IL-17 is produced in periodontal lesions and is involved in Th1 modulation and also enhances inflammatory reactions through mediators from gingival fibroblast in periodontal disease(Takahashi *et al*.,2005).

Bartemes and Kita (2018) evaluated for the presence of IL-17 in gingival crevicular fluid (GCF) and from cell cultures of gingival tissue in patients with periodontitis and reported its presence in GCF and gingival cell cultures suggesting a role for IL-17 in periodontal pathogenesis.

Another study done by Chen *et al.*(2015) investigated Th1/Th2/Th17 cytokine levels in plasma and GCF in chronic periodontitis patients and healthy controls. The results showed a stronger correlation between IL-17/IL-4 and IL-17/IL 10 in periodontitis patients than in healthy controls.

Cheng *et al.(*2014) in a review proposed the possible role of IL-17, and IL-17 producing CD4+ T cells (also called Th17 cells) in inflammatory periodontal disease.They suggested the crucial role of IL-17 and Th17 cells in periodontal disease and also the proof from animal studies, indicating the potential role in gingival inflammation and bone resorption in periodontitis.

1.5.Cell Line Culture

A cell culture that is derived from one cell or the set of cells derived from the same type and in which under certain conditions the cells will proliferate indefinitely in the laboratory. Cells are separated in two classes : Eukaryotic and Prokaryotic Cells.The clone or clones of cells derived from a small piece of tissue develope in culture. Cell is the basic structural unit of life that are bounded by plasma membrane i.e divided on the basis of the presence of cell or plasma membrane. Cell lines were the clones of animal or plant cells that grow on a suitable nutrient media in the laboratory which has various applications in the field of biochemistry and cell cell biology and biotechnology (Chaudhary& Singh, 2017).

Origin of cell line-1950-55. Hela cell, is a cell type in an immortal cell line that was used in scientific research. It is the oldest cell and most commonly used human cell line(Rahbari *et al.*,2009).Early, the primary cell line said to be named after a "Helen Lane" to conclude the fact that cells were taken without her knowledge or consent by Gey. Despite this attempt, her name was used by

the press within a few years of her death. These cells were treated as cancerous, as they are obtained from a biopsy taken from a lesion on the cervix as part of diagnosis of cancer. A conflict still continues on the classification of these cells.Culture that were derived from main tissue is known as primary culture. A primary culture has becomes a cell line when it is transferred into the other culture vessel. Adherent cultures, the cells were separated using a protease, such as trypsin, and/or a chelating agent, such as EDTA, and subdivided — that process was known as passaging(Marx .,2014).

For cells that will grow in suspension, the culture was split into new culture vessels. Under these circumstances the specialized culture conditions are used, within a few passages a relatively uniform population of proliferative cells was selected. This population was probably representative agent of the cells that divide when the tissue of origin is suffered, and will carry on growing until the end of the natural proliferative lifespan were reached and senescence occurs. As far as the cells proliferate, they show little or no law of tissue-specific differentiation. However, given the suitable signals, they can regenerate a functional tissue. Culture derived from primary subculture is known as cell lines and from continous culture or derived from passage of cell lines is known as subclones (Luong, *et al.*,2011).

The term cell line refers to the propagation of culture after the first subculture. In other words, once the primary culture is sub-cultured, it becomes a cell line. A given cell line contains several cell lineages of either similar or distinct phenotypes. It is possible to select a particular cell lineage by cloning or physical cell separation or some other selection method. Such a cell line derived by selection or cloning is referred to as cell strain. Cell strains do not have infinite life, as they die after some divisions(Marx .,2014).

The gingiva, both anatomically and functionally, is a unique structure with gingival fibroblasts (GFs) as the predominant cells of the gingival connective tissue. The existence of various subpopulations of GFs has been

reported (Fournier *et al*., 2013, Fournier *et al.*, 2010) However, these subpopulations are phenotypically different, sharing fibroblast-like structures and requiring identical growth conditions *in vitro* (Fournier *et al*., 2013).

A distinct property of the gingival cells is their role during scarless wound healing. Upon damage to oral tissues, the inflammatory response is manifested by a unique cytokine response from the GFs. At the same time, the GFs display fetal fibroblast-like properties including those related to migration and the production of migrating stimulation factors (David *et al*., 2014, Haekkine*n et al*., 2000). This healing capacity of the gingiva and its regenerative capacity has resulted in extensive research to identify the resident stem cell population within the gingiva with the ability to self-renew (Politis *et al*., 2016).

Gingival tissue represents an ideal source of tissue biopsies and GFs due to its accessibility and significantly reduced donor site morbidity compared to other dental tissues (Jin *et al*., 2015, Mostafa *et al*., 2008, Zhang *et al*., 2009). The literature offers overwhelming evidence to support the hypothesis that a subgroup within the GF cell population possesses mesenchymal stem cell (MSC) properties – and are thus called gingival mesenchymal stem cells (GMSCs) (Gardin *et al.*, 2016). Whether sorted (enriched) or unsorted, several studies have demonstrated that these GMSCs are able to differentiate into more than one lineage *in vitro* including osteogenic, chondrogenic, and adipogenic (Fournier *et al.*, 2010, Marynka-Kalmani *et al.*, 2010, Mitrano *et al.*, 2010).

1.6. Harvey-Ras gene expression

Rat sarcoma virus (RAS) is a protooncogene and its three family members are Harvey-Ras (H-Ras), Neuroblastoma Ras (N-Ras) and Kristen Ras (K-Ras having isoforms A and B) (Murugan *et al*.,2012). This family encodes Ras proteins having inherent guanosine triphosphatase (GTPase) activity and stimulates downstream signaling cascade via Raf-MEK-ERK, PI3K/AKT or c-Jun N-terminal kinase (JNK) pathways involved in cellular

proliferation, migration, adhesion and differentiation after growth factor stimulation such as EGFR (Krishna *et al*.,2018).

This gene and its signaling pathway is frequently mutated in oral cancer and mostly the mutations (T81C, Q61R, G12V and G13R) are reported in H-Ras (Akiyama *et al.*,2016). These mutations are mostly reported in smokers, betel quid chewers and also show ethnic variations (Krishna *et al*.,2018).

The studies have highlighted the role of H-Ras mutations in treatment failure or development of resistance to EGFR tyrosine kinase inhibitors such as cetuximab and erlotinib in oral cancer patients (Hah *et al.*,2014). The proposed mechanism of therapeutic resistance to EGFR TKIs include constant stimulation of downstream signaling pathways by mutated RAS gene in oral cancer via special group of genes such as CCND1, c-MYC, BCL-XL and BCL-2 (Rampias *et al*.,2014).

Harvey-Ras (H-Ras) is an important guanosine triphosphatase protein for the regulation of cellular growth and survival. Altered Ras signaling has been observed in different types of cancer either by gene amplification and/or mutation. The H-Ras oncogene mutations are well reported, but expression of the H-Ras gene is still unknown (Krishna *et al.*,2018).Ras gene family members play a relevant role in cancer, especially when they are mutated (Sciacchitano *et al*.,2021).

A number of transforming cellular oncogenes have been identified and isolated from different types of human tumor. Categorization of these oncogenes provides an understanding of cancer at the molecular level. In this context, attention has focused on the Harvey-Ras (H-Ras) gene in oral cancer. The RAS gene family consists of three functional genes, H-Ras, Kristen Ras (K-Ras: isoform A and isoform B) and Neuroblastoma Ras (N-Ras) encoding four highly similar, small and conserved Ras proteins (or p21 proteins), which located on the inner surface of the plasma membrane(Han, *et al.*,2017).

The Ras proteins have intrinsic guanosine triphosphatase (GTPase) activity that transduces the growth signal from the cell surface to intracellular effectors through mitogenic activating protein kinase (MAPK), c-Jun N-terminal kinase (JNK) and p38-kinase pathways, which regulate normal cell proliferation function(Krishna *et al.*,2015). The RAS GTPases activation regulates through cycle between GDP bound inactive and GTP bound active state with the help of guanine nucleotide exchange factors and GTPase-activating proteins (GAPs)(Kratz *et al.*,2007).

Previous research has improved the understanding of the structure, processing and signaling pathways of RAS in cancer cells and opened up new avenues for inhibiting RAS function (Ferrer *et al.*,2018;Marín-*Ramos et al.*,2019).

Abnormally activated RAS proteins regulate the function of major signaling pathways involved in the initiation and development in one-third of human cancers, RAS proteins act as a cellular switch that is turned on by extracellular stimuli, resulting in the transient formation of an active, GTP-bound form of RAS that activates various signaling pathways which regulate basic cellular processes (Khan, *et al.*, 2019; Li *et al.*,2018; Lindsay *et al.*,2018).

2.1. Subjects of the Study

This study involved (150) patients were sample collected from periodontal patients, the samples include saliva ,cotton swab and paper point from each periodontitis patients were admitted to specific dental health center and outpatient clinics of dentistry in Al-Hillah city/ Iraq during the duration from (April 2022 to September 2022).These patient were diagnosed by the dentist. Each patient were underwent-detailed history regarding age (the age of patients ranged from 5 to70 years) including both males and females., symptoms of infection .

2.1.1 Ethical Approval

The necessary ethical approval from ethical committee of the hospitals and patients and their followers must obtained. Moreover, all subjects involved in this work are informed and the agreement required for doing the experiments and publication of this work is obtained from each one prior the collection of samples.

2.2.Materials

2.2.1. Laboratory Apparatuses and Instruments

The main scientific apparatus, and technical instruments with disposable materials respectively, those were employed during the course of this study listed down in Tables (2-1) and (2-2).

Table (2-1): Scientific Laboratory Apparatus.

N	Item	Company	Country
1	Autoclave	Herayama	Japan
2	Bacteriological cabinet	Labogene	Denmark
3	Benson burner	Membrane	Germany
4	Camera	Nikon	Japan
5	Candle – jar	Memmert	Germany
6	Centrifuge	Kokusan	Japan

7	Deep freezer 20-°C	Jermaks	Germany
8	Electrophoresis	Biometra	USA
9	Incubator	Memmert	Germany
10	Light microscope	Stermite Olympus A &D	Japan
11	Micro centrifuge	Hamburg	Germany
12	Micropipettes(different size of Micropipettes)	Gillson Instruments	France
13	Nano drop	Biometra	USA
14	Oven	GS	Taiwan
15	PH meter	Orient	USA
16	Refrigerator	Concord	Italy
17	Sensitive Balance	Kern	Germany
18	Thermocycler	BioRad	USA
19	UV transilluminater	Wised	Korea
20	Vortex	IKA	USA
21	Water path	Polyscience	USA
22	ELISA Reader and Washer	BioTek	USA

Table (2-2): Technical Instruments and Disposable Materials.

N	Item	Company	Country
1	Eppendrof Tube	Biobasic	Canada
2	Glass Slides	Sail brand	China
3	Medical Gloves	Broche	PRC
4	Microscopic Cover Slide	Gitoglas	China
5	Parafilm	Bemis	USA
6	Pettri dishes	Himedia	India
7	Plastic Test Tubes 10ml	Afco	Jordan

8	Sterile Swabs	Afco	Jordan
9	Syringes	Dolphin	Syria
10	Test Tube Rack	Himedia	India
11	Tips	Afco	Jorden
12	Wooden Sticks	Supreme	China

2.2.2. Chemical Materials

Itemized down in **Table (2-3)**, the main chemicals utilized in this study.

Table (2-3): Chemical Materials and Reagents.

N	Item	Company	Country
1	Agarose	iNtRON	Korea
2	**Agar**	**Himedia**	**India**
3	**Dextrose**	**Himedia**	**India**
4	Ethanol 70%	GCC	England
5	Ethidium Bromide	Biotech	Canada
6	Glycerol	B.D.H	England
7	Gram stain kit	Sigma	Germany
8	KOH	Schuchariot	Germany
9	Normal Saline	Pharmaline	Egypt
10	Nuclease Free water	Bioneer	Korea
11	TB-Buffer	Biotech	Canada
12	Tetramethyl–p–phenylen Diamin Dihydrochloride	Fluka	UK
13	Phenol	BDH	England
14	Proteinase k	Promega	USA
15	Sodium chloride	BDH	England
16	Tris – Hcl	BDH	England
17	Interleukin(17) kit	BT-Lab	Korain

2.2.3. Biological Material

The main biological materials utilized in this study was listed in table(2-4).

Table (2-4): Biological Materials.

N	Item	Company	Country
1	Blood Agar Base	Oxoid	UK
2	CHROM agar candida	Liofilchem	Italy
3	Brain Heart Infusion Broth	Oxoid	UK
4	Columbia Agar	Himedia	India
5	Sabouraud Dextrose Agar	Hi media	India
6	Potato dextrose agar	-	Prepare in laboratory
7	*P. gingivalis* agar (P.GING)	-	Prepare in laboratory

2.2.4. Polymerase Chain Reaction Kits

In table (2-5) chemical materials used in PCR experiment in this study with their companies and countries of origin are listed.

Table (2-5): PCR Kits with their Remarks.

No.	Kit	Company	Country
1	**Genomic DNA Extraction Kit (Bacteria)**	**iNtRON**	**Korea**
	Buffer CL		
	Buffer BL		
	Buffer WA		
	Buffer WB		
	Buffer CE		
	Spin column/ Collection tube 2ml		
	Proteinase K 22mg/ml		
	RNase A 10mg/ml		

2	**Maxime PCR PreMix kit**	**Bioneer**	**Korea**
	Taq DNA polymerase		
	dNTPs (dATP, dCTP, dGTP, dTTP)		
	Tris-HCl pH 9.0		
	KCl		
	$MgCl_2$		
	Loading dye		
3	**TransSript Green One step qRT-PCR Super mix**	TRAN	
	TransSript Green One step RT\RI Enzyme Mix		
	2x PerfectStar Green One step qPCR Super Mix		
	Passive Reference Dye(50x)		
	RNase- free water		
4	DNA Lader(100bp)	NEXmark™	Korea
5	**Master Mix**	Promega	USA

2.3. Methods

2.3.1. Sample Collections

After obtaining the permission from the patients for examination and sampling the proper specimens collected for bacteriological and fungal analysis are described below. These specimens were collected in proper ways to avoid any possible contamination under use Rubber dam.

Samples were obtained from the necrotic pulp or most diseased sites with individual sterile paper points for *P.gingivalis* ,and cotton swab for

C.albicans,.paper point which were placed in necrotic pulp for 15 sec, In the present study three paper point were used for the collection sample (Singamaneni *et al* ;2010).

Then sterile paper points placed in tube contain 5 ml of BHI broth, after that cultured on Blood agar plates and on selective media anaerobically ;in the anaerobic incubator with using jar and gas back at 37°C for 7-14 days plus (10% Co_2).Then subjected to identification according to the cultural properties, microscopic examination and Biochemical test (MacFaddin,2000 and Forbes *et al* .,2007).The cotton swab were streaking on on S. agar & chromo agar for isolation *C.albicans* as shown in Figure(2.1).

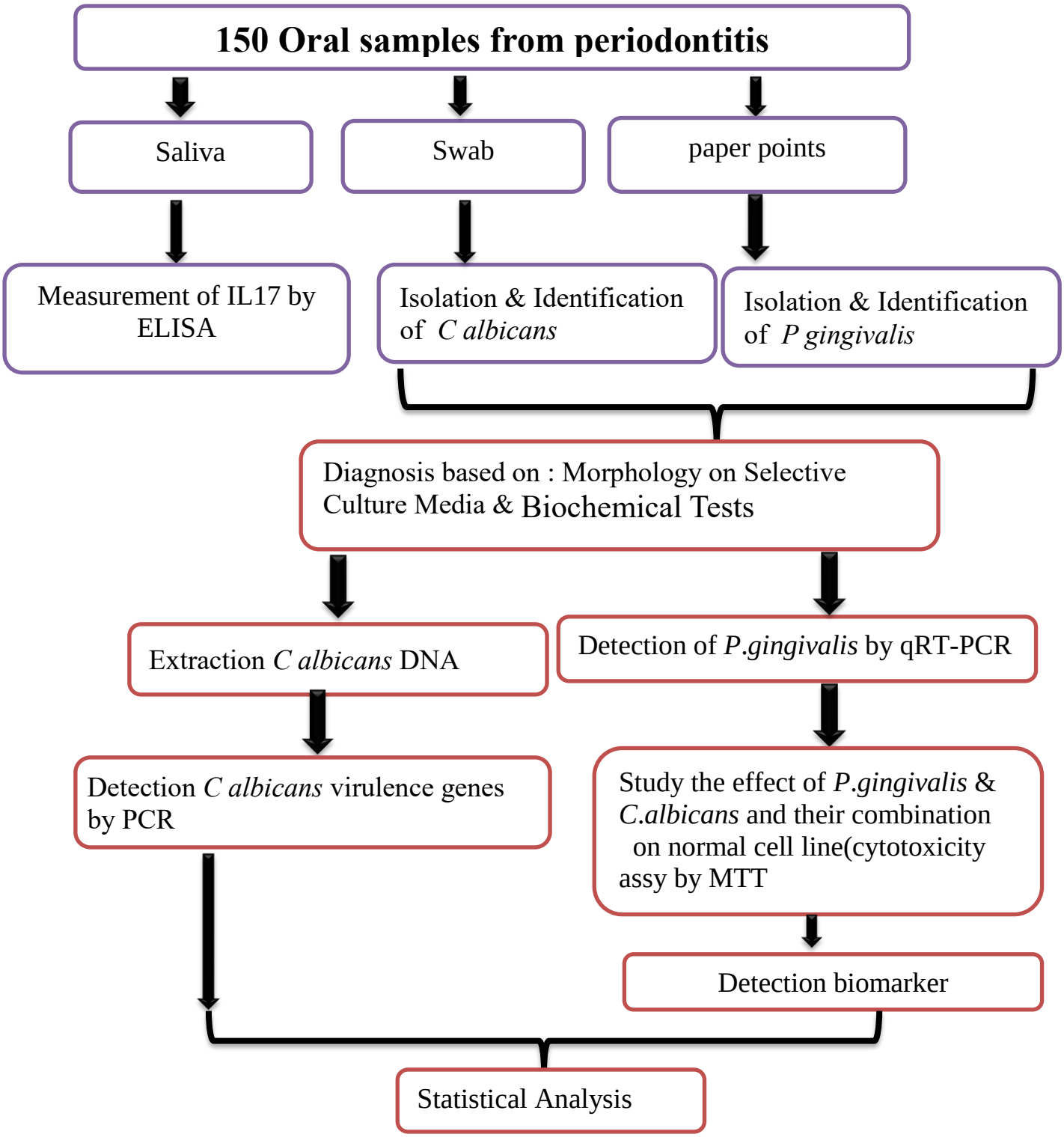


Figure (2-1): Demonstrating the Study analysis.

2.3.2. Preparation of Materials

2.3.2.1 Preparation of Stains ,Solutions and Reagents

2.3.2.1.1 Gram Stain

Ready to use stains and reagents: crystal violet, gram iodine, ethanol 70% and safranin stain, which were used for staining bacterial isolates for microscopic examination (Forbes *et al.*, 2007).

2.3.2.1.2 Catalase Reagent (3%)

This reagent was prepared by adding a 3ml of 6% H2O2 solution to 1 ml of D.W and stored in a dark container , to identify catalase producing bacteria (Forbes *et al.*, 2007).

2.3.2.1.3 Kovac's Reagent

It was prepared by dissolving 5g of p-dimethyl-l-aminobenzal-dehyde in 75 ml of isoamyle alcohols, then 25 ml of concentrated HCl was added carefully and gradually, and kept at 4°C until use. The reagent was used for detection the presences of indole (McFadden, 2000).

