Biological constituents from Sudanese medicinal plants hinder the pathogenicity of *Porphyromonas gingivalis* TDC60

[*Porphyromonas gingivalis* TDC60の病原性を阻害するスーダン薬用植物の成分]

A DISSERTATION

By

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List of abbreviations

P.g	*Porphyromonas gingivalis*
Rgp	arginine-specific gingipain
Kgp	lysine-specific gingipain
Fim	fimbriae
LPS	Lipopolysaccharides
GAM	Gifu Anaerobic Medium
Boc	*t*-butyloxycarbonyl
VPR	L-valyl-L-prolyl-L-arginine
VLK	L-valyl-L-leucyl-L-lysine
MCA	methylcoumaryl-7-amide
ODS	hydrosphere column C18 filled with octadecyl silyl
TLC	thin-layer chromatography
HPLC	high-performance liquid chromatography
IC_{50}	the half-maximal inhibitory concentration
MIC	Minimum inhibition concentration
HTs	hydrolyzable tannins
PAs	Proanthocyanidins
HRMS	high-resolution mass spectrometry
TMS	Trimethylsilyl
$CDCl_3$	chloroform-d

CHAPTER ONE

General introduction

1.1 Oral and Periodontal diseases

The primary health problems worldwide continue to be a result of chronic diseases with oral ailments (Petersen et al., 2005; Petersen and Ogawa, 2012). Therefore, periodontal diseases are among the most important global oral health problems (Petersen et al., 2003). Periodontal diseases are a combination of periodontal tissue disorders by microorganisms that adhere to and grow on the teeth surfaces (dental plaque). Together with an over-aggressive immune response against them, they are commonly believed to be the causes of periodontitis. The inflammation refers to the pathologic state of the gingival and the supporting structures of the periodontium, which include gingival, alveolar bone, periodontal ligament, and cementum. They are commonly found in most human populations and result in significant morbidity and loss of the teeth in severe conditions. The Periodontal diseases are commonly having two main groups (Fig.1.1), a group of gingival diseases, and another group is periodontitis (Wiebe and Putnins, 2000)**.** The inflammation of the gingival tissues caused by the accumulation of dental plaque followed by redness, swelling, and bleeding of the tissues, which clinically characterized as gingival disease. Gingivitis will not progress to periodontitis unless there is perturbation in the local conditions or generalized host susceptibility (Offenbacher, 1996).

On the other hand, periodontitis is clinically known by the formation of a periodontal pocket, which is the leading cause of that soreness. This pocket is an ideal surface for further bacterial colonization and the formation of subgingival plaque. The irreversible plaque-induced inflammation of the periodontal tissues leads to the destruction of the periodontal ligament and

alveolar bone and the migration of the epithelial ligament and teeth losses (Palombo, 2008; Williams et al., 1922). The crucial of periodicities diseases is in line with the results of a numerous of recent studies that are linking between chronic periodontitis conditions and systemic diseases such as diabetes mellitus, AIDS, leukemia, Down's syndrome (Komatsu, 2014; Mehta, 2015), cardiovascular diseases (e.g., heart attack, coronary artery disease, and stroke) (Saremi et al., 2005; Friedewald et al., 2009), and pre-term low birth weight babies (Ali and Abidin, 2012).