2.3.2.2. Preparation of Culture Media

The media were shown in Table (2-4) for isolating and diagnosing bacteria , fungi and yeasts according to the instructions of the manufacturer installed on the packages or according to the references of the scientific references and pH modification where necessary using the KOH and HCL the diluted dose were measured using pH strip. All the media were sterilized in a temperature of 121 C° , under pressure of 15 PSI for 15 minutes and the most important of these media is :

2.3.3.1.1. Blood agar medium

Blood agar medium performed according to manufacturer by dissolving 40 gm blood agar base in 1000 ml D.W. The medium was autoclaved at 121ºC for 15 minutes at15 pound/inch$_2$, cold to 45ºC and 5% of fresh human blood was added. It was used as enrichment medium for the bacterial isolates and to determine their ability to hemolysis RBCs (Wanger *et al.*, 2017).

2.3.3.1.2. Brain heart infusion broth(BHI)

This medium prepared by melting 37g in 1000 ml of Distille water (D.W.) into and heated to boiling to dissolve completely, as well adding chloramphenicol and streptomycin to make the fungal growth medium more competitive and preventing the growth of bacteria, sterilize at 121Ċ° for15min (Washington, 2012). This medium is useful for the grow for stored wide range of microorganisms.

2.3.3.1.3 Sabouraud Dextrose Agar medium (SDA)

It was prepared by suspending 65 g of SDA medium in 1000 ml of D.W and regulating the pH to 6.5 according to the instruction of the manufacturers 0.05 gm of Chloromenphenicol is added for each to prevent growth of bacteria. 0.5 gm of Cyclohexomide is added to prevent growth of saprophytic fungi. Then mixed and sterilized by autoclave. This medium is used for culturing and maintaining the pathogenic fungi and yeast isolates(Raines *et al*.,2013).

2.3.3.1.4 CHROM agar candida medium

The chrom agar is prepared by suspending 47.7 g of chrom agar in 1000 ml of D.W. and heated to the point of effervescence (for yeast cultivation), as instructed by the manufacturers. It was then poured into a plastic 9 cm petri dishes. The media is used for researching and diagnosing the Candida spp. Their appearance is based on color (Nadeem *et al.*, 2010).

2.3.3.1.5 Potato Dextrose Agar medium

This medium was prepared by following the instructions of the manufacturing by suspending 39 gm. dissolved in 500ml and complete the volume for 1000m and add 0.5 gm. of both antibacterial chloramphenicol , streptomycin and erythromycin then mixed and autoclaved at 121°C\15 minutes(MacFaddin, 2000).

2.3.3.1.6 Preparation of Sabouraud Dextrose Broth medium (SDB):

According to the manufactures instruction this medium is prepared by suspending 30 gm. of medium in 1000 ml of distilled water. Then 0.05 gm of Chloramphenicol is added and sterilized by autoclave (Odds, 1991).

2.3.3.1.6 Peptone water Medium

This medium was prepared by dissolving 8 gm peptone in 1000 ml of distilled water, and autoclaved at 121oC for 15 minutes at15 pound/inch2. It was used for the demonstration of the bacterial ability to decompose the amino acid tryptophan to indole (MacFaddin, 2000).

2.3.3.1.7 Selective media of *P.gingivalis*

The supragingival biofilms from clinically diagnosed chronic periodontitis patients were gently removed with sterile cotton pellets and (GCF) samples were collected using sterile paper points inserted in periodontal pockets during (30-60) seconds and (2-4) soaked paper points for each subject, were placed in 1.5 ml microcentrifuge Eppindorf tube containing 1 ml Sodium Thioglycolate Transport Fluid (STTF) .The periodontal samples were transferred to the microbiology laboratory during a period about four hours and subjected to *in vitro* cultivation on (P.GING) medium is locally prepared and it consists of Columbia Agar Base, supplemented with Sheep blood, Hemin, L-cystein, Vitamins K1,K3 and other selective agents for the isolation of *P. gingivalis* from other periodontal pathogenes in the periodontal samples as mentioned in the following table(2-6). and detection procedures Jousimies-Somer *et al*., (2002) and NCCLS, (2004).

After that , the periodontal sample tubes were incubated vertically at 37°C for 48 hours, then, 100 µl aliquot from each periodontal sample was streaked on *P. gingivalis* agar (P.GING) medium which is an enriched selective medium for the isolation and presumptive identification of *P. gingivalis* according to Jousimies-Somer *et al*., (2002) and NCCLS, (2004).

Table (2-6) Cultural requirements of *P. gingivalis* in (P.GING) selective medium according to Jousimies-Somer *et al*., (2002) and NCCLS, (2004).

Compositions	Dosage	Origin
Columbia Agar base	42.5 g /L.	Himedia , India .
L-Cystein	1mg /ml (1g /L.)	(BD BBLTM).
Hemin	5µg /ml (5 mg /L.)	Sigma Chemical Co.
Vitamin K_1	1µg /ml (1mg /L.)	Hoffman-LaRoche Ltd/ France
Vitamin K_3 (Menadione)	1µg /ml (1mg /L.)	Hoffman-LaRoche Ltd/ France
Agar Bacteriological powder	6.5 g./L.	(Oxoid) Basing stoke, U. K.
Bacitracin	10.0 mg/L.	Himedia Laboratories/ India
Colistin methane sulfonate	15.37 mg/L	Himedia Laboratories/ India
Nalidixic Acid	15.0 mg/L.	Himedia Laboratories/ India
Sheep Blood	50.0 ml/L.	Local sheep
Distilled Water	1000.0 ml	Local product
Blood agar base	39 gm/L.	Oxoid, no.2,Basing stoke, U. K.

Selective medium plates were incubated in a tightly packed anaerobic atmosphere jar using gas pack (OXOID Limited, Basingstoke, Hampshire, England) at 37°C for 7-14 days. In order to obtain pure culture, displayed colonies were recultivated on other selective blood agar plates (Oxoid, Basingstoke, United Kingdom) supplemented with all the additives in table (3-8) at the same circumstances.

2.3.3. Identification of Bacterial Isolates

2.3.3.1Cultural Characteristics

Each primary positive culture was identified depending on the morphological properties such as (Colony size, shape, color , translucency, edge and elevation of texture) (Wanger *et al.*, 2017).

2.3.3.2 Microscopic Examination

A single colony was selected by a loop from culture media and spread on a clean slide and fixed with heat to be attained using the gram stain according to. The morphology of bacterial cells was investigated by Gram- stain to observe the shape and arrangement of cells under oil immersion (100X magnification) microscopically (Brenner *et al*. 2005).

2.3.3.3 Cultivation on Blood Agar

All gram negative anaerobic bacilli inoculated on blood agar give black-pigmented colonies . All *P.gingivalis* gave catalase negative and Indole positive.

2.3.4 Biochemical Test

2.3.4.1Catalase test

A colony of organisms was transferred by sterile wooden stick to a clean, dry slide and mixed with few drops of 3% H_2O_2. A positive result indicated by the evolutions of bubbles as a result of the presence of catalase that hydrolyzes hydrogen peroxide to water and oxygen (Thille, 2016).

2.3.4.2 **Indole test**

The amino acid tryptophan is found in nearly all proteins, bacteria that contain tryptophanase can hydrolyze tryptophan to its metabolic products namely indol, pyruvic acid and ammonia to satisfy nutritional needs .The appearance of red ring on the surface medium was regarded as a positive result (MacFaddin, 2000).

2.3.4 Identification of Isolated Fungi

The identification tests, including cultural, morphological and biochemical characteristics were done for each isolate.

2.3.4.1 Cultural Characteristics

Each primary positive culture was identified depending on the morphological properties such as (Colony size, shape, color and natural of pigments, edge, texture) .

2.3.4.2 Microscopic Examination

Fungi isolates are examined microscopically, the fingerprint of the champignon in the colony is taken by adhesive tape, transparent adhesive tape is used, touches the surface of the fungal colonies and then pastes the tape on a glass slide containing a drop lacto phenol blue cotton. Slides examined under magnification 10X, 40X and 100X as described by (Pitt and Hocking, 2009; Rai, 2016; Samson and Pitt, 2000).

2.3.4.3. Culture Characters According to CHROM agar

This test is performed by inoculating the medium CHROM agar Candida, which was previously prepared from Candida isolates cultivated on SDA for 24 h. Single colonies of any yeast isolates are then picked and stretched on medium chrom agar , then incubated at 30 C for 24-48 hours (Sivakumar *et al*., 2009) Chrome agar test is used for presumptive identification of Candida species by different color production on this medium(*C. albicans*= green/ blue green, *C. dubliniensis* = dark green, *C. parapsilosis* = cream white, *C. krusei*=pink) (Sivakumar *et al*., 2009).

2.3.5 Preservation

2.3.5.1 Bacterial Isolates

1- Short Time Preservation

Single pure colony of bacterial isolate was inoculated on Brain heart infusion broth . Incubated at 37ºC for 24 hrs, sealed well and stored at 4ºC in the refrigerator one month for the plates and three months for the slants.

2- Long Time Preservation

The bacterial isolate was inoculated into the Brain heart infusion broth and incubated at 37°C for 24 hrs then the broth culture was preserved by adding glycerol to a final concentration of 20% and stored at -20ºC for 12-18 months (Forbes *et al*., 2007).

2.3.5.2 Preservation of *C albicans* Isolates

The isolates was inoculated into the Brain heart infusion broth and incubated at 37°C for 24 hrs then the broth culture was preserved by adding glycerol to a final concentration of 20% and stored at -20ºC for 12-18 months (Forbes *et al*., 2007).

2.3.6. Molecular Methods

2.3.6.1. Preparation of Molecular Materials

2.3.6.1.1. Preparation of 1X TBE Buffer

The preparation of 1X TBE buffer was performed by dilution of a concentrated 10X TBE buffer, this dilution was accomplished as 1:9 (v/v); 1 volume of 10X TBE: 9 volumes of distilled water. This solution was used to prepare agarose gel and as a transmission buffer in electrophoresis process. (Sambrook and Russel, 2001).

2.3.6.1.2. Preparation of Agarose Gel

The agarose gel was prepared according to the method of Sambrook and Rusell (2001) by adding 1-1.5gm agarose to 100ml of 1x TBE buffer. The solution was heated to boiling (using water bath) until all the gel particles dissolved. The solution was allowed to cool down within 50-60ºC, and mixed with 0.5µg/ml ethidium bromide (Sambrook and Russel, 2001).

2.3.6.1.3. Rehydration of Primers

Lyophilized primer pairs were rehydrated by DNA rehydration solution 1X (pH 8.0) Tris- EDTA buffer (TE-buffer). Initially, primer storage-stock tube prepared and then the working solution would prepared from primer stock tube. Consistent with the instructions of the producer (Bioneer/Korea), TE buffer was added to produce 100 picomole/microliter concentration of primer stock solution. The working solution prepared from stock as 1:9 (v/v) by dilution with TE buffer to get 10 picomole/microliter.

2.3.6.1.4 Ethedium Bromide

Prepared by dissolving 0.25 g from ethidium bromide in 50 ml D.W to get a final concentration of 0.5 mg/ ml (Sambrook and Russel ,2001).

2.3.7. Genomic Fungal DNA Extraction

Fungal genomic DNA was extracted from SDB. The extraction was done by using (Presto™ Mini gDNA Fungi Kit) according to company instructions as following steps:

1. One milliliter of (18 hours) incubated cultured fungal cells (up to 1 × 108) was transferred to 1.5 milliliters micro-centrifuge tube, then, was centrifuged at 10000 rpm for 2 minute then the supernatant was discarded.
2. Totally, 180 microliters GT buffer was added to the tube and the cell pellet suspended by vortex. After that, 20 microliters of Proteinase K was added, and the mixtures was incubated at 60ºC for 10 minutes. During incubation periods, the mixtures tubes were inverted every 3 minutes.
3. Totally, 200 microliters of GB buffer were added to each tube and mixed via vortex for 10 seconds. Then, the tubes were incubated at 60ºC for 10 minutes with inverted the tubes every 3 minutes throughout the incubation periods.

4- Totally, 200 microliters of absolute ethanol were added and immediately mixed via vortex. Then, precipitates if happen was broken by pipetting.

5- The GD column was placed in a 2 milliliters collection tube and the mixtures was transferred (including any precipitate) to the GD column. Then, the mixtures were centrifuged at 10000rpm for 1 minute. The 2 milliliters collection tubes that contained the flow-through were discarded and placed the GD column in a new 2 ml collection tube.

6- Totally, 400 microliters of W1 buffer were added to the GD column then centrifuged at 10000 rpm for 1 minute. The flow-through was discarded and placed the GD column back in the 2 milliliters collection tube.

7- Totally, 600 microliters Wash Buffer was added to the GD column. Then, it was centrifuged at 10000 rpm for 1 minute. The flow-through was discarded and

placed the GD column back in the 2 milliliters collection tube, after that, the tubes were centrifuged again for 2 minutes at 12000 rpm to dry the column matrix.

8- The dried GD column was transferred to a clean 1.5 milliliters micro-centrifuge tube and 100 microliters of pre-heated elution buffer was added to the center of the column matrix.

9- The tubes were let stand for at least 3 minutes to ensure that, the elution buffer was absorbed by the matrix. Then, it was centrifuged at 10000 rpm for 1 minutes to eluted the purified DNA

10- .All samples were stored at (- 10 to -20) ^{o}C .

2.3.8. Genomic Bacterial DNA Extraction

DNA extraction by using G-Spin^{TM} kit (iNtRON/ Korean) and performed as follows:

1- Transfer the appropriate number of Bacterial cell (up to 1×10^{9}) to a 1.5ml microcenterifuge tube and centrifuge at full speed.(14.000 rpm or 10.000× g) for 1 minute. Then Discard the supernatant.

2- CL Buffer (200µl) , lysozyme (100µl), RNase (5µl) and proteinase K (20µl) were applied to the tube and resuspended the cell pellet by vigorously shaking the vortex, after that incubated the tube at 25C for 10 minutes, and the tubes inverted each three minutes during periods of incubation.

3- BL Buffer (200µl) was applied to each tube and blended vigorously for 5 seconds. the tubes were Then incubated in 60^{o}C water bath for ten minute and inverted each three minutes during incubation period.

4- 200 µl Absolute ethanol has been applied to the clear lysate and immediately bended with vigorous shaking, after that precipitates have been broken up by pipetting.

5-The spin column was put in a collection tube(2ml)and all the mixture (including any precipitate) was transported to the spin column . After that the tube centrifuged at 15,000 rpm for two minute and the collection tube 2 ml containing the flow-through was discarded and put the spin column in a new collection tube 2 ml.

6- WA Buffer (700µl) was applied to spin column, after that the tube centrifuged at 15,000 rpm for thirty second. The flow through was removed and the spin column returned to the 2 ml collection tube.

7- 700µl of WB Buffer was applied to the spin column. After that centrifuged at 15,000 rpm for thirty second. The flow-through was removed and the spin column breturned to 2 ml collection tube. Then, the tubes were again centrifuged for three minute at 15,000 rpm to dryed the column matrix .

8- A dry spin column was transported to a clean 1.5 ml microcentrifuge tube and 100 ML of initial heating elution buffer were applied to the middle of the column matrix.

9- A tubes were left standing to at least three minute to ensure that elution buffer was absorbed by the matrix.
The extracted DNA was then centrifuged at 15.000 rpm for 30 seconds.

10- The purified DNA was preserved at -20°C.

2.3.9. Determination DNA Concentration and Purity

The extracted genomic DNA was checked by using Nanodrop spectrophotometer, which measured DNA concentration (ng/µL) and check the DNA purity by reading the absorbance at (260/280 nm) as following steps:

1. After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, DNA).

2. A dry paper-wipe was taken and clean the measurement pedestals several times. Then carefully pipet 2µl of ddH2O onto the surface of the lower measurement pedestals for blank the system.

3.The sampling arm was lowered and clicking OK to initialized the Nanodrop, then cleaning off the pedestals and 1µl of extracted DNA carefully pipet onto the surface of the lower measurement pedestals ,then check the concentration and purity of extracted DNA.

2.3.10 Detection of Virulence Gene of *C albicans* by PCR

DNA was used as a template for specific PCR to detect (ALS1, HWP1,EAP1) virulence gene . A pair of specific primer were used for the amplification of a fragment gene shown in table(2-7). A single reaction mixture contained 2.5ml of each primer (forward and reverse) , 5 ml of DNA extract , 5 ml of master mix and 5 ml of nuclease free water to obtain a total volume 20 ml . The amplicon were run in 1-1.5 a garose gel.

Table (2-7): Primer and condition for amplification *Candida albicans virulence genes*.

C albicans Genes	Sequence of Primer (5' -------- 3')		PCR amplicon size	Condition	References
ALS1	**F**	GACTAGTGAACCAACAAATACCAGA	318	**Initial Denaturation** (94Cº for 5 min No.of cycle 35) **Denaturation (**94Cº for 1 min) **Annealing (**55Cºfor 30sec) **Extension (**72Cºfor 1min) **Final extinction (**72Cºfor 5min)	**(Melek *et al*.,2013(**
	R	CCAGAAGAAACAGCAGGTGA			
HWP1	**F**	ATGACTCCAGCTGGTTC	503	**Initial Denaturation** (94Cº for 5 min No.of cycle 35) **Denaturation (**94Cº for 30 sec) **Annealing (**55Cºfor 40sec) **Extension (**72Cºfor 1min) **Final extinction (**72Cºfor 5min	**(Ardehali *et al*.,2019)**
	R	TAGATCAAGAATGCAGC			
	F	TGTGATGGCGGTTCTGTTC	66	**Initial Denaturation** (94Cº for 5 min No.of cycle 35) **Denaturation (**94Cº for 30 sec) **Annealing (**55Cºfor 30sec) **Extension (**72Cºfor 30Sec) **Final extinction (**72Cºfor 5min	
EAP1	**R**	GGTAGTGACGGTGATGATAGTGACA			

2.3.11 PCR Products Investigation

Successful PCR amplification was confirmed by agarose gel electrophoresis by visualization against UV light (Sambrook and Russell, 2001).

Agarose gel was prepared according to (2.3.3.3.2.). Then the comb was fixed at one end of the tray for making wells used for loading DNA sample. The agarose was poured gently into the tray, and allowed to solidify at room temperature for 30 min . The comb was then removed gently from the tray. The tray was fixed in an electrophoresis amplicon was transferred into each well of agarose gel, and in one well we put the 5µl DNA ladder.

The electric current was allowed to pass at 70 volts for 50min. UV trans-illuminator was used 280 nm for the observation of DNA bands, and the gel was photographed using digital camera.

2.3.12 Real Time -PCR for Detection *P.gingivalis* .

DNA was used as a template for RT-PCR to detect *P.gingivalis* . A pair of specific primer were used for the amplification of a fragment gene shown in table(2-8).

Table (2-8):The Sequence of Primer and probe that was Used in the Present Study for Detection *P.gingivalis*

Genes	Primer Sequence (5' ----- 3')		Probe (5' ----- 3')	Reference sequence
P. gingivalis	F	TGGTTTCATGCAGCTTCTTT	CGTACCTCATATCCCGAGG	PG1370b
WaaA	A	TCGGCACCTTCGTAATTCTT	GGCTG	

2.3.13 PCR Master Mix Preparation and Condition

PCR master mix *P . gingivalis* gene was prepared by using (Trans Script One Step **qRT-PCR Super Mix**) and this master mix done according to company instructions as following table(2-9):

Table (2-9): Contents of the qRT -PCR reaction mixture with their volumes

PCR master mix	Volume
DNA tamplate	4µL
forward primer (10µM)	1µL
reverse primers (10µM)	1 µL
One step qPCR SuperMix	9.5 µL
TransScript Greenone step RT/RI Enzyme Mix	0.5 µL
Rnase –free water	4
Total	**20µL**

Thermocycles condition

Rotagene Q (Qia gene , Germany)
95-5 min
95-20 Sec 40 cycle
60-30 Sec 40 cycle
Data collected at green channel.

2.3. 14. Atotal RNA extraction :

2.3.14. 1.A.totale RNA Mini Kit:

The RNA extraction from Cell line specimens were carried out according to the manual of manufacturer of Geneaid company **:**

The Genomic RNA extracted kit for tissue Component show in Table (2- 10).

Table (2-10) RNA extraction kit for tissue contents

Component	Volume Final
Lysis Buffer	10 ml
RB Buffer	2ml
DNase I Reaction Buffer	200 µl
W1 Buffer	2 ml
Wash Buffer (Added Ethanol)	1.5 ml (6 ml)
RNase-free Water	1 ml
RB Column	4
2 ml Collection Tube	8

Protocols:

1. A volume of 50µg of cell was added to a sterile 1.5 ml microcentrifuge tube. Mixed by inversion.
2. A volume of 1 ml of RBC Lysis Buffer was added to the tube and incubated on ice for 10 minutes (briefly vortex twice during incubation).
3. Centrifuge at 3,000 x g for 5 minutes then remove the supernatant completely.
4. A volume of 400 µl of RB Buffer was added, and the tube was incubated at room temperature for 5 minutes.
5. A volume of 400 µl of Wash Buffer was added to the RB Column. Ethanol was confirmed to be added to the RB Column and centrifuge at 14000 x g for 30 seconds. Flow- through was discarded and the RB Column was placed in the 2 ml collection tube.

6. DNase 1 solution was prepared in a 1.5 ml microcentrifuge tube (RNase-free) as follow:

Table (2-11) The DNase I prepared contents

Content	Volume
D Nase 1	5 µl (2 U/µl)
DNase 1 Reaction Buffer	45 µl
Total Volume	50 µl

7. By pipette DNase 1 solution was mixed carefully.
8. A volume of 50 µl DNase 1 solution was added into the RB column and Incubated for 15 minutes at room temperature (20-30ºC).
9. A volume of 400 µl of W1 Buffer was added into the RB Column, centrifuge at 14000 x g for 30 seconds. Flow-through was discarded then the RB Column was placed in the 2 ml collection Tube.
10. A volume of 600 µl of Wash Buffer was added into the RB Column. Centrifuge at 14000 x g for 30 seconds .
11. A volum of 600 µl of Wash Buffer was added into the RB Column, Centrifuge at 14000 x g for 30 seconds . The RB Column was placed back in the 2 ml collection tube and centrifuge at 14000 x g for 3 min to dry the column.
12. The dried RB Column was place in a clean 1.5 ml microcentrifuge tube.
13. A volume of 50 µl of RNase-free Water was added into the column. It was left for at least 1 minute to ensure that the RNase-free water was absorbed. Centrifuge at 14,000 x g for 1 minute to elute the purified RNA.
14. DNA digestion in Solution the DNase 1 reaction in a 1.5 ml microcentrifuge tube (RNase-free)was prepare as follows :

Table (2-12) The DNase I reaction contents

Content	Volume
RNA in RNase-free Water	1-40 µl
DNase I	0.5 µl/µg RNA
DNase I Reaction Buffer	5 µl
RNase-free Water	Added to final
Total Volume	50 µl

15.By pipette DNase 1 solution was mixed carefully.

16.The microcentrifuge tube was incubated at 37ºC for 15-30 minutes.

17.The tube containing RNA was kept at -20 °C.

2.3.14.2 qRT-PCR for Detection *H-RAS* gene Expression

RNA was used as a template for qRT-PCR to detect the expression ***H-ras gene***.

A pair of specific primer and primer for housekeeping gene were used for the amplification of a fragment gene shown in table(2-13).

Table (2-13):The Sequence of Primer that was Used in the Present Study for Detection *H-RAS gene Expression*

Genes	Primer Sequence (5' ----- 3')		size	Condition	Reference
Acti	F	'CGTGCGTGACATTAAG GAGAAG		Temperature 45 Time 5 min	**(Azab *et al*.,2012)**
	R	GAAGGAAGGCTGGAA GTG			
H-ras	F	TGAGGACATCCACCAG TACA	118	Temperature 45 Time 5 min	**(Krishna *et al*.,2018)**
	R	CGAGATTCCACAGTGC			

One step (TRANS super mix)

Rotagene Q (Qia gene , Germany)

Thermocycles condition (H-ras gene)

45c -30 Min cDNA synthesis

95-5 min

95-20 Sec 40 cycle

60-30 Sec 40 cycle

Data collected at green channel.

2.3.15 Cytology Study(Cell line and cell culture)

2.3.15.1 Chemicals and reagents

Table (2-14): The chemicals and reagents that are used in tissue culture of oral epithelial cel (HGF1)

No.	Items	Company	Country
1	Trypsin/EDTA	Capricorn	Germany
2	DMSO	Santacruz Biotechnology	USA
3	RPMI 1640	Capricorn	Germany
4	MTT stain	Bio-World	USA
5	Fetal bovine serum	Capricorn	Germany

2.3.15.2 Instruments

Table (2-15): The instruments that are used in tissue culture of HGF1

No.	Item	Company	Country
1	CO_2 incubator	Cypress Diagnostics	Belgium
2	Microtiter reader	Gennex Lab	USA
3	Laminar flow hood	K & K Scientific Supplier	Korea
4	Micropipette	Cypress Diagnostics	Belgium
5	Cell culture plates	Santa Cruz Biotechnology	USA

2.3.15.3 Description of HGF1 cell line

The HGF1-PI 1 cell line was derived from an explant culture of gingival biopsy taken from a normal 28-year-old Caucasian female in NCBI. Obtain from Institute Pasteur in Iran .

2.3.15.4 Handling Procedure for Frozen Cells

1- The vial was thawed by gently agitation in a 37C water bath, and to reduce the possibility of contamination the Oring and cap were keep out of water, the thawing was rapid approximately 2 minutes.

2- The vial then removed from the water bath as the contents are thawed and decontaminate by spraying with 70% ethanol and all of the operations carried out under strict aseptic conditions.

3- The contents of the vial transferred to a centrifuge tube containing 9.0ml complete culture media and then spine at approximately 125 xg for 5-7 minute and the supernatant was discarded.

4- The cell pellet re-suspend with RPMI 160 and dispense into a T25 cm culture flask (prior to addition of the vial contents, the culture vessel containing the complete growth media placed into the incubator for at least 15 minutes to allow the medium to reach its normal pH(7.0-7.6)).

5- The culture incubated at 37C in a suitable incubation with 5% CO2 .

2.3.15.5 Sub culturing procedure

1- The culture media aspirated from the culture vessel without disturbing the cell monolayer**.**washing with PBS **.**

2- Trypsin-EDTA added to the culture vessel, the culture vessel gently rocked to ensure complete coverage of the Trypsin-EDTA over the cells.

3- The cells observed under a microscope to confirm they are dissociating from each other and are rounding up. the culture vessel gently taped from several sides to promote cell detachment. Cells that are difficult to detach can be put in 37oC for several minutes to facilitate dispersal.

4- Equal volume of the complete media added into the culture vessel to neutralize the trypsin-EDTA, the culture suspension then swirled or pipetted to ensure the neutralization is complete.

5- The culture suspension transferred to a sterile centrifuge tube and the cell suspension Centrifuged at 1500 rpm for 3 minutes.

6- The supernatant aspirated after checking all cells are pulled down into the pellet. The cell pellet re-suspended in pre-warmed fresh complete media.

7- The newly seeded culture vessel was placed in a 37ºC, 5% CO2 incubator. Incubated for at least 24 –48 hours before processing the cells for downstream experiments.

8- The culture media was renewed every 2-3 days if the cells did not reached 80% confluence.

2.3.16. Cytotoxicity Determination Using Methyl Thiazolyl Tetrazolium (MTT) Assay

This assay done according to (Abdul-majeed,2000 ; Freshney,2001) Briefly, cells were seeded at 1×10^5 cells/mL in 96 well micro titer plates in RPMI medium. The cells were incubated overnight for attachment candida albicans(st1,st2,st3)and *P.gingivalis*(st1,st2, st3) and combination *C.albicans* and *P.gingivals* were added in triplicate and incubated for 72 hrs. Thereafter, the cells were treated with Methyl Thiazolyl Tetrazolium (MTT) at concentration 2mg/ml for 3hr.

After the incubation time, all the contents of well aspirated. DMSO was added to each well after incubation period and the absorbance was measured at 570 nm using microplate reader. For visualizing the shape of cells under inverted microscope, 120 µL of cell suspensions were seeded to suspensions in 96-well micro-titration plates at density $1x10^4$ cells mL^{-1} and incubated for 48 hrs. at 37ºC. Then the medium removed and added 50 µL *C.albicans*(st1,st2,st3) *P.gingivals*(st1,st2,st3)and componation After *C.albicans* and *P.gingivals* exposure periods, the plates were stained by 50 µL

with Crystal violet and incubated at 37ºC for 10-15 min, the stain was washed gently with tap water until the dye was removed. The cell observed under inverted microscope at 20 and 40x magnification microscope filed and photographed with digital camera.

2.3.17: Cytotoxicity assays:

2.3.17.1: MTT Assay:

2.3.17.2.: Principle:

The general purpose of the MTT assay is to measure viable cells in relatively high throughput (96-well plates) without the need for elaborate cell counting. Therefore the most common use is to determine the cytotoxicity of several drugs at different concentrations. The principle of the MTT assay is that for most viable cells mitochondrial activity is constant and thereby an increase or decrease in the number of viable cells is linearly related to the mitochondrial activity. The mitochondrial activity of the cells is reflected by the conversion of the pale yellow tetrazolium salt (MTT dye) into dark purple formazan crystals by NADH (Figure 2.2) which can be solubilised for homogenous measurement. Thus, any increase or decrease in viable cell number can be detected by measuring formazan concentration reflected in optical density (absorbance) using a plate reader at 570 nm. The darker the solution, the greater the number of viable and metabolically active cells (Meerloo *et al;* 2011).

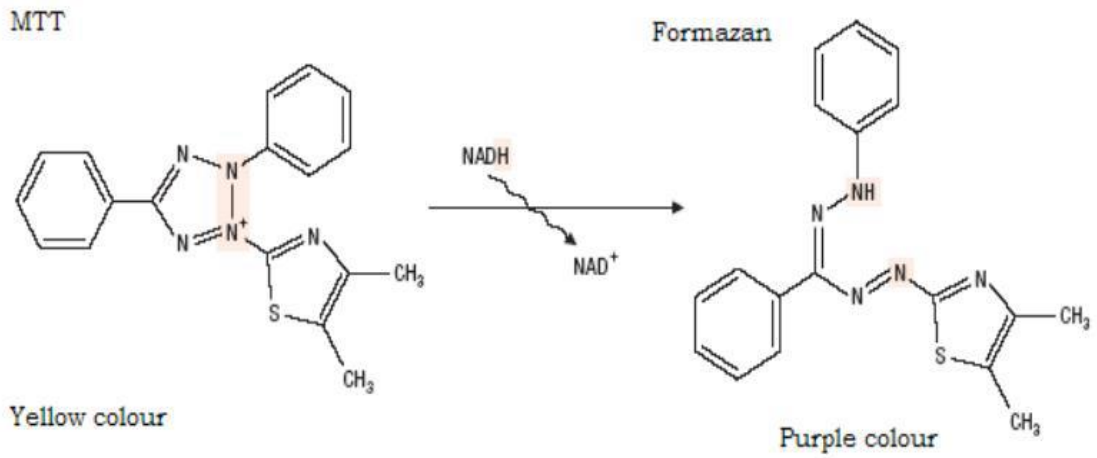


Figure 2.2: Principle of MTT Assay (Sukhramani et al; 2011)

2.3.17.3: Procedure (Meerloo *et al;* 2011):

1- At the end of the drug exposure period, the medium was removed from the wells and then the cells were washed with PBS. A blank control was carried to assess unspecific formazan conversion.

2- A volume of 1.2 ml of MTT solution (5 mg/ ml) was added to 10.8 ml medium to obtain final concentration of 0.5 mg/mL. Then, 200 µl of the resulting solution was added in each well.

3- The plate was incubated for 3 hours at 37°C until intracellular purple formazan crystals were visible under the inverted microscope.

4- The supernatant was removed and 100 µl DMSO was added in each well to dissolve the resultant formazan crystals.

5- The plate was incubated at room temperature for 30 minutes until the cells have lysed and purple crystals have dissolved.

6- Absorbance was measured by a microplate reader at 570 nm. The absorbance reading of the blank must be subtracted from all samples. Absorbance readings from test samples must then be divided by those of the control and multiplied by 100 to give percentage cell viability or proliferation. Absorbance values greater than the control indicate cell proliferation, while lower values suggest cell death or inhibition of proliferation. Percent of cell viability or percent of inhibition was calculated by the following formula:

% viability = (AT – AB) / (AC – AB) × 100%

Where, AT = Absorbance of treated cells (drug).

AB = Absorbance of blank (only medium).

AC = Absorbance of control (untreated).

% Inhibition = 100 – % viability

2.3.17.4 Apoptosis Effect Assay

The induced death of HGF-1cell was performed using Acridine orange – Ethidium bromide (AO/EtBr) dual staining method (Kuan Liu.,*et al.,* 2015). Briefly, the cells in 96- well plates were treated with C.albicans, P.gingivalis and combination and incubated for 16 hrs. The cells were detached and washed twice using PBS, and transferred to a clear 96-well plate. Dual fluorescent dyes (10 μL) were added into the cells at equal volumes. Finally, the cells were visualized under fluorescence microscopy.

2.3.17.5 : IC50 Calculate : To calculate IC50, you would need a series of dose-response data (e.g., drug concentrations x_1, x_2, ...,x_n and growth inhibition y_1, y_2, ...,y_n). The values of y are in the range of 0-1.(Cell Biology , online website)**.**

Linear Regression

The simplest estimate of IC50 is to plot x-y and fit the data with a straight line (linear regression). IC50 value is then estimated using the fitted line, i.e.,

$$Y = a * X + b,$$

$$IC50 = (0.5 - b)/a.$$

2.4. Detection The level of IL-17

2.4.1 Materials Used in ELISA Technique

Table 2-16 *: Cytokines (IL-17) kit contains:*

No	Items	Specifications
1	Standard solution (160ng/L)	0.5ml x1
2	Pre-coated ELISA plate	12 * 8 well strips x1
3	Standard diluent	3ml x1
4	Streptavidin-HRP	6ml x1

5	Stop solution	6ml x1
6	Substrate solution A	6ml x1
7	Substrate solution B	6ml x1
8	Wash buffer Concentrate (25x)	20ml x1
9	Biotinylated Human IL-17B antibody	1ml x1
10	Plate sealer	2 pics

3.4.2 Preparation of Solutions for ELISA Technique

Washing Buffer : Dilute 20ml of Wash Buffer Concentrate 25x into deionized or distilled water to yield 500 ml of 1x Wash Buffer. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

Standard : Reconstitute the 120ul of the standard (160ng/L) with 120ul of standard diluent to generate a 80ng/Lstandard stock solution. Allow the standard to sit for 15 mins with gentle agitation prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (80ng/L) 1:2 with standard diluent to produce 40ng/L, 20ng/L, 10ng/L and 5ng/L solutions. Any remaining solution should be frozen at -20°C and used within one month.

Dilution of standard solutions suggested are as follows:

320ng/L	Standard No.5	120ul Original standard + 120ul Standard diluent
160ng/L	Standard No.4	120ul Standard No.5 + 120ul Standard diluent
80ng/L	Standard No.3	120ul Standard No.4 + 120ul Standard diluent
40ng/L	Standard No.2	120ul Standard No.3 + 120ul Standard diluent
20 ng/L	Standard No.1	120ul Standard No.2 + 120ul Standard diluent

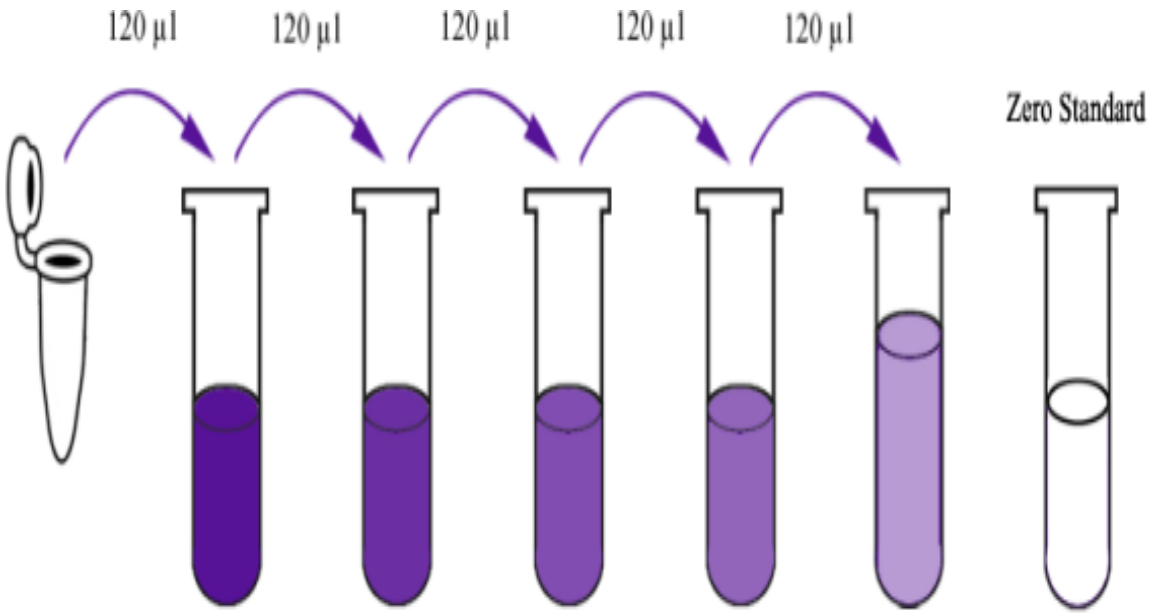


Figure (2.3) Dilution of standard

Standard concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
640ng/L	320 ng/L	160ng/L	80ng/L	40ng/L	20 ng/L

Biotinylated Human IL-17B antibody: Calculated the required amount before the experiment (100µL/well). In actual preparation, you should prepare 100~200µL more. Centrifuged the stock tube before use, diluted the concentrated Biotinylated Detection Ab to the working concentration using Biotinylated Detection Ab Diluent (1:100). **Streptavidin-HRP:** Calculated the required amount before the experiment (100µL/well). In actual preparation, you should prepare 100~200µL more. Diluted the Concentrated HRP Conjugate to the working concentration using Concentrated HRP Conjugate Diluent (1:100).

2.4.3 Detection of IL 17 Assay Procedure

Principle of test :

This ELISA kit uses Sandwich-ELISA as the method. The assay uses This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with Human IL-17B antibody. IL-17B present in the sample is added and binds to antibodies coated on the wells. And then biotinylated

Human IL-17B Antibody is added and binds to IL-17B in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated IL-17B antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of Human IL-17B. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

Procedure :

1. Prepare all reagents, standard solutions and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature.
2. Determine the number of strips required for the assay. Insert the strips in the frames for use. The unused strips should be stored at 2-8°C.
3. Add 50ul standard to standard well.
4. Add 40ul sample to sample wells and then add 10ul Human IL-17B antibody to sample wells, then add 50ul streptavidin-HRP to sample wells and standard wells (Not blank control well). Mix well. Cover the plate with a sealer. Incubate 60 minutes at 37°C.
5. Remove the sealer and wash the plate 5 times with wash buffer. Soak wells with 300ul wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirate or decant each well and wash 5 times with wash buffer. Blot the plate onto paper towels or other absorbent material.
6. Add 50ul substrate solution A to each well and then add 50ul substrate solution B to each well. Incubate plate covered with a new sealer for 10 minutes at 37°C in the dark.
7. Add 50ul Stop Solution to each well, the blue color will change into yellow immediately.
8. Determine the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

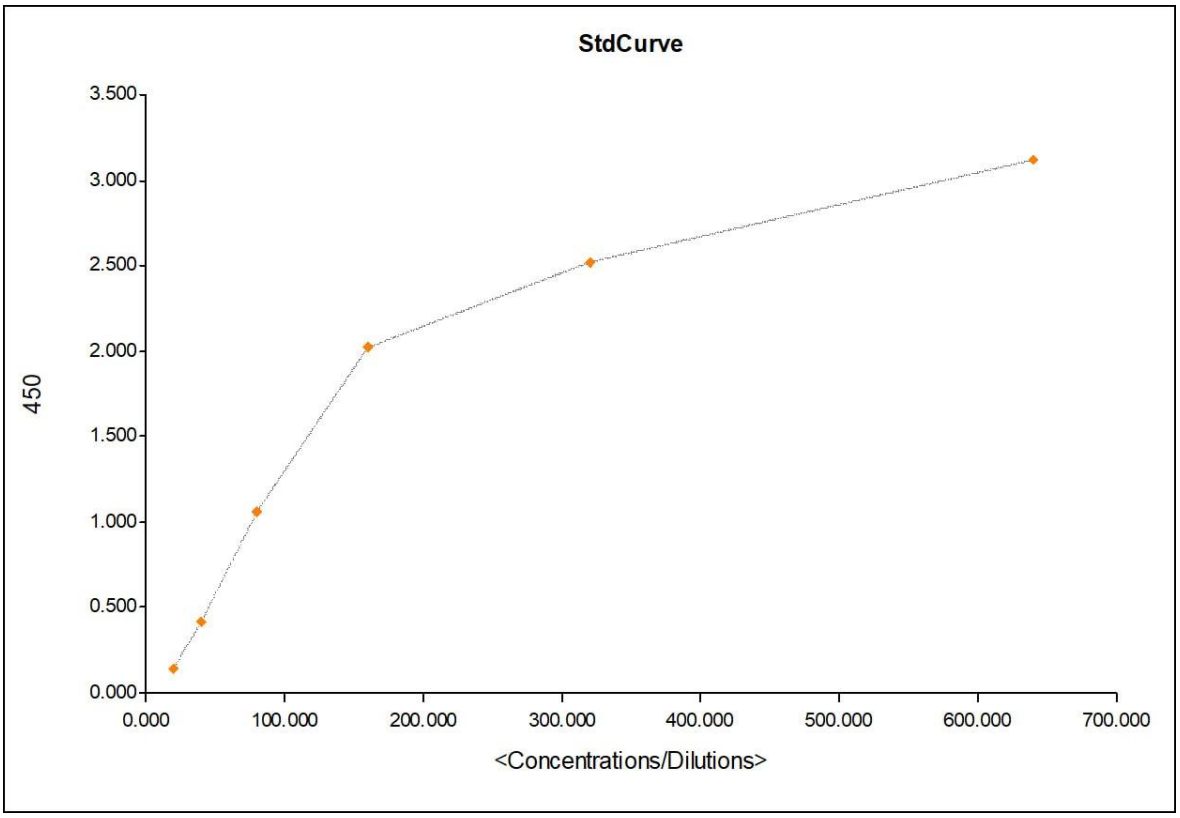


Figure (2-4) Standard curve of IL-17

2.5. : Statistics analysis

Statistical analysis was conducted by using (SPSS 23). Determining the statistical differences among different groups was made using the Pearson Chi-square test .Probability of ($P \leq 0.05$) was considered to be statistically significant (TEAM, 2010).

Also using two software programs, data were presented, summarized and analyzed. These programs were Microsoft Office Excel 2010 and the Graph Pad prism ,7 version using (t-student test) , One Way ANOVA test and (Tukey's Multiple Comparison Test) at the level of significant alpha ≤ 0.05.

3. Result and Discussion

3.1. Isolation and Identification of *Candida albicans*

From A total of 150 patients with oral infection swab , paper point and saliva were taken from the same patient . The result of culture showed 25(16.6%) samples identify as *candida albicans* and 125 (83.3%) samples negative for *C albicans* as shown in figure (3.1). The negative percentage may be due to other causes ,like aerobic bacterial and viruses that not detection in this study.

The result of the percent study was similar to study done by (Oka et al.,2022) who was detected *C. albicans* in 22 (25.6%) of the 86 patients . Also study done by (Khaleefa *et al.*,2020) showed the isolates of oral samples were 12 (24*%) C. albicans* and 18(36.0%) non *C. albicans.*

Previous study done by (Mohammed *et al*.,2017) shown that 22(47%) out of 47 *Candida* isolates from oral swabs were *C. albicans* and 25(53%) were non *albicans*.

The current result is also different to that was reported by AL-Ruaby and Kadhum (2019) who found that the isolation rate of *C. albicans* from oral swabs that were collected from human in Wasit province was 43.3%, and all these isolates were detect by PCR.

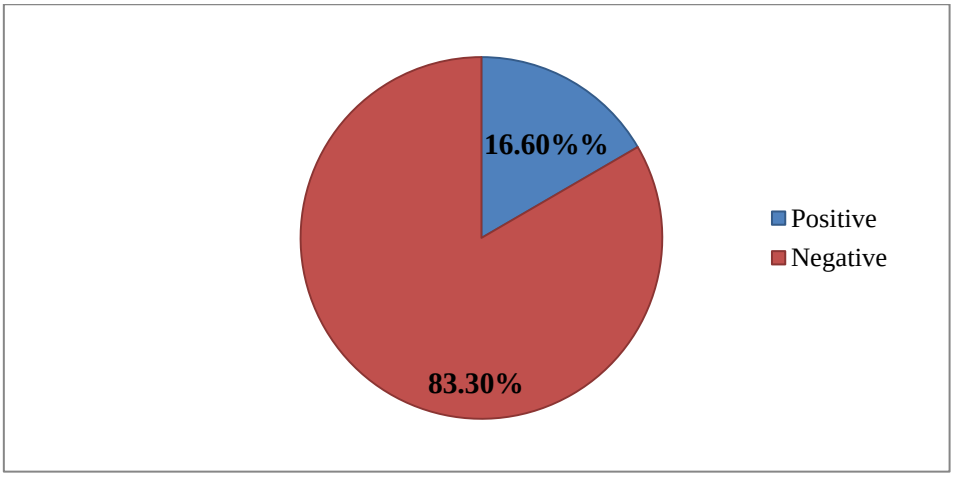


Figure (3.1): Percentage of *C albicans* isolates in oral infection samples.

All these samples were cultured on different media. The suspected plaque samples were transported to the Laboratory of microbiology department of medicine collage by using PBS. After the recording of patient information, the obtained samples were cultured on a sabouraud chloramphenicol medium and chroma agar media for the isolation of *Candida species* .

Then identification of *C.albicans* from other *Candid spp* depending on its morphological properties (colony form, size , color, borders, and texture)(Bhavan *et al.*,2010). *C albicans* colonies on the Sabouraud chloramphenicol medium are creamy whitish and smooth (Figure 3.2).

These results were agreed with (Bhavan *et al*., 2010). Especially, the colonies of *C. albicans* on SDA appeared white to creamy in color with round edges, soft and smooth associated with curved top, with yeast odor. The yeast growth reached to the typical form within three days.

The current result was consistent with that reported by (Singh and Chakrabarti, 2017) who detected cream-colored shiny and circular colonies of *C.albicans* in presence of suitable cultivation conditions.

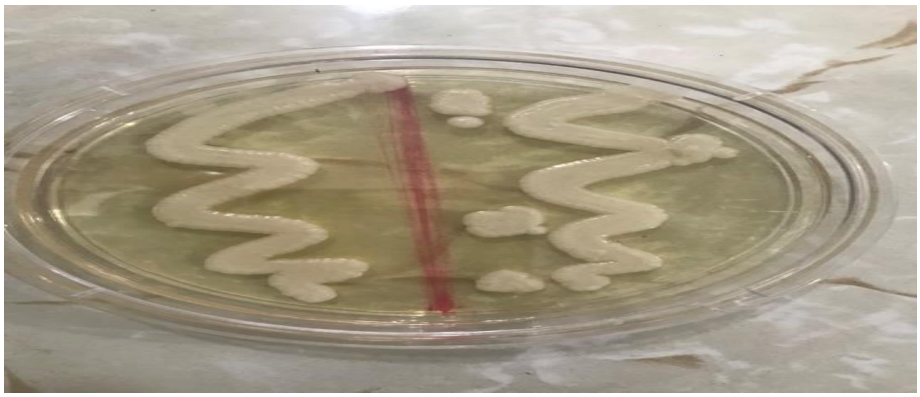


Fig. (3-2): Colonies color of *C albicans* on Sabouraud chloramphenicol medium at 30˚C for 24-48h.

C. albicans has a preponderant role among other species of it genus in periodontal disease , the presence of its hyphae has been demonstrated in the connective tissue of periodontal patients in associated with highly

invasive anaerobic bacteria such as *prophyromonas gingivalis* (Unniachan *et al*.,2020).

In a study conducted by DelaToree *et al,* chronic periodontitis patients were reported to have a higher Candida colonization rate than those without chronic periodontitis ,it was found a relationship between colonization of candida and the severity of periodontitis (DelaToree *et al*.,2018).

(Jabri *et al* .,2022) found that 20% of periodontitis patient were positive for *C albicans*. *C albicans* can grow either aerobically or anaerobically which may explain their presence in deep periodontal pockets (Colombo *et al*.,2016).

The role of *C. albicans* in periodontitis pathogenesis is yet unclear. Indeed, this yeast could alter the oral microbiome and, therefore, influence significantly bacteria colonization(Janus *et al*.,2017; Bartnicka *et al*.,2019). Coadherence between *Candida albicans* and some bacteria may help the formation of complex biofilms with mixed species affecting (Xu *et al*.,2014; Wu *et al*.,2015), it was suggested also that *C. albicans* promotes bacterial invasion of host cells by anaerobic bacteria such as *P. gingivalis* and, thus, induces infections by anaerobic bacterial diseases.

In addition, *C. albicans* can grow either aerobically or anaerobically (Dumitru *et al*.,2004), which may explain their presence in deep periodontal pockets (Wu *et al*.,2015, Colombo *et al*.,2016). Other studies reported that *C. albicans* is capable of adhering to epithelial cells and induce inflammation (Hube and Naglik ,2001).

3.1.1 Identification of *C. albicans* by CHROME agar medium

The result of this test of the colonies produce different color on chrome agar medium . The result in the present study show the ability of C albicans grow on chrome agar and *C. albicans* produce the light green color on this medium as shown in figure(3-3) the result are considered with (Nadeem *et al*., 2010; Vignesh Kanna *et al* 2017).

Candida chromogenic agar which was used according to the manufacturer's guidelines. Green colonies were identified as *C.albicans* (Ganguly *et al*.,2011).

CHROM agar Candida contains enzymatic chromogenic substrates, which combine with certain enzymes secreted by the types of *Candida* when they grow on this medium, which leads to different colors depending on the Candida species, this test is useful in the laboratory diagnosis of yeast (Murray *et al*., 2005).

It is a rapid diagnosis testing for *Candida* species, colonies growth is observed after 24-48 hours. Detection of *Candida species* and recognition of their growth; Compared to traditional methods, it has the advantage of being technically simple, fast and cost effective (Vijaya *et al*., 2011).

Figure (3-3): Colonies color of *C albicans* on CHROM agar medium .

3.1.2 Isolation and Identification of *P. gingivalis* according to Culture Characters

The results of isolation and identification of *P.gingivalis* indicated that 15 (10%) samples were as *P.gingivalis* as shown in table(3.1) .

Table (3.1) : Percentage of isolation *P gingivalis* in oral infection samples

Samples	Positive %	Negative%	Total
P.gingivalis	15(10%)	135(90%)	150(100%)
C albicans + *P.gingivalis*	4(2.66%)	146(97.33%)	150(100%)

The Identification of *P* gingivalis isolates depends mainly on the cultural , biochemical characteristics and microscopic examination. The result in Table (3.1) demonstrates that *P gingivalis* is an aerobic, Gram-negative small coccobacilli. The colonies on blood agar forms black spots, black pigmented colonies, due to it takes part in Iron transport, the way it does this is by using a hemin as a device to help it transport iron. When this builds up the black pigmentation appear as show in figure (3.4) (Ogrendik *et al*.,2005;Al-Kafagee *et al.*,2013).

P gingivalis is cultured on selective media (P.GING) it is enriched selective media for the isolation and presumptive identification of *P gingivalis* (Jousimies-Somer et al.,2002;NCCLS,2004).On these media the bacteria appears to be required Nalidixic acid ,Colistin and Bacitracin requirement ,these reqirment considered as a good enrichment agents , to provide the bacteria with the needed nutritional factors (Grenier and Dang,2011).

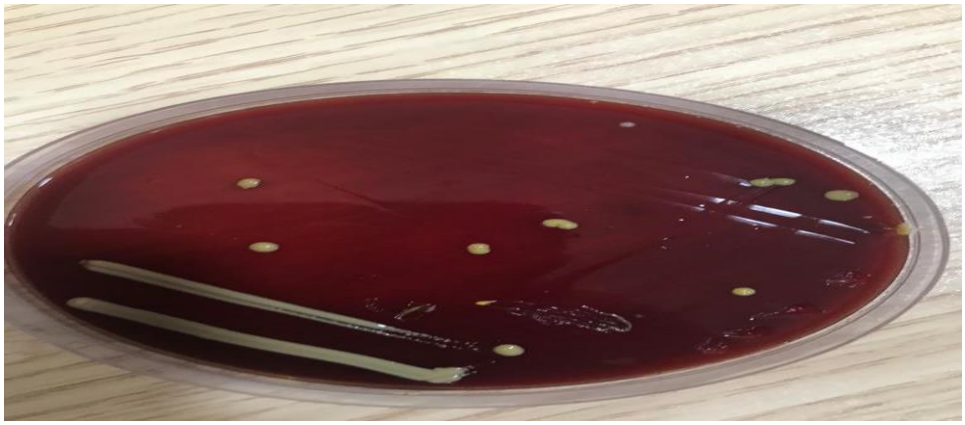


Figure (3.4): Colonies of *P gingivalis* on Blood agar

P gingivalis showed a positive reaction for Indole and negative reaction for catalase (MacFaddin,2000) .The diagnostic feature summarized on table (3.2). However, the identification isolates of *P gingivalis* confirmed by PCR.

Table (3.2):Biochemical Tests and the Microscopic Examination of *P.gingivalis* .

NO	Test	Result
1	Gram stain	G-Ve, Coccobacilli
2	Catalase	-Ve
3	Indole	-Ve
4	Grow on(P.GING)	+Ve
5	Grow on Blood agar	Black pigmented colonies

A previous study done in Iraq by (AL-Bdery and Al-Yasseen, 2018) found that out of 150 subgingival dental plaque samples only 78 isolates were belonged to *P. gingivalis*, which appeared as a small to large colonies convex, semi mucoid, translucent after 48 hr of incubation anaerobically and formation of black pigmented colonies after 7 days of incubation anaerobically on blood agar supplemented with 5% sheep blood, hemen and vitamin K and all isolates were negative to oxidase, catalase, methyl red and simmon citrate while it's

gave positive results to indole test and Alk/Alk without gas and H_2S production on TSI agar.

According to the culturing identification 15(10%) *P.gingivalis* were detected only this result is agrees with(Gomes *et al*.,2005) , they found that *P. gingivalis* was rarely isolated by culture methods (1%).

However , other studies have indicated that the prevalence rate of *P. gingivalis* in healthy subjects was 36.8% and in the periodontitis patients was 87.1%(Amano *et al*.,2000;Bostanci and Belibasaki,2012*)*.

Another study done by Hajishengallis,(2011)who found that *P.gingivalis* isolation rate from subgingival sample was(4%).

This anaerobic bacterium is a natural member of the oral microbiota ,yet it can become highly destructive and proliferate to high cell number in peroidental lesions(Clais *et al*.,2014).

Research by Bostanci and Belibasakis ,(2012) on *P*.gingivalis as a periodontal pathogen has provided a special attention should be paid to clinically relevant properties of *P.gingivalis*,such as pathogenicity and it possible relation systemic disease.

A previous study done by Atanasova and Yilmaz ,(2014) found that the rate of *P.gingivalis* in subgingival plaques reach to 60% of cases.

A synergistic interaction between *Candida albicans* and oral bacteria promoting the virulence of polymicrobial biofilms was reported (Koo *et al*.,2018; Montelongo-Jauregui and Lopez-Ribot ,2018). Indeed, regarding the specific case of periodontitis, the consumption of oxygen by *Candida albicans* seems to create an oxygen tension that helps *P. gingivalis* growth (Sztukowska *et al*.,2018) and support *P. gingivalis* ability to invade host cells (Tamai *et al*.,2011).

A study done by (Oka *et al.*,2022) found co infection *C. albicans*/*P. gingivalis* in 12 patients (14%) higher than the result in this study which was 4(2.66%).

Results of (Oka *et al.*,2022) suggest that co-infection of *C. albicans* and *P. gingivalis* rather than *C. albicans* infection alone contributes to active periodontitis. *C. albicans* may be involved in periodontitis in cooperation with periodontopathic bacteria.

The viability and hemagglutination activity of *P. gingivalis* was enhanced in the *P. gingivalis/C. albicans* mixed biofilm under low heme conditions, suggesting that *C. albicans* can enhance the virulence of *P. gingivalis* under the conditions of insufficient heme (Guo *et al*.,2020).

The capacity of *P. gingivalis* to invade gingival fibroblasts and epithelial cells was enhanced by mannans derived from *C. albicans* (Tamai *et al*.,2011). Therefore, *C. albicans* is thought to play a supportive role in the progression of periodontitis in the presence of *P. gingivalis*. Additionally, *C. albicans* may be associated with periodontopathic bacteria other than *P. gingivalis*

In the diagnosis of *P.gingivalis* infection ,currently rely on the traditional culture method requires the isolation ,culture and identification of the microorganism ,and has the disadvantages of being time –consuming insensitive , and cumbersome with the development of molecular biology techniques , rapid diagnostic studies of *P.gingivalis* have progressed rapidly (Ge *et al.*,2022) .

3.2 Molecular diagnosis of *P.gingivalis* by quantitive Real Time PCR

From 15(10%) positive culture to *P.gingivalis* only 10(6.66) isolates give positive result to *P.gingivalis* specific gene (waaA) by q RT-PCR as shown in figure(3.5).

Several diagnostic methods can be used to detect bacterial species that have been identified as periodontal pathogen including *P.gingivalis* . These methods include bacterial culture, enzyme assays , immunoassays nucleic acid probes, DNA-DNA hybridization and PCR(Kulkarni *et al*.,2018).

Study by (Kugaji, *et al*.,2019) detected *P. gingivalis* by qRT-PCR Chronic Periodentitis group in a rate 79.16%, whereas 29.17% samples were positive in the Healthy group.

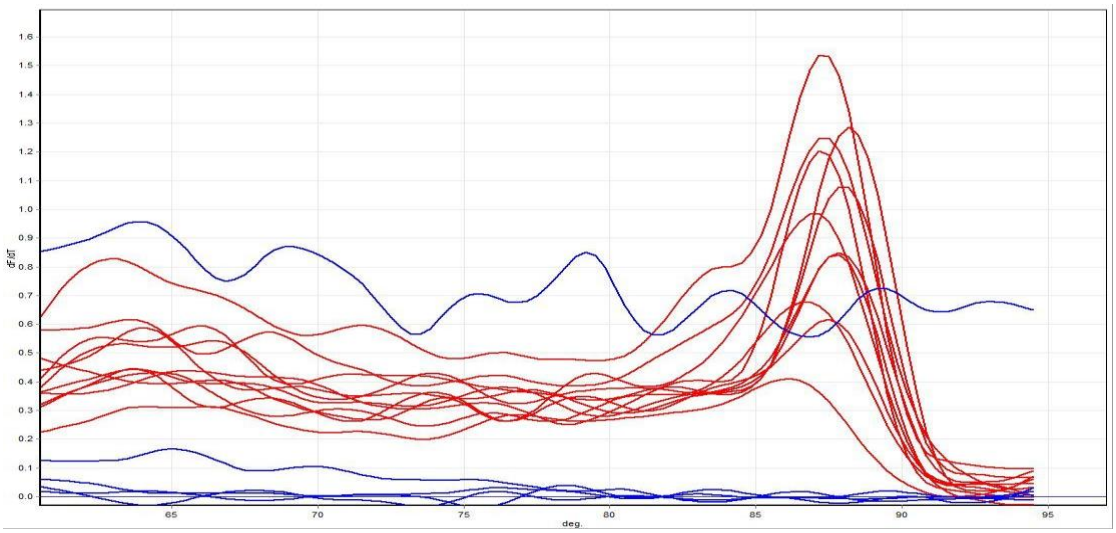


Figure (3-5) :Detection of *P. gingivalis* by real time PCR , amplification and melting curve , the red curve represent positive detection.

Also these result correlated with other result(ALwarid,2017)that indicate the percentage of *p.gingivalis* by PCR (17.7%);but this result is not agreement with (Gomes *et al*,2005) that indicate the isolate of *p. gingivalis* by PCR is (38%) . Also there is other study state that the percent of *P.gingivalis* is 3% (Ercan,2006).

Joshi (*et al*.,2016) found the percentage of prevalence of *P. gingivalis* was 66% in the chronic periodontitis (CP)group in a study which was carried out in Indian subjects. There are also researchers found that the prevalence of *P. gingivalis* more than 80% in the chronic periodontitis(CP)group (Chen *et al*.,2005; Chaudhary *et al*.,2013).

In few other studies prevalence of *P. gingivalis* in the chronic periodontitis (CP) group which was ranging from 45% to 53%(Boutaga *et al*.,2006; Boutaga *et al*.,2003).

The difference in the prevalence of bacteria in the CP group could be due to different geographical locations, inclusion criteria, technique used for detection, and variation in sample size between other previous studies.

The PCR examine gave more positive results than culture techniques when traditional culture methods are used, laboratories may need 7-14 days to identify anaerobic strict bacteria, followed by biochemical and other tests to identify the microorganism; the time required for identification can be even longer for slow-growing microorganisms or samples with low microbial counts, while PCR can offer information in only a few days and faster (Siqueirs and Rocas,2003).Previous studies found higher prevalence rate (49.1%) of *P. gingivalis* was reported by using Real-Time PCR (Avila-Campos 2003; Herrera *et al*., 2008).

On studying the microbiology of subgingival plaque samples from patients with severe chronic periodontitis, were reported a much lower detection rate (25.9%) of *P. gingivalis* yielded from anaerobic culturing (Boyanova *et al*.,2009).

However, all the mentioned references agreed on that the rate of detection is higher in disease than health. Presence of this bacterium in low number of healthy individuals (9/35) and in a significantly higher number of subjects in Chronic Periodontitis group indicates that it is an opportunistic pathogen. Healthy periodontium is maintained through a good oral hygiene of the individual. Opportunity for higher growth rate of *P. gingivalis* is usually generated through plaque accumulation in the sub-gingival area in which the growth of early plaque colonizers (gram positive cocci and rods) provide necessary growth factors such as attachment sites, substrate, reduced

oxygen tension and an area away from host's oral immunity(Nelson *et al*.,2003; Kawada *et al*.,2004).

Although microbiological culture technique is still considered as a gold standard for the detection of *P. gingivalis*, RT-PCR provided several advantages over the conventional methods. In addition to quantitative evaluation, RT-PCR offers the advantage of eliminating false positives which could otherwise play abysmal role in conventional detection techniques.

3.3 Distribution of Patients According to Age

As showed in figure (3-6), A total of 150 patients are diagnosed as periodontist the mean age of patients in the study group are ranging from (5-69) years old.

The highest percentage of infection was 43(28.66%) among patients in the age range (55-70) , 30(20%) among patients in the age (25-35) years, 26 (17.33%) among patients in the age (35-45)years , 20 (13.33 %) among patients in the age (5-15)years , 17 (11.33%) patients in the range of age (45-55), while the range of age (15-25) scored the lowest rate of infection 14 (9.33%).

The high infection rate at age group range from 55 to 70 years may be due to that the disease may come from constant neglect of not caring for the health of the teeth ,in addition to smoking and alcohol abuse this lead to lower immunity .Previous study by(Jabri *et al*.,2022) found that 16% of the studied population aged between 12 and 25 years old had periodontitis. All over the world, studies suggested that the prevalence of periodontitis is 15–30% in adults, and sometimes higher (Baelum *et al.*,2003; Holtfreter *et al*.,2009) among African adolescents, the prevalence of aggressive periodontitis was estimated as 3.4% to 6.5% (Albandar *et al*.,2002; Elamin *et al.*,2011). In a

Moroccan population (Kissa *et al*.,2016) found a prevalence of periodontitis was 11.3% (94/830) in students aged range from 12 to 25 .

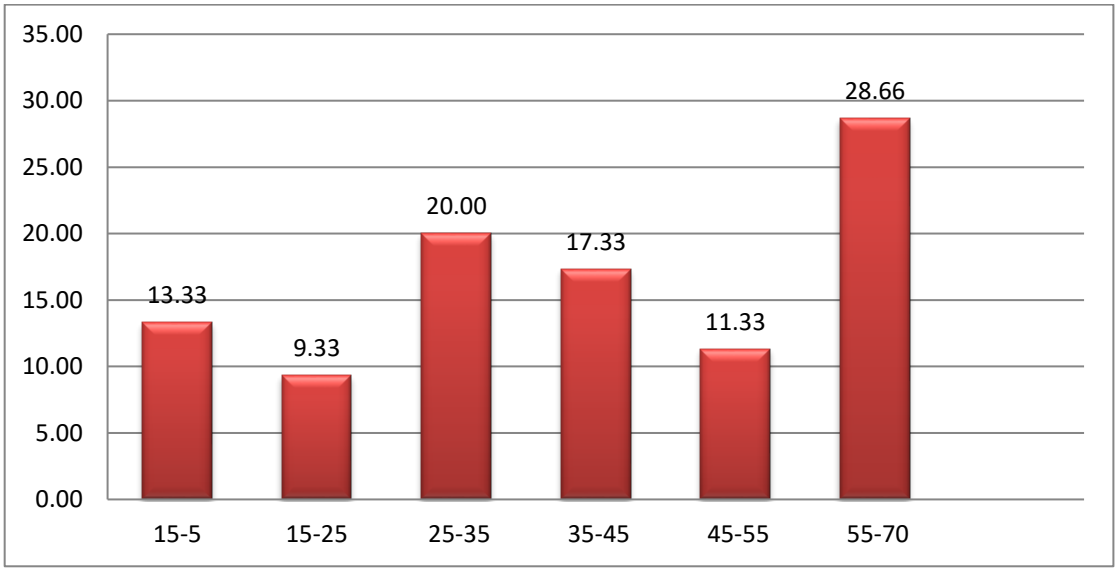


Figure (3-6): Frequency Distribution Of Periodontitis Patients According to Age group.

The higher age group (25-35) in which *C albicans* was isolated at a rate 10(40%), 7 (32%) in the age group (15-25) and 5(20%) *C albicans* isolated from age group (55-70) , 2(8%) also *C albicans* isolated in the age group (35-45) while in the age group (45-55) scored the lowest rate of *C. albicans* isolated 1(4%) as show in figure (3-7).

Previous study conducted by (Oka *et al*.,2022) found a higher *C. albicans*-positive rate (35.3%) in Patients in 80 years old compared with other participants.

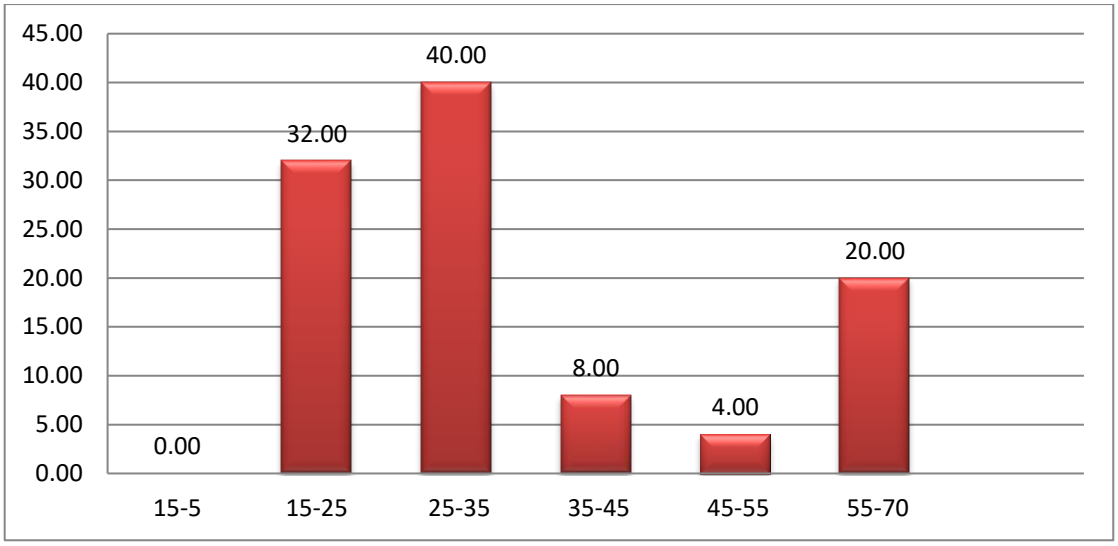


Figure (3-7): Frequency Distribution Of *C albicans* isolation By Age group.

P.gingivalis isolated in high rate 6(4%) from age group (25-35) and 4(2.66) in the age group (15-25) while only 2 (1.33%) isolate obtained from the age group (45-55) and 3 (2%) isolate from age group (55-70)as show in figure (3.8).

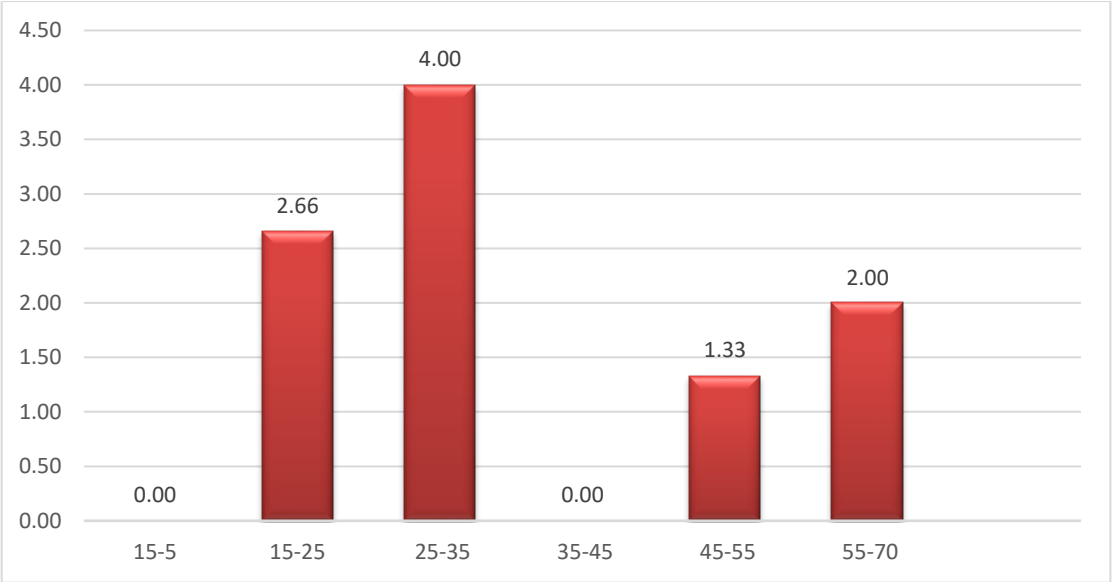


Figure (3-8): Frequency Distribution Of *P. gingivalis* isolation By Age group.

The present study agree with study done by (Al-Rawi.,2012) who revealed that there are effects of age and sex on isolation rate and the results

indicated that percentage of *P.gingivalis* was detected in 20-30 years old and males were more infected than females.

The current result similar to study conducted by (Kugaji, *et al*.,2019) who found a higher rate of detection of *P. gingivalis* In the older age groups. Also (Oka *et al.*,2022) found Patient in their 80 years old showed a higher rate of co-infection of *C. albicans and P. gingivalis* (23.5%) compared with other participants.

The older age groups were detected with more number of positive cases compared to younger age groups, which could mean the older age group is at higher risk to get infected with *P. gingivalis*. The pathogenicity of *P. gingivalis* is attributed to different virulent factors which directly or indirectly destabilize the immunogenic responses from host and help the bacterium to invade the host tissue.

3.4: Molecular study of *Candida albicans* Virulence Gene

The result show that the agglutinin-like sequence 1 (*ALS1*) gene detect in 13 (52%) isolates by using PCR with amplicon size 318 bp when compared with allelic ladder as shown in figure(3.9) and (table 3.3).The result in the current study Similar to a study conducted by (Ali,2014) who isolated the *ALS1* gene in a rate 12 from twenty five isolates.

Also the current study detected other *C. albicans* virulence gene *(HWP1)*and it was found 17 (68%) isolates out of twenty five isolates were positive to *HWP1* gene with amplicon size 503 bp as shown in(figure 3.10) and (table 3.3). Also the current study detected other *C. albicans* virulence gene ***(EAP1)*** which found that 19 (76%) isolates were positive out of twenty five isolates with amplicon size 66 bp as shown in(figure 3.11) and table 3.3).

Table 3.3: Percentage of *C. albicans virulence genes (ALS1 , HWP1 and EAP1)detected by PCR.*

Gene	Positive Number	Negative Number	Total
C. albicans ALS1	13(52%)	12(48%)	25(100%)
C. albicans HWP1	17(68%)	8(32%)	25(100%)
C. albicans ***EAP1***	19(76%)	6(24%)	25(100%)

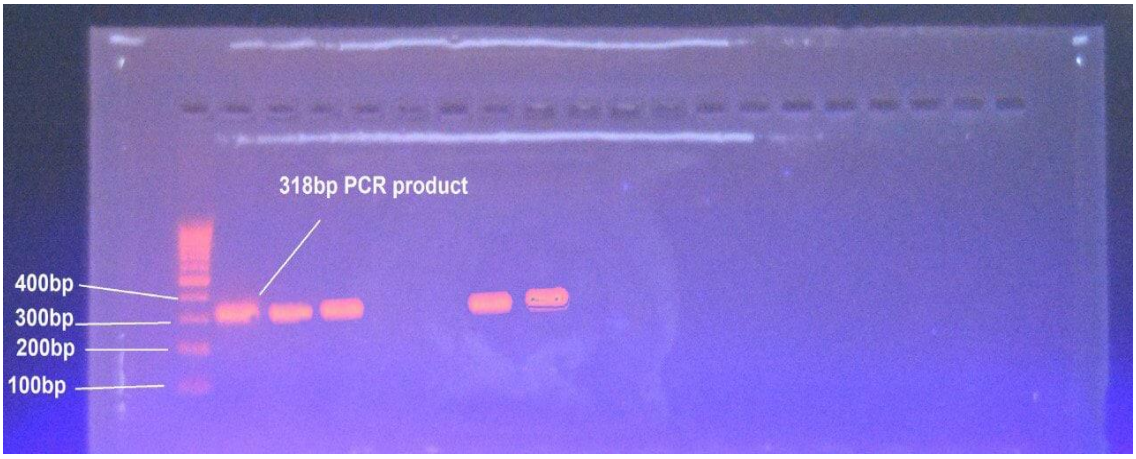


Figure (3-9): Gel electrophoresis of single PCR products of ALS1 genes **(318pb)** with in *Candida albicans* on 1% agarose gel at 70volt / cm for 45 minutes. Lane 1: 100bp DNA ladder,1,2,3,7and 8, positive for this gene and other show negative .

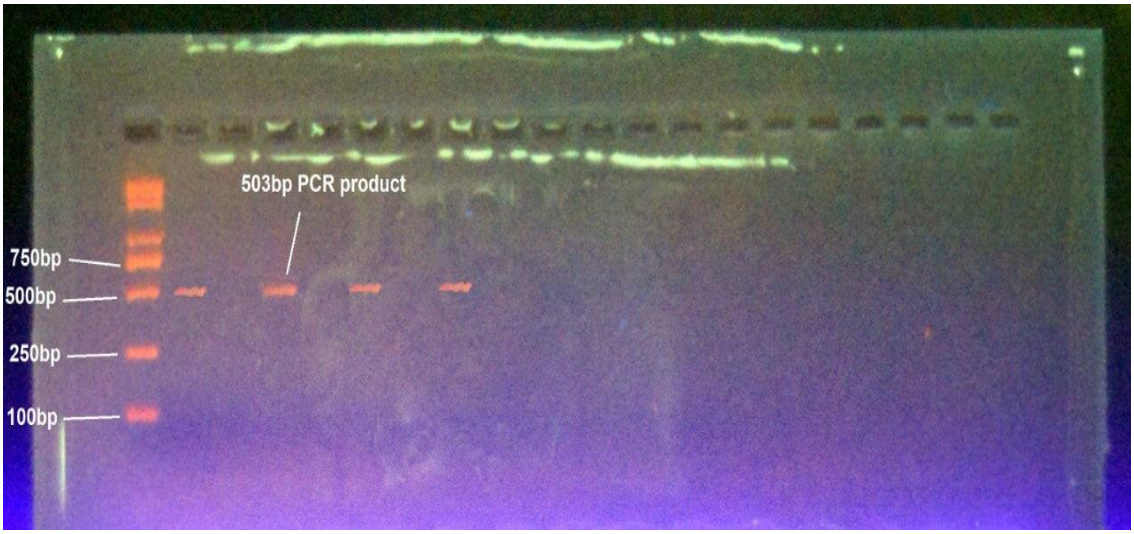


Figure (3-10): Gel electrophoresis of single PCR products of HWP1 genes (**503pb**) in *C albicans* on 1% agarose gel at 70volt / cm for 45 minutes. Lane 1: 100bp DNA ladder.1,3,5and 7 positive for this gene and other negative.

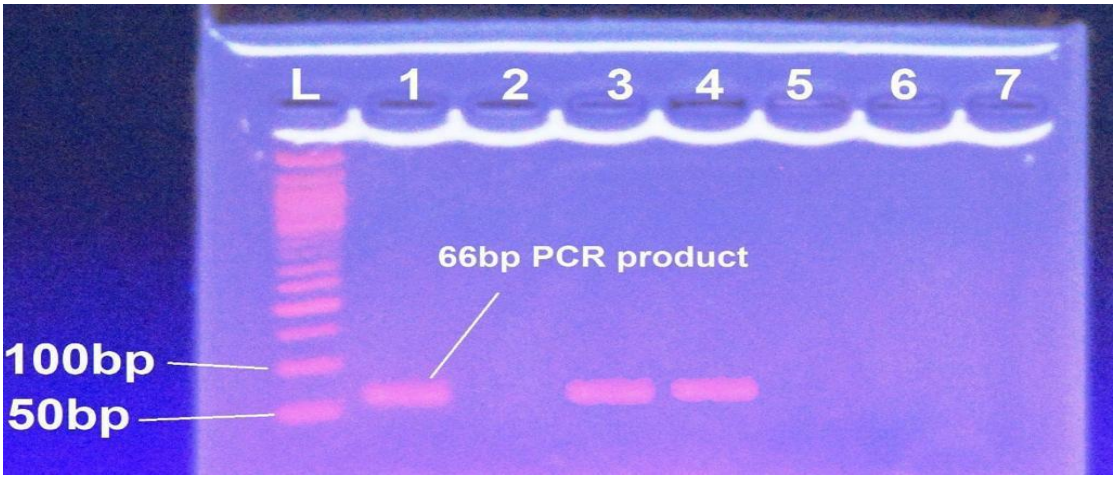


Figure (3-11): Gel electrophoresis of single PCR products of EaP1 genes (**66pb)** in *C albicans* on 1% agarose gel at 70volt / cm for 45 minutes. Lane 1: 100bp DNA ladder ,1,3 and 4 show positive result for this gene while other negative .

It was widely documented that *C. albicans* has a large number of virulence factors that causing disease, including phenotypic switching, filamentation, adherence and secreted hydrolyses. Some of these pathogeneses are associated with gene families, particularly, *ALS1*, secreted aspartyl proteinase, and lipase families (Gropp *el al*., 2009; Sun *et al*., 2010).

In both commensal and pathogenic lifestyles, *C. albicans* utilizes a set of proteins called adhesins to prime adherence between *C. albicans* and host cells or with inanimate surfaces **(**Maras *et al*., 2021).*C. albicans* isolation , which was encodes a cell surface protein that mediates adherence of *C.alblcans* to endothelial cell such as mentioned in the study of (Nadeem *et al*.,2010).

The most important *C. albicans* adhesins virulence genes are ALS proteins (ALS1-7 and ALS9)(Hoyer *et al*.,2008). ALS1 was reported as the most commonly expressed genes of the ALS gene family(Nas *et al*.,2008).

The result of present study disagree with study conducted in Iraq by (Mohammed *et al*.,2017) were found that 100% of *C. albicans* isolates

from oral and vaginal infection are ALS1 positive. This variations might be due to associated with the number of samples studied or with the virulence of the strains analyzed and with the methodology employed or the microorganism may be contain other gene responsible for the same virulence factors .

study of (Monroy -Pérez *et al*.,2016) show that ALS1gene was detected in 39/39 (100%) of *C. albicans* isolates and HWP1was detected in 35/39 (89.7%) and all strains were positive for HWP1 35/35 (100%) expressed this gene during infection.

The current detection results were much higher than other Iraqi thesis by (Ali ,2014)who found that the gene detection of each of ALS1 and HWP1was done by PCR methods. Out of 25 *C. albicans* twelve isolates were positive for ALS1 gene and only nine samples positive for HWP1 gene, eight isolates were positive for both genes by multiplex PCR method.

The study of (Inci *et al*., 2013) found that the presence of the ALS1 gene was detected in 53.9% of all strains, while the HWP1 gene was present in 5.3% which are much lower than present findings. The high frequency of detected ALS1and HWP1in this study may be related to the high pathogenicity of *C. albicans* that isolated from patients in ruled in this study.

The different in the results in this study with others studies could be related to differences in *Candida* strains, Candida pathogenicity, sampling numbers, sites and degree of infection.

The ALS proteins that were coded by Als1-Als9 genes are essential extracellular components to adhesion and colonization (Murciano *et al*.,2012, Moeckli ,2014). Moreover, SAP4 and HWP1 are other virulence factors that were shown has a significant role in hyphae Production, host tissue damage

and biofilm development, respectively (Khodavandi *et al*.,2011, Inci *et al*.,2013).

HWP1 protein is revealed that contributed in *C. albicans* covalently binding to epithelial cells and data of published studies have shown that HWP1 surface protein and ALS1/3 association is necessary to initiation and development of biofilm formation. A complementary role was proposed for ALS1/3 and HWP1 genes in biofilm formation (Sundstrom .,2002).

Also, the finding of many studies suggested that several genes included HWP1and HWP2 have the main role in biofilm formation, but between them, HWP1 was the essential factor(Inci *et al*.,2013, Nobile *et al*.,2008).The *HWP1* is a main adhesin protein, commonly expressed on the germ tube and hyphal surface of Candida species as a substrate attach covalently to host cells transglutaminases and cross-links of this genus to epithelial cells of mucosa (Chaffin, 2008; Romeo and Criseo, 2008).

HWP1 is proposed as an essential substrate that could contribute to covalent attachment of *C. albicans* to host cells (Sundstrom ,2002). This result supports the hypothesis that HWP1 gene has a significant role in the initiation and development of candida infections at the various tissue site. (Peters *et al*., 2012 , Schlecht *et al.*,2015).

Moreover, in vivo model of biofilm formation, it has been proposed that *HWP1* adhesin retains Candida in the biofilm (Mirhendi *et al*., 2011). Additionally, detecting the presence of the *HWP1* gene in *C. albicans* isolates that was recovered from clinical specimens will help to ascertain the role of this gene in colonization, which is a vital stage in infection process. Therefore, it has been found that *HWP1* gene was an excellent marker for

the identification of *Candida species*, as well as, the phylogenetic analysis of the most clinically significant *Candida* species(Abastabar *et al*., 2016).

The result in this study is inconsistent with NAS *et al.* (2008) who found that *HWP1* gene was detected in 73% of the isolates .However, the current result is in concordant with the result of a molecular study that was carried out by Mohamed *et al*. (2017) in Baghdad who reported that the detection rate of *HWP1* gene was 100% of isolates.

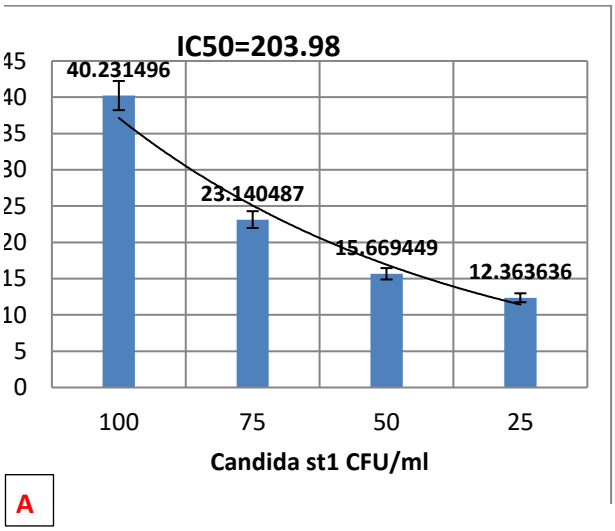
Study done by (li *et al.*, 2007) describe the role of the *C. albicans EAP1* gene, which encodes a glycosylphosphatidylinositol-anchored, glucan-cross-linked cell wall protein, in adhesion and biofilm formation in vitro and in vivo. Deleting *EAP1* reduced cell adhesion to polystyrene and epithelial cells in a gene dosage-dependent manner. Furthermore, *EAP1* expression was required for *C.albicans* biofilm formation in an in vitro parallel plate flow chamber model and in an in vivo rat central venous catheter model. *EAP1* expression was upregulated in biofilm-associated cells in vitro and in vivo. Our results illustrate an association between Eap1p-mediated adhesion and biofilm formation in vitro and in vivo.

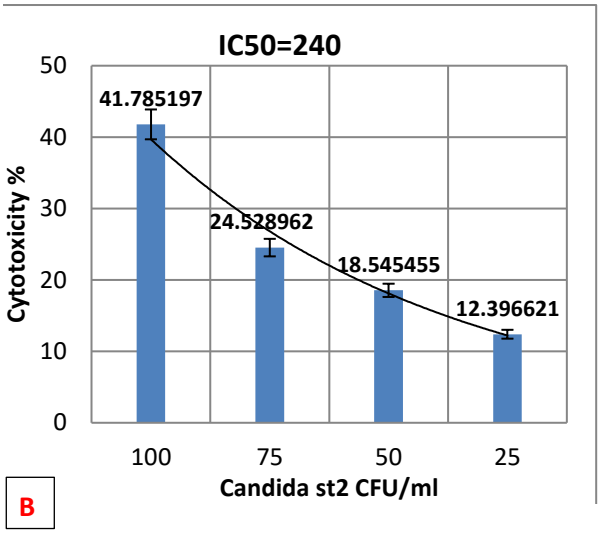
3.5 Cytology Study

3.5.1Determine the Cytotoxicity activity of *Candida spp.*

I. Determine the cell cytotoxicity by using MTT assay

The cytotoxicity potentials of *Candida* with 3 isolates were evaluated by MTT assay against HGF-1 cell line culture after 48 hrs., which appear that Candida spp exhibited selective cytotoxicity against HGF-1 cell line with inhibitory concentration (IC50) 203.98 µg/ml, 240 µg/ml as showed in figure (3.12-a, b)respectively for strain-1 and strain-2 and, while the IC50 of the Candida strain-3 HGF-1 cell line was 54.45 µg/ml as illustrated in figure (3.12.C)





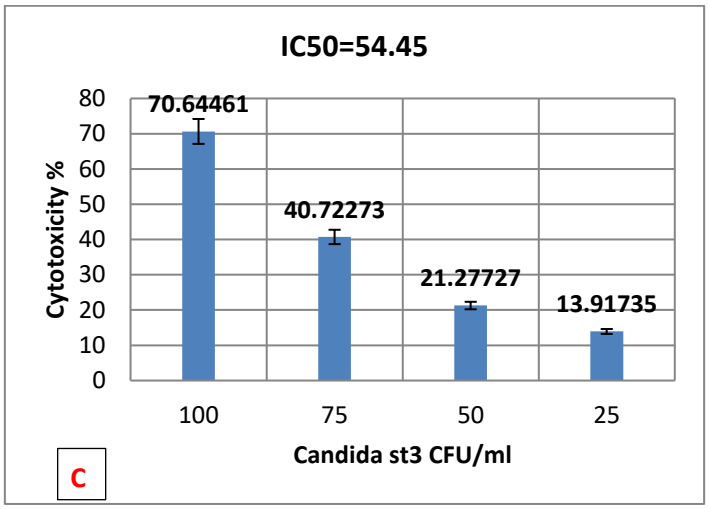


Figure (3.12) The cytotoxic effect of Candida spp (against HGF-1 cell line **after 48 hrs of exposure.**

A. The IC50 of the Candida spp strain-1 against HGF-1 cell line B. The IC50 of the Candida spp strain-2 against HGF-1 cell line. C. The IC50 of the Candida spp strain-3against HGF-1 cell line. Both strain1 and 2 shows IC50 more than the concentration used the experiment, while the strain3 was more impact on the cells than strain1 and 2

Candidalysin is the first and currently only ,peptide toxin identified in human fungal pathogen , causes cell damage in mucosal and systemic infection molds(Ito *et al.*,2020).*C albicans* candidalysin damages human oral epithelial cell in mannar is independent of apoptotic caspases (Blagojevic *et al.*2021).

Also candidalysin activates the NLRP3 inflammasome and induces cytolysisin both murine and human mononuclear phagocytes,cell death(Kasper *et al*.,2018).

The role of *Candida albicans* in the process of carcinogenesis tends to be complex, such as the role of virulence factors, the host genome, influence on the immune response, and oral dysbiosis, as noted in a review conducted by (Di Cosola *et al*., 2021)

.Increased colonization of *Candida albicans* is one of the strong associations with oral epithelial dysplasia and neoplastic transformation leading to the Oral Squamous Cell Carcinoma (OSCC)process (McCullough *et al*.,2000). The number of colonies and excessive density of *Candida albicans* can damage host cells and promote the development of carcinogenesis (Gallè *et al.*,2013).

Study condected by (Ayuningtyas *et al*.,2022) found the presence of *Candida albicans* in the form of colonies and biofilm formation found in the healthy mucosa group compared to moderate, severe dysplasia and OSCC showed high statistical significance.

Candida albicans also have the potential to induce OSCC by producing carcinogenic compounds. Certain strains of *Candida albicans* and other yeasts play an essential role in developing oral cancer by creating endogenous nitrosamines. *Candida albicans* can convert both nitrite and/or nitrate into nitrosamines and other substances to produce acetaldehyde (Gayathri *et al.*,2015). Acetaldehyde can induce tumor development in various ways. This carcinogen binds to proteins and DNA, changes its structure and function, and the reduction in the antioxidant activity of glutathione increases the content of reactive oxygen species (ROS) in the cells. These changes can lead to genomic instability, inhibiting the apoptotic system and tumor development (Ramirez-Garcia *et al*.,2014).

C albicans has the ability to convert alcohol to acetaldehyde,which has carcinogenic role in oral cavity. This conversion is facilitated by *Candida albicans* Alcohol dehydrogenase 1 (CaADH1) mRNA gene (Alnuaimi *et al*.,2015).

Chronic contact with microorganisms and their products such as endotoxins, enzymes are toxic for host cells, which can either trigger mutations or alter the signaling pathways to influence on cell proliferation or the survival of the epithelial cells (Kurago *et al*.,2008).

Candida albicans can produce carcinogens such as acetaldehyde, nitrosamines, and enzymes (proteases, lipases, esterases, and phospholipases) that can promote cancer formation (Alnuaimi *et al*.,2016, Ramirez-Garcia *et al*.,2014; Kurago *et al*.,2008).

One of the proteins identified in the mannoprotein infraction of *Candida albicans*, which increases tumor adhesion by triggering inflammation in endothelial cells, is alcohol hydrogenase (ADH1), which is associated with a cancer-stimulating mechanism by acetaldehyde production. *Candida albicans* use the enzyme alcohol hydrogenase (ADH1) to convert alcohol and other substances, such as carbohydrates into carcinogenic acetaldehyde (Ramirez-Garcia *et al*.,2014).

Nitrosamines produced by *Candida albicans* individually or in combination with other carcinogenic compounds can activate specific proto-oncogenes that can cause the development of cancer lesions that lead to changes in dysplastic conditions in oral epithelium and cancer (Sanjaya *et al*.,20211; Ramirez-Garcia *et al*.,2014).

3.5.2 The Effect of *P gingivalis* on HGF1 Cell line .

The cytotoxicity potentials of *P gingivalis* with 3 strains were evaluated by MTT assay against HGF-1 cell line culture after 48 hrs., which appear that *Gingivalis spp* exhibited selective cytotoxicity against HGF-1 cell line with

inhibitory concentration (IC50) 192 µg/ml 72.46band 63.73, as for strain 1,2 and 3 respectively showed in figure (3.13).

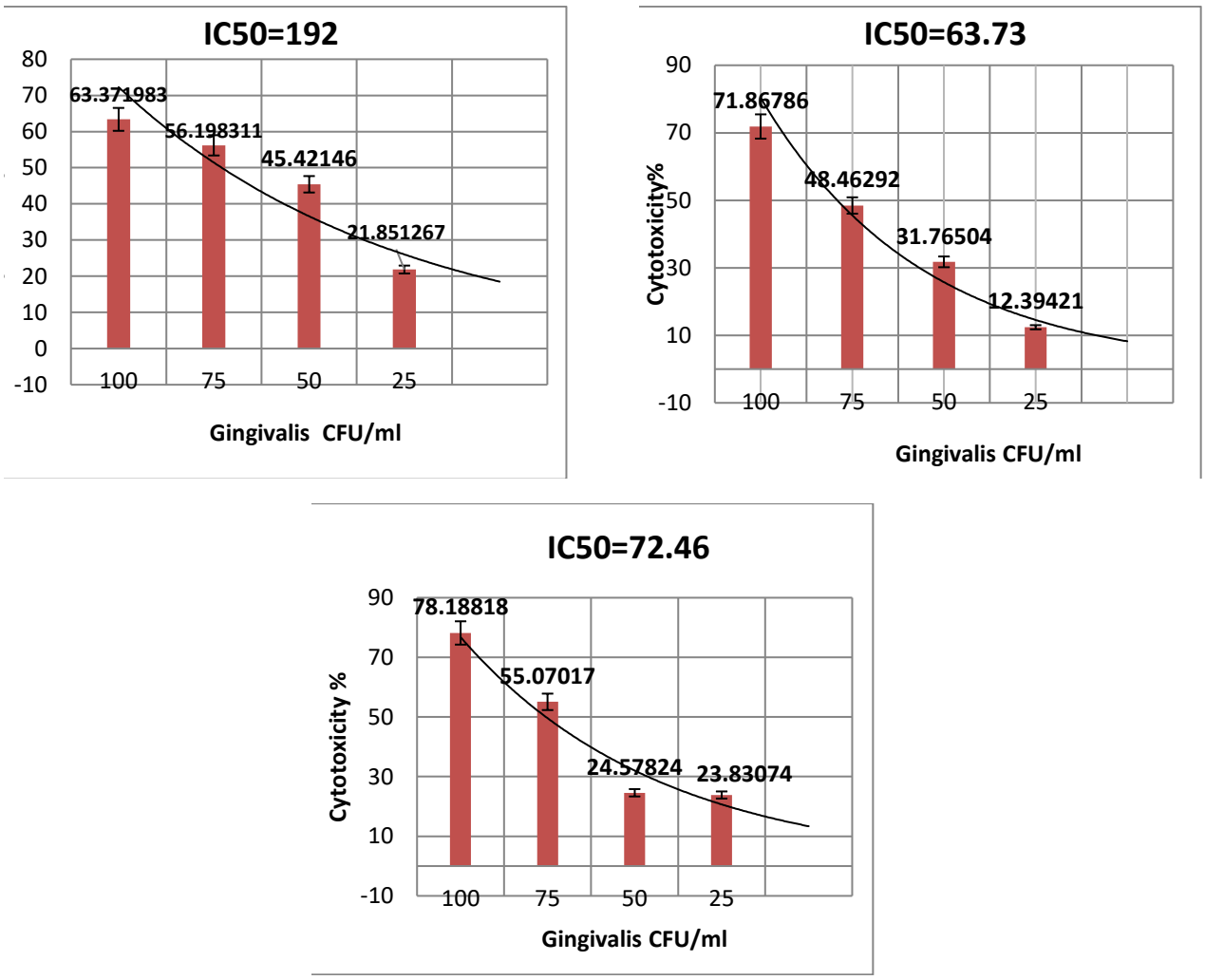


Figure (3.13) The cytotoxic effect of *Gingivalis spp* (against HGF-1 cell line **after 48 hrs of exposure.**

A. The IC50 of the *Gingivalis spp* strain-1 against HGF-1 cell line B. The IC50 of the *Gingivalis spp* strain-2 against HGF-1 cell line. C. The IC50 of the *Gingivalis spp* strain-3 against HGF-1 cell line. sStrain1 IC50 more than the concentration used the experiment, while the strains 2 and strain3 wrer more impact on the cells than strain1.

Porphyromonas gingivalis has been recovered in abundance from oral squamous cell carcinoma (OSCC). Recently established tumorigenesis models have indicated a direct relationship between *P. gingivalis* and carcinogenesis. The bacterium upregulates specific

receptors on OSCC cells and keratinocytes, induces epithelial-to-mesenchymal (EMT) transition of normal oral epithelial cells and activates metalloproteinase-9 and interleukin-8 in cultures of the carcinoma cells. In addition, *P. gingivalis* accelerates cell cycling and suppresses apoptosis in cultures of primary oral epithelial cells. In oral cancer cells, the cell cycle is arrested and there is no effect on apoptosis, but macro autophagy is increased. *Porphyromonas gingivalis* promotes distant metastasis and chemoresistance to anti-cancer agents and accelerates proliferation of oral tumor cells by affecting gene expression of defensins, by peptidyl-arginine deiminase and noncanonical activation of β-catenin.

The pathogen also converts ethanol to the carcinogenic intermediate acetaldehyde. Although coinfection with other bacteria, viruses, and fungi occurs in periodontitis, *P. gingivalis* relates to cancer even in absence of periodontitis. Thus, there may be a direct relationship between *P. gingivalis* and orodigestive cancers (Olsen and Yilmaz,2019)

P. gingivalis secretes a variety of metabolic end products as a result of its asaccharolytic metabolism; however, study of the carcinogenic potential of these is scant. Volatile sulfur compounds, such as hydrogen sulfide, methyl mercaptan, dimethyl sulfide, and dimethyl disulfide, are cytotoxic, and hydrogen sulfide in particular may also be genotoxic and stimulate cell proliferation(Nguyen *et al*.,2020).

Short-chain fatty acids, such as butyrate and propionate, are produced in abundance by *P. gingivalis* and influence the physiology of epithelial and immune cells through serving as energy sources(Blacher *et al.*,2017; Meijer *et al.*,2010) .

Hence, an imbalance in the levels of short-chain fatty acids in the tumor microenvironment has the potential to impact cell proliferation and differentiation; however, the matter requires experimental investigation. Butyric acid produced by *P. gingivalis* can contribute to activation of the

Epstein-Barr virus lytic cycle. Butyric acid inhibits histone deacetylases, thus increasing histone acetylation and the transcriptional activity of the Epstein-Barr virus *BZLF1* gene, which encodes ZEBRA, a master regulator of the transition from latency to the lytic replication cycle(Imai *et al*.,2012).Another study done by (Johansson *et al*.,1996)to analyze the cytotoxicity of some bacterial species associated with periodontal diseases. The specificity of cytotoxicity was estimated against cells of various origin and from different individuals.

3.5.3 Determine the Cytotoxicity activity of the combination of *P.gingivalis and Candida spp*

The cytotoxicity potentials of combination of *P .gingivalis* and *Candida spp* were evaluated by MTT assay against HGF-1 cell line culture after 48 hrs., which appear that combination exhibited selective cytotoxicity against HGF-1 cell line with inhibitory concentration (IC50) 80.3699 µg/ml, as showed in figure(3.14).

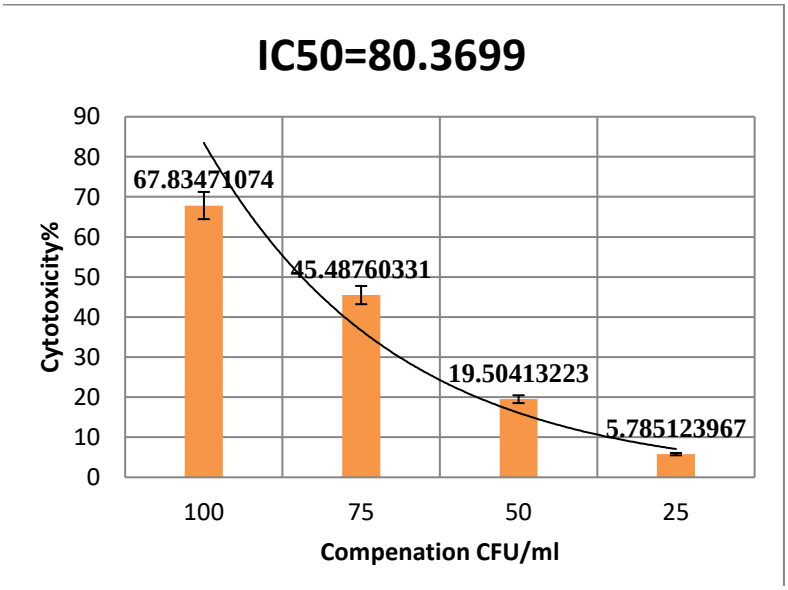


Figure (3.14) The cytotoxic effect of combination of *Gingivalis spp* and *Candida spp* (against HGF-1 cell line after 48 hrs of exposure.

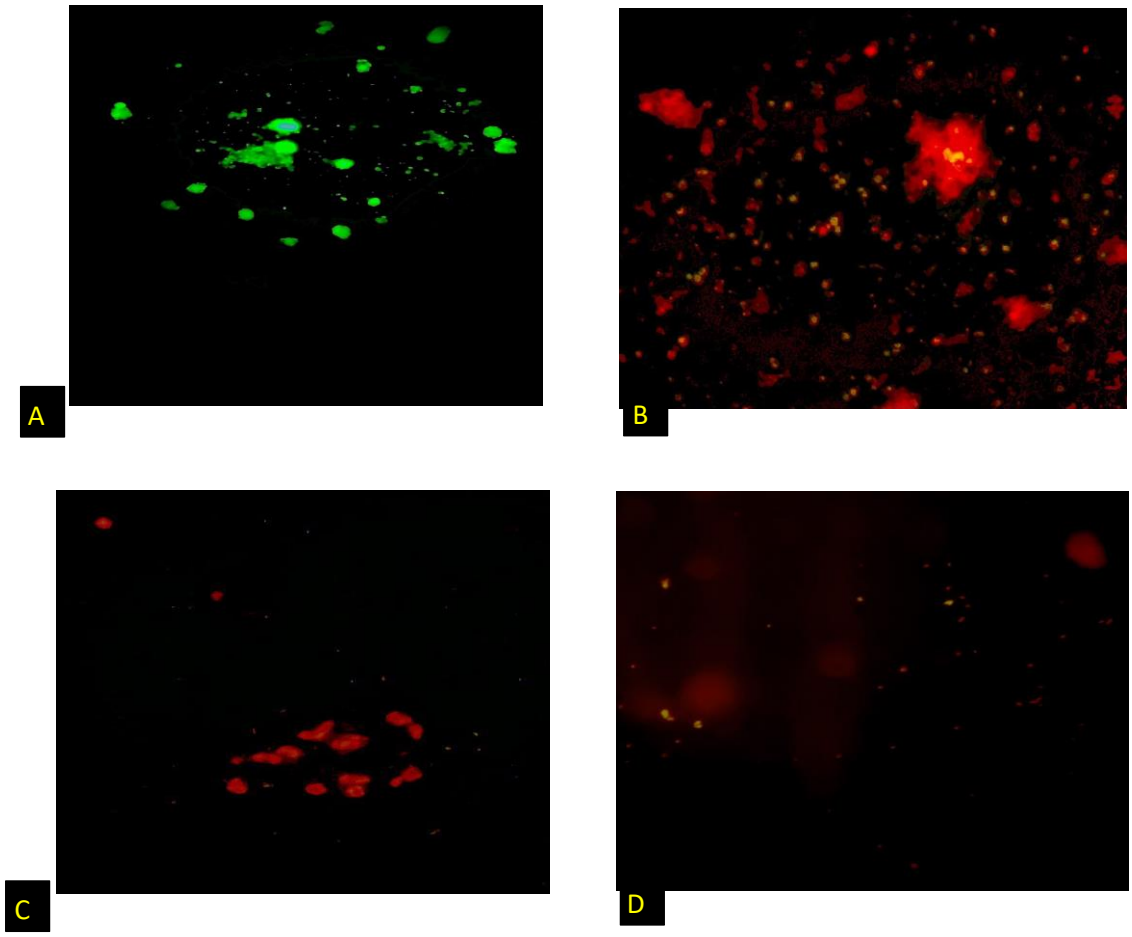


Figure (3.15): The fluorescence microscope image to HGF-1 of healthy human oral cavity cells stained with AO / EtBr and represent the apoptosis method. (A)Untreated HGF-1 cells (100x) . (B) HGF-1 cells treated with Gingivalis strian3 with 100ug/ml after 48 h (100 x) .(C) HGF-1 cells treated with Candida strain3 with 100ug/ml after 48 hr (40 x).(D) HGF-1 cells treated with combination of *Gingivalis spp* and *Candida spp* with 100ug/ml after 48 hrs. (100 x).

The effect of a mixed infection of *Candida spp* and *P.gingivalis* on the inhibition of epithelial cell migration was also studied. The inhibition of cell migration challenged with a mixed infection was stronger than the inhibition caused by one of both microorganisms separately. The inhibiting effect might partly be attributable to the oxygen-reducing effect of both *Candida spp.* Within a biofilm, bacterium–fungus interactions influence the overall survival and proliferation of the respective species (Martin *et al.*,2011).

C. albicans promotes growth and biofilm formation of anaerobic bacteria under aerobic conditions (Janus *et al*.,2016, van Leeuwen *et al*.,2016). An explanation for this might be that Candida creates a pro *P. Gingivalis* anaerobic microenvironment by using oxygen for its own metabolic processes(metabolic interaction).

Epithelial cell death was not excluded as a mechanism of inhibition of epithelial cell migration in a study done by (Haverman *et al*.,2017). However, during all experiments, the epithelial cells were strongly attached to the surface, and the cells looked morphologically viable. Moreover, in a previous study, using the same model, epithelial cell viability was confirmed (Laheij *et al*.,2013).

Therefore, epithelial cell death would not appear to be the mechanism that is responsible for the inhibition of cell migration by *Candida spp*. That was observed in this study.

The study done by (Haverman *et al*.,2017)found that the presence rather than the concentration *of P. gingivalis* was important for the additional inhibitory effect on cell migration when both *Candida spp.* were present. First, it is possible that the inhibitory effect on epithelial cell migration of *Candida* and *P. gingivalis* is at its maximum within the model that was used. Another explanation might be that one Candida cell can only interact with a certain amount of *P. gingivalis,* which means that after a certain threshold, extra *P. gingivalis* does not result in an additional effect.

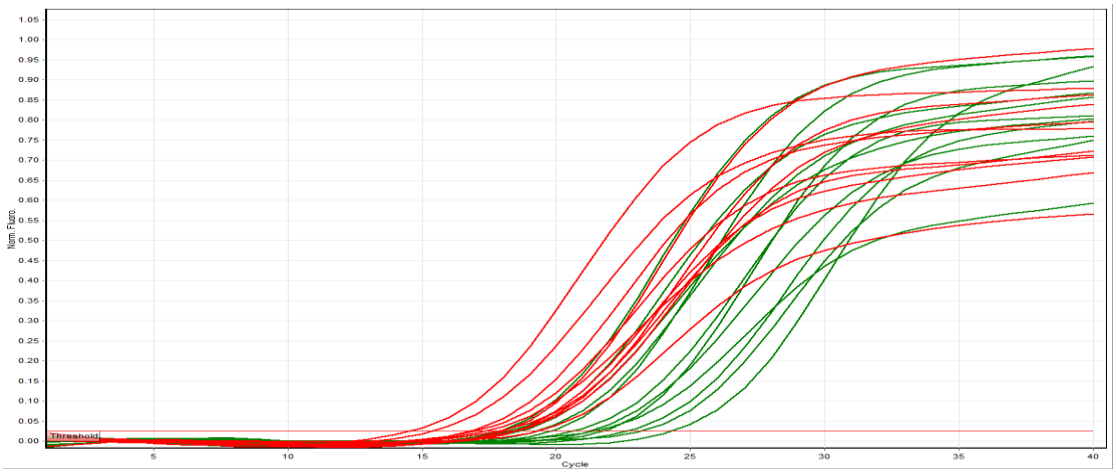
3.6 Determine the gene Expression of Harvey -Ras Gene by RT-qPCR

Gene expression of the *Ras* gene was determined to assess its role in the carcinogenic effect. Quantitative-Real Time-polymerase chain reaction (qRT-PCR) was used to determine the level of *Ras* RNA

transcripts,.Results in (Fig. 3-16) showed that the PCR cycle was proportional to the amount of PCR products after measuring the fluorescence during each cycle. Gene expression estimated by qRT-PCR red trace *Ras* gene , Green house keeping gene .

A total from 4 tissue cell culture infected with candida albicans with different concentration(25%,50%, 75%,100%) , 4 tissue cell culture infected with *P.gingivalis* and 2 tissue cell culture infected with *C albicans and P.gingivalis* ,and 2 sample as control group.

RNA was extracted to study the gene expression by of H-Ras gene using real time PCR(Relative gene expression) (2ddcT) methods ,in this method the level of expression H-ras gene in test samples as well as control samples normalize with house keeping gene for test sample as show in figure 3.16).



(Figer 3-16) : H- Ras gene Expression level .This is the first run for samples (red target gene) , green house keeping gene (GAPDH).

The present study found that the expression of H-Ras gene significant decrease in the patient group (both patients who infected with candida or infected with p.gingivalis or infected with both types)when compared with control group(cell line not infected with any

microorganism) so the gene expression is decrease more than (4.5)fold in compared with normal tissue line as show in figure(3-17).

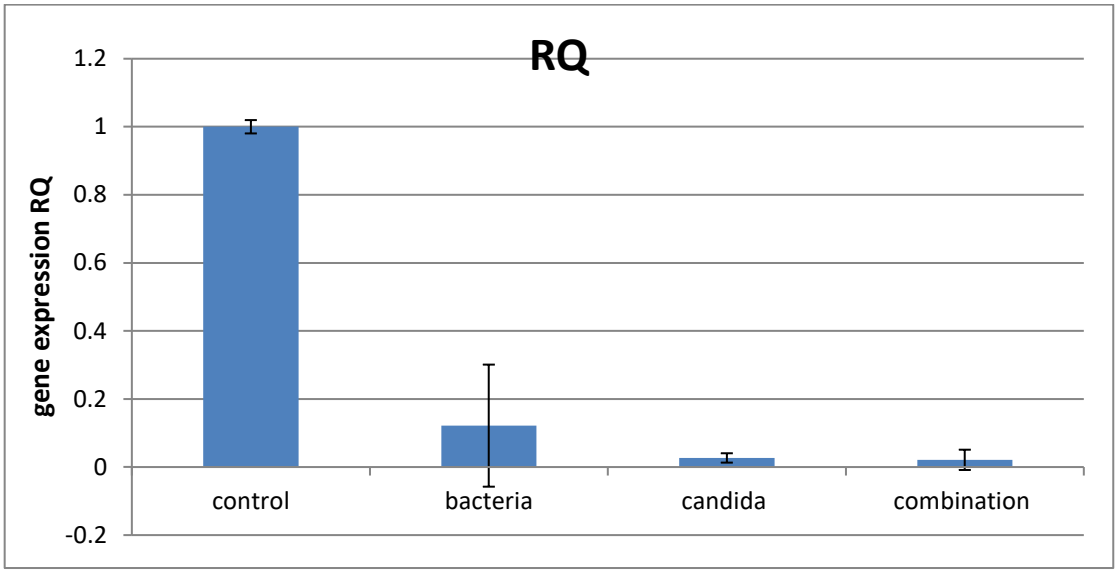


Figure (3-17) :Ras gene expression among samples versus the reference gene (GAPDH).

Also the results in this study show that the CT in normal tissue line less than the value in tissue infected with candida or with p.gingivalis as show in table (3.4).

Table 3.4: ΔΔCt for isolates

	sample	gene Ct	house Ct	ΔCt	ΔΔCt	RQ
Bacteria	1	20.45	27.4	-6.95	2.35	0.196146
Bacteria	2	18.46	23.2	-4.74	4.56	0.042394
Bacteria	3	19.47	28.1	-8.63	0.67	0.628507
Bacteria	4	17.94	21.1	-3.16	6.14	0.01418
Bacteria	5	19.93	25.27	-5.34	3.96	0.064257
Bacteria	6	17.42	21.27	-3.85	5.45	0.022876
Bacteria	7	21.71	27.2	-5.49	3.81	0.071298
Bacteria	8	22.46	28.22	-5.76	3.54	0.085971
Bacteria	9	14.6	18.67	-4.07	5.23	0.026645
Bacteria	10	18.07	20.66	-2.59	6.71	0.009552
Bacteria	11	18.95	22.57	-3.62	5.68	0.019505
Bacteria	12	18.81	26.27	-7.46	1.84	0.279322
Candida	13	18.69	22.27	-3.58	5.72	0.018972

Candida	14	16.8	21.77	-4.97	4.33	0.049721
Candida	15	21.41	25.87	-4.46	4.84	0.034915
Candida	16	21.7	26.17	-4.47	4.83	0.035158
Candida	17	21.55	24.78	-3.23	6.07	0.014885
Candida	18	20.53	25.51	-4.98	4.32	0.050067
Candida	19	22.68	25.28	-2.6	6.7	0.009618
Candida	20	22.67	25.43	-2.76	6.54	0.010746
Candida	21	23.49	27.71	-4.22	5.08	0.029564
Candida	22	22.13	25.59	-3.46	5.84	0.017458
Candida	23	21.43	25.61	-4.18	5.12	0.028756
Candida	24	24.11	27.88	-3.77	5.53	0.021642
Combination	25	18.49	24.34	-5.85	3.45	0.091505
Combination	26	26.1	28.95	-2.85	6.45	0.011438
Combination	27	18.49	22.67	-4.18	5.12	0.028756
Combination	28	25.39	27.77	-2.38	6.92	0.008258
Combination	29	26.5	27.31	-0.81	8.49	0.002781
Combination	30	19.33	18.28	1.05	10.35	0.000766
Combination	31	17.87	21.42	-3.55	5.75	0.018581
Combination	32	17	19.3	-2.3	7	0.007813
Control	33	18.19	27.51	-9.32	-0.02	1.013959
Control	34	20.72	30	-9.28	0.02	0.986233

The Ras pathways is one of the most prevalent oncogenic alterations in both human and animal in vivo oncogenic mutation have been shown to occur at exon 12 ,13 and 61 resulting of possible point mutation for each RAS is forms (miller and miller,2012).

The main member of the RAS gene family-KRAS,HRAS and NRAS-encode proteins that have a pivotal cytoplasmic role in cell signaling ,when RAS gene are mutated ,cell grow uncontrollably and evade death signals ,also RAS mutations also make cell resistant to some available cancer therapies (simanshu *et al* 2017).

RAS proteins are important for normal development active RAS drives the growth , proliferation and migration of cell ,in normal cell RAS receives signal those signals rapidly switch between the active GTP form inactive GDP form stste(prior *et al*.2020).

Mutated RAS is stuck in the active state and drives cells to become cancerous, there are distinctive patterns in the mutation frequencies associated with each RAS gene and cancer type (prior *et al*. 2020)

The result in this study , show decrease in H-RAs gene expression this may be due to the gene have a mutant so it detected in low value and this results make patient more susceptible to cancer type due to which infected with candida or *P.gingivalis* .

The significant differences in the RAS signaling capacity can result depending on the tissue isoform mutation combination (pershing *et al*.,2015).

Results in (Fig. 3-17) indicated that there is a significant differences in *RAS* gene expression between bacteria and *Candida albicans* with control. when the isolates is treated with cell line the results indicated inhibition gene expression with candida albicans higher than the *P .gingivalis* and control.

The Current result showed that high significant difference (P value 0.05%) in concentration (75%) with the third isolated of bacterial (0.043) while the four isolates of bacteria show significant difference (0. 54%) in a concentration (100%) .

Also the present study showed significant difference(0.043) in second concentration (50%) isolates of *C albicans* while the combination between *C albicans* and *P gingivalis* show no significant with all concentration as show in table (3.5) and (3.6).

Table (3.5): Concentration of isolates and combination

Descriptives								
RQ								
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1.00	2	1.0001	.01961	.01386	.8239	1.1762	.99	1.01
2.00	12	.1217	.17940	.05179	.0077	.2357	.01	.63
3.00	12	.0268	.01376	.00397	.0180	.0355	.01	.05
4.00	8	.0212	.02977	.01053	-.0037	.0461	.00	.09
Total	34	.1162	.25188	.04320	.0284	.2041	.00	1.01

Table (3.6). Concentration of isolates and combination:

Multiple Comparisons						
Dependent Variable: RQ						
LSD						
(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	.87838*	.08398	.000	.7069	1.0499
	3.00	.97330*	.08398	.000	.8018	1.1448
	4.00	.97886*	.08693	.000	.8013	1.1564
2.00	1.00	-.87838*	.08398	.000	-1.0499	-.7069
	3.00	.09493*	.04489	.043	.0033	.1866
	4.00	.10048	.05019	.054	-.0020	.2030
3.00	1.00	-.97330*	.08398	.000	-1.1448	-.8018
	2.00	-.09493*	.04489	.043	-.1866	-.0033
	4.00	.00555	.05019	.913	-.0969	.1081
4.00	1.00	-.97886*	.08693	.000	-1.1564	-.8013
	2.00	-.10048	.05019	.054	-.2030	.0020
	3.00	-.00555	.05019	.913	-.1081	.0969

*. The mean difference is significant at the 0.05 level

*1.00 mean control , 2.00 Bacteria , 3.00 Candida and 4 the combination .

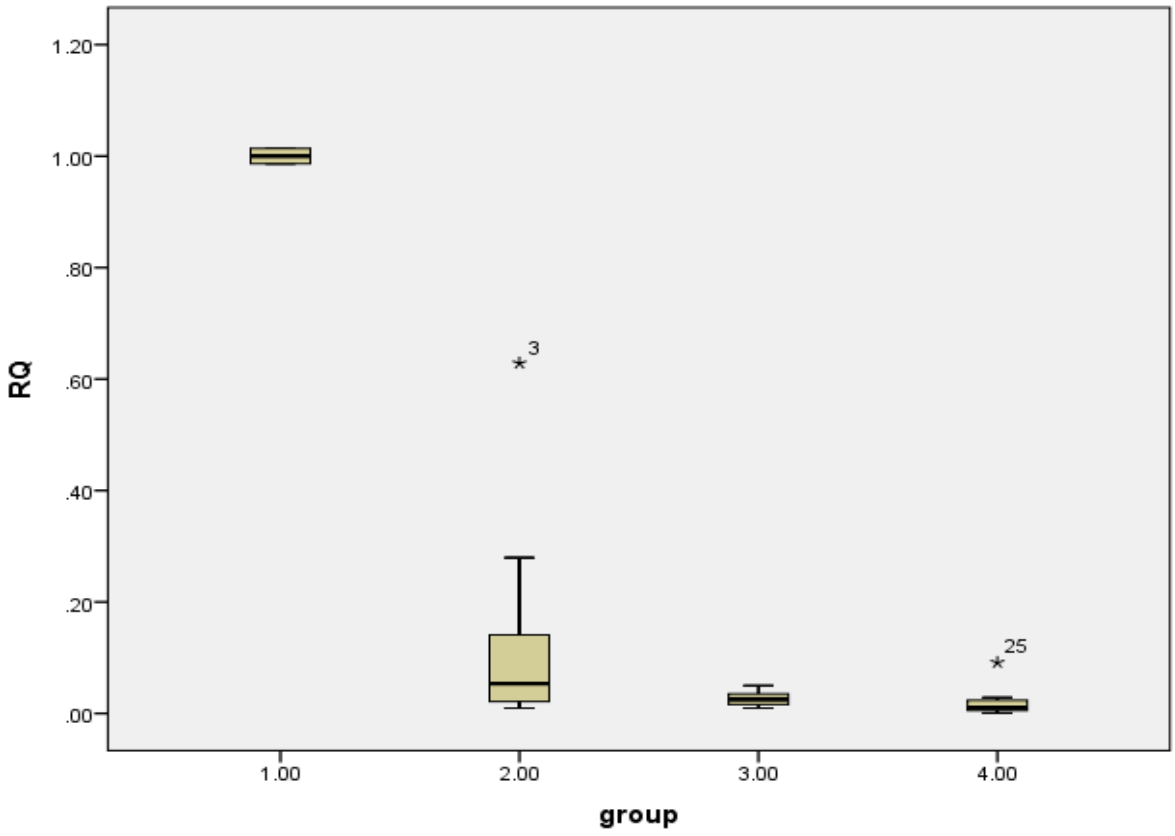


Fig 3-18: B ox plot Represent the value gene expressions of Ras gen different group at four 1=control ,2=bacteria ,3=candida ,4=compensation

A study proposed by (Krishna *et al.*,2018) found that the H-Ras protein was significantly overexpressed in the oral carcinoma group compared to the normal group (P = 0.03) . H-Ras positivity increased in cases affected with buccal mucosa site and higher grade of carcinoma. Relative mRNA level of H-Ras was significantly elevated in oral carcinoma as compared with the control group ($P \leq 0.001$). Protein and mRNA levels of H-Ras in case group was poorly correlated .

Also (Krishna *et al.*,2018) concluded that H-Ras oncogene expression was markedly higher in oral carcinoma, and it can be a prognostic marker and target for an effective molecular therapy.

Mutations in any one of three members of the Ras family are common events in human tumorigenesis. Several studies reported that most

oncogenic mutations predominantly affect the K-Ras locus and express altered protein in oral carcinoma and some other cancer.(Vitale-Cross *et al.*,2004; Camps *et al.*,2005; Vaughn *et al.*,2011) .

The exact interpretation of this finding remains unclear, but previous Indian studies based on etiological factors and mutational status of Ras oncogenes indicate that rare mutations or transcriptional splicing in H-Ras gene might be related to overexpression or mutant type H-Ras protein in tumoral tissues. More studies are required on the functional and mutational gene expression of H-Ras in oral carcinogenesis.

3.7 Interleukine 17 (IL-17B) Assessment

The results of this study showed there was no significant p value (0.398) of IL-17 in saliva of periodontitis patient compared with control group as shown in Table(3.7) and figure (3.20) . .

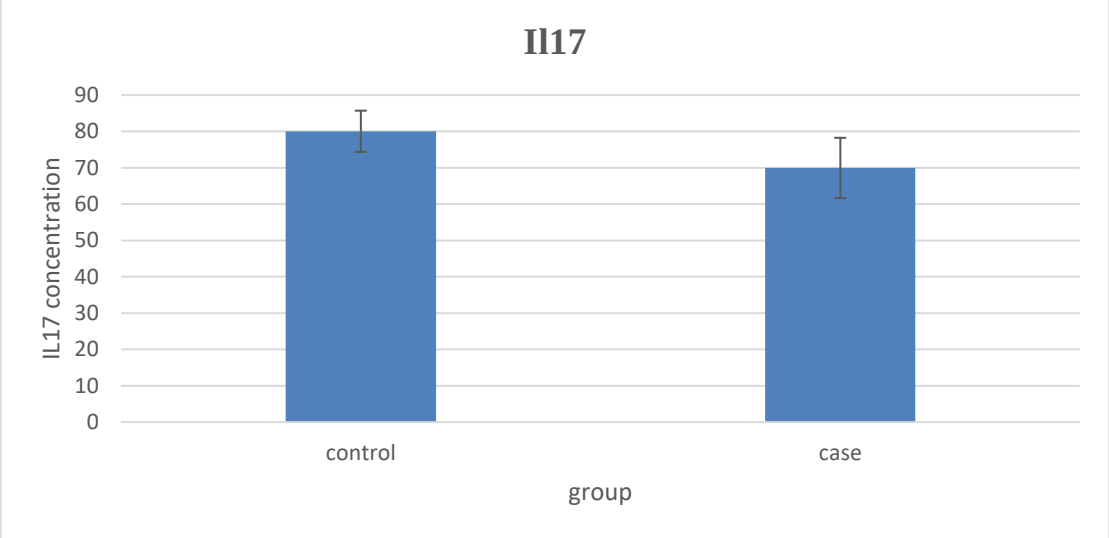


Figure (3.20): The level of salivary IL-17 in case and control groups

Table (3.7): The level of Salivary IL-17 in case and control group.

Group Statistics					
	group	**Mean**	**Std. Deviation**	**Std. Error Mean**	**P value**
Il17	control	80.0288	17.96528	5.68112	0.398
	case	69.9388	34.24481	8.30559	

*Non significant

Interlukin-17 stimulates the release of cytokines which particularly attract neutrophils to the inflammatory site. However, AgP known to have divergent PMN function shows defect in neutrophils chemotactic response. It might possible that despite stimulation of IL-17, inflammation may remain unnoticed in AgP due to lack of chemotactic response. Study conducted in a Vermin model reveal the significance of IL-17 in mobilization of neutrophil in the control of the any bacterial infection(Ye *et al*.,2001).

A previous study done by (Azman *et al*.,2014) demonstrated that Serum, saliva and gingival crevicular fluid (GCF) IL-17A levels were higher in periodontitis patients and correlated positively with clinical parameters of attachment loss, pocket depth and bleeding on probing and In vitro, IL-17E inhibited *Porphyromonas gingivalis* and IL-17A induced expression of chemokines by reducing phosphorylation of the NF-κB p65 subunit.Also concluded that IL-17E may have opposing roles to IL-17A in periodontitis pathogenesis. IL-17E can negatively regulate IL-17A and periodontal pathogen induced expression of chemokines by oral keratinocytes.

While the results showed there was a significant increase in IL-17 concentration incomperation to treatment with *C albicans* alone and

combination with *P .gingivalis* at P value (0.001) as show in table (3.8) and figure(3.21) .

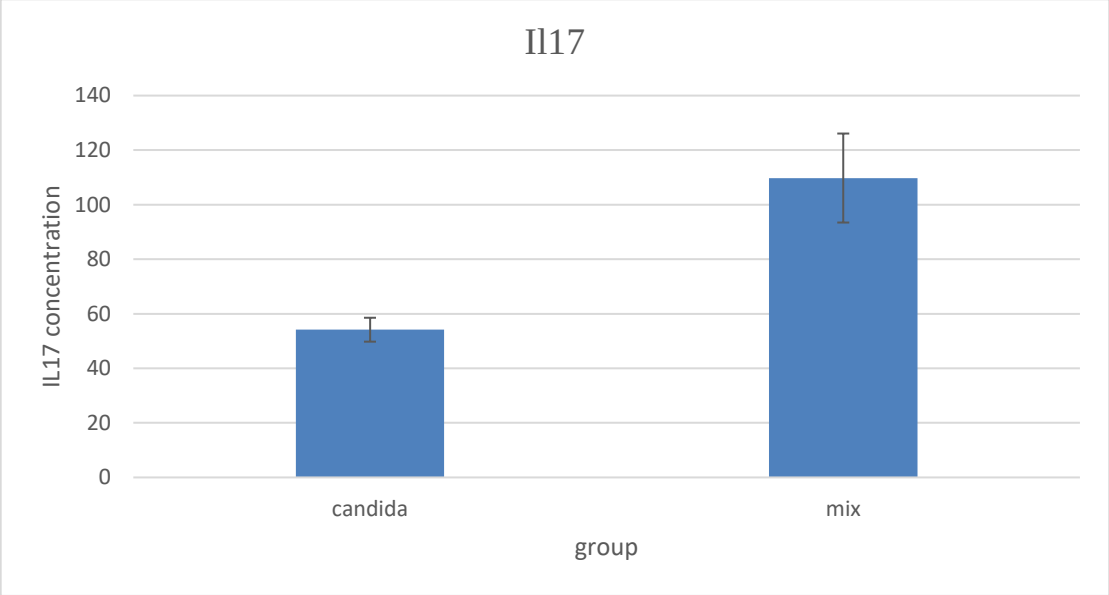


Figure (3.21): IL17 Salivary concentration among candida and mixed infection

Table (3.8): IL17 Salivary concentration among candida and mixed infection

Group Statistics					
	Group	Mean	Std. Deviation	Std. Error Mean	P value
	Candida	54.1665	14.53969	4.38388	0.001
	Combination	109.7686	36.47849	16.31368	

IL-17A plays a protective and destructive role in the progression of chronic periodontitis. When periodontal infection occurs, neutrophils can quickly move out of the blood vessel and are the first effector cells that reach the infected site. IL-17A can regulate neutrophils to leave the bone marrow and enter the blood circulation, and recruit neutrophils to the infected site of periodontal tissues (Hajishengallis,2015;, Hajishengallis,2020).

Studies have mentioned that many defense mechanisms were included in response to candidiasis, and IL17 is the most strongly recommended in such immune response, It was reported that IL-17 and INF-γ play an important roles in protection against fungal infections, when they effectively enhanced neutrophils and macrophages in killing of fungi during the innate responses of the host (Fidel.,2011) .

Result of (Cardoso *et al.*,2009) demonstrated elevated levels of IL-17, TGF-β, IL-1β, IL-6, and IL-23 messenger RNA and protein in diseased tissues as well as the presence of Th17 cells in gingiva from patients with periodontitis.

Another study by (Chen *et al*.,2015) found that IL-35 and IL-17 were significantly higher in GCF from patients with periodontitis than healthy participants (P <0.01, P <0.05, respectively).

CP represents long lasting inflammation of periodontal tissues where microbes are a major etiological factor and the hallmark of periodontal diseases is bone loss. Virulence factors produced by *P. gingivalis* such as Arg- and Lysgingipain proteinases are key factors for host tissue invasion which leads to activation of immune-infammatory processes. Subsequently, various molecules (proteases, MMPs, cytokines, etc.) are activated leading to destruction of connective tissue attachment and alveolar bone loss (Chen *et al*.,2015).

In the study of (Batool *et al*.,2018) was high in CP compared to healthy controls and it gradually increased with the severity of disease. Corroborating our data, previous studies have documented increased level of IL-17A in saliva, serum, and gingival cerevicular fluid (GCF) in periodontitis compared to healthy subjects and also reported important role

of IL-17 in gingival infammation and bone loss (Prakasam and Srinivasan, 2014; Azman *et al*.,2014; Cheng *et al*.,2014).

(Batool *et al*.,2018) Concluded Salivary levels of IL-6 and IL-17 were signifcantly higher in patients with calculus associated CP compared to healthy subjects. These cytokines increased as the disease progressed from mild to moderate and severe form. Therefore, can conclude that the salivary level of IL-6 and IL-17 may help in the subcategorization of periodontitis.

According to (Rohaninasab *et al*.,2013; Abusleme and Moutsopoulos2017; Zenobia and Hajishengallis, 2015). IL-17 levels in the GCF of patients with periodontitis were higher compared to healthy control patients and IL-17 played a role in the pathogenesis of chronic periodontitis. This can occur because the role of IL-17 affects the immunity and contributes to making clinical disease, and also the identification and characteristics of other molecules play a role.1-3 The presence of concentrations of IL-17 is a consequence of the early stages of gingival inflammation, but it does not cause periodontitis lesions.

Neutrophils phagocytize bacteria and sterilize through oxygen dependent and independent mechanisms to achieve immune activation and protection. However, the main bactericidal substances of neutrophils are superoxide ions and lysosomal enzymes, whose excessive release will damage the surrounding cells and tissues and aggravate the inflammatory response. At the same time, the inflammatory cytokines produced and released by neutrophils in the process of phagocytosis of bacteria will also aggravate inflammation and promote the local inflammatory response of periodontal tissues, leading to damage and destruction of the periodontal tissues.

(Yu et al.,2007) have shown that IL-17A plays a major protective role in bone loss in *Porphyromonas gingivalis* induced periodontitis, although a large number of researches have shown that IL-17A is closely related to bone erosion in rheumatoid arthritis.

In addition, IL-17A can also act synergistically with IL-1 and TNF-α to induce gingival fibroblasts to produce MMP-1 and MMP-3, which plays an important role in the tissue destruction in periodontitis (Beklen et al.,2007).

IL-17A can promote keratinocytes to secrete antimicrobial peptides and play a defensive role, but whether it plays a role in the progression of periodontitis has not been confirmed. In the mouse model of ectopic tracheal transplantation, IL-17A is involved in the pathogenesis of obliterative bronchiolitis by regulating M1 macrophage polarization (Meng *et al*.,2017), but whether IL-17A mediates periodontal tissues destruction by promoting M1 macrophage polarization and secreting inflammatory factors remains to be studied.

IL-17 is found in high amounts in periodontal disease. IL-17 aggravates periodontal disease by activating gingival fibroblasts to produce inflammatory cytokines. There is abundant documentation that suggests major tissue destruction in periodontitis which results from the recruitment of host cells through the activation of monocytes/macrophages, lymphocytes, and fibroblasts cell(Cardoso *et al*.,2009) .

IL-17 is more frequently detected in periodontitis patients than in gingivitis patients. Takahashi *et al*. have suggested that IL-17 is produced in periodontitis, which may be involved in Th1 modulation and which

increase inflammatory reactions through gingival fibroblast-derived mediators and thus, IL-17 has a potential role in the pathogenesis of the periodontal disease(Oda *et al.*,2003; Takahashi *et al*.,2005).

IL-17 has an action on alveolar bone cells. It has been documented that T-cells can be directly involved in bone metabolism through T-cell-derived cytokines which includes IL-17(Takayanagi *et al.*,2000).

IL-17 has the ability to stimulate osteoclast cells and activate receptor activator of nuclear factor kappa-B ligand production by osteoblasts(Sato *et al.*,2006).

Aggressive periodontitis (AgP) is generally seen in teenagers and young adult. It is the most severe form of periodontitis which can lead to significant periodontal inflammation and premature tooth loss in maximum number of cases at early age. There is abundant literature suggesting aberrant polymorphonuclear leukocytes (PMN) function as a key pathogenic mechanism in AgP exhibiting defective *in vitro* PMN chemotactic response and enhanced oxidative metabolic responses(Baeten et al.,2013).

Evidence suggest that the rate of bone destruction is about the three to four times faster than in chronic periodontitis. Early age of onset is one of the main characteristics features of AgP; however, patients with AgP are clinically healthy(Andrukhov *et al*.,2011).

IL-17 may play a significant role in AgP because of the functional impairment of PMN and because of the association of IL-17 pathways with the recruitment of neutrophils which results in enhanced inflammation and bone resorption(Ay *et al*.,2011).

It has been hypothesized that IL-17 has a key role in regulating neutrophils *in vivo* and neutrophils play a crucial role in controlling periodontal infection. Neutrophils are considered as the first line of defense against a broad range of periodontal pathogens. Functionally intact neutrophils are necessary for defense in any inflammatory condition. Patients suffering from defects in neutrophils function suffer from recurrent and severe infection including AgP. Neutrophils are also potentially harmful if turned against host tissue(Linden *et al.*,2005).

The constant phases of neutrophil mobilization are key components of innate immunity contributing to host defense(Ibbotson et al.,2001; Shiohara et al.,2004).

IL-17 is important for neutrophil homeostasis and therefore for periodontal health. Any alteration from normal neutrophil activity (in terms of numbers or activation status) can potentially cause periodontitis(Darveau,2010; Hajishengallis, E., and Hajishengallis, G. (2014).

The functional pathways of the IL-17 cells in periodontitis are still not sufficiently understood, and thus, more research is required. There is abundance literature available with IL-17 levels in chronic periodontitis but miniscule information with IL-17 in relation to AgP cases. More studies in all ethnic groups are necessary which can be used in the development of individualized diagnostic and treatment plans of periodontitis, especially in case of AgP.

4.1 Conclusions

According to the results of the present study, the following conclusions could be elucidated:

- Detection of *C.albicans* virulence gene (ALS1, EAP1 HWP1) in some isolates by PCR techniques.
- Detection cytotoxicity potentials effect cell line study to3 isolates of *C albicans* were evaluated by MTT assay against HGF-1 cell line culture after 48 hrs., which appear that C albicans exhibited high toxicity (IC50 of the Candida strain-3 HGF-1 cell line was 54.45 μg/ml.).
- The cytotoxicity potentials Detectby cell line study effect to *P .gingivalis* in 3 isolates which found that they were evaluated by MTT assay against HGF-1 cell line culture after 48 hrs., which appear that *P .gingivalis* exhibited selective cytotoxicity against HGF-1 cell line with inhibitory concentration (IC50) 192 μg/ml.
- Cytotoxicity potentials detected for combination of *P .gingivalis* and *Candida albicans* by cell line study were evaluated by MTT assay against HGF-1 cell line culture after 48 hrs., which appear that combination exhibited selective cytotoxicity against HGF-1 cell line with inhibitory concentration (IC50) 80.3699 μg/ml.
- Gene expression of the H-Ras gene indicated that there was a significant differences in RAS gene expression between bacteria and candida albicans with control.
- IL-17 detected by ELISA technique indicated high concentration in patients infected with candida and *P. gingivalis.*

4.2 Recommendations

Depending on the finding of this study, the following recommendations are drawn:

- ❖ further research should be undertaken in this field to establish the exact pathogenic mechanism of this opportunistic fungus in periodontal diseases and also confirm the results using a long-term follow-up by evaluating the effect of periodontal treatment on this opportunistic fungus.
- ❖ Further studies will be necessary to identify the molecular pathways that promote the alteration in epithelial cells during *C. albicans* and *P .gingivalis* oral infection
- ❖ Further study relationship between existence of specific type of bacteria and its oncogenic effects
- ❖ More studies are required on the functional and mutational gene expression of H-Ras in oral carcinogenesis.
- ❖ Study the role of others' specific pro and anti-inflammatory cytokines levels and their gene polymorphism, which may have impact on the disease management.
- ❖ The application of new typing methods like PFGE type or sequencing typing methods may result in a better discrimination of isolates

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