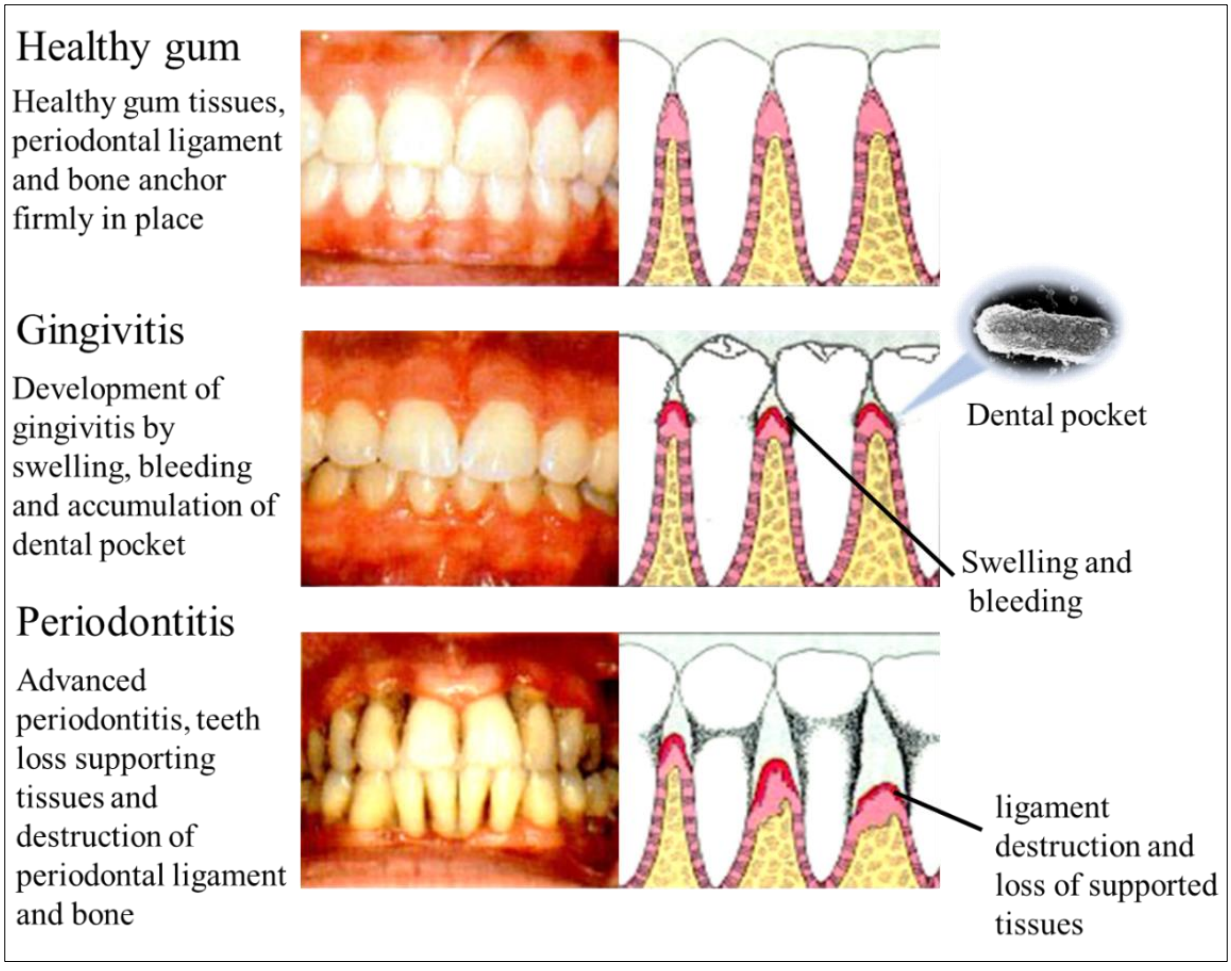

Fig.1.1 Periodontal disease stages (https://evolve.community.uaf.edu)

1.2 Oral microorganisms

The atmosphere of the oral cavity surface with saliva and a slight temperature range from 34 to 36◦C, and nearby neutral pH is a suitable environment for most microorganisms (Marcotte and Lavoie, 1998; Parahitiyawa et al., 2010). Various analytical approaches illustrated that there are more than 700 species with no less than six billion bacteria believed to be the bacterial community of the human mouth (Theilade, 1990; Aas et al., 2005), along with fungi, mycoplasma, protozoa, and even viruses (Pennisi, 2005). Generally, oral bacteria can be classified as gram-positive and gram-negative bacteria, or more specific, and according to their oxygen requirements into anaerobic or facultatively anaerobic. The primary isolated microorganisms from the human oral cavity are listed in Fig.1.2.

Although the diversity of the oral microbiota community, it is well known that oral microbiota and the oral cavity are a stable community known as the climax community. Hence, an imbalance of oral microbiota leads to the occurrence of oral diseases such as caries and periodontal diseases, resulting in the multiplication of possibly pathogenic microorganisms. Numerous studies have pointed out the correlation between destruction of periodontal tissue, teeth loss in severe conditions, and the changes of microbial species in the gingival sulcus from gram-positive, facultative, fermentative microorganisms to predominantly gram-negative, anaerobic, chemoorganotrophic, and proteolytic organisms (Eloe-FadroshandRasko, 2013). Therefore, periodontal breakdown occurs as a result of multifaceted interactions between bacterial microbiota and the host defense mechanisms, leading to the disruption of the balance between bacterial aggression and the host protection system (Hajishengallis et al., 2012). Based on this fact, several studies have confirmed that the primary etiological agents of periodontal diseases are generally gram-negative rods, which include *Actinobacillus actinomycetemcomitans*, *Tannerella forsythia*, *Prevotella*, *Fusobacterium*,

and *Porphyromonas gingivalis.* These microbial species are not capable of causing destructive events individually. However, to be involved in the periodontal disease progression nonetheless, the etiology requires several steps of interaction between these microbial members with other surrounding factors to establish their positions in the oral cavity (Marcotte and Lavoie, 1998; Maiden et al., 2003; Paster et al., 2006).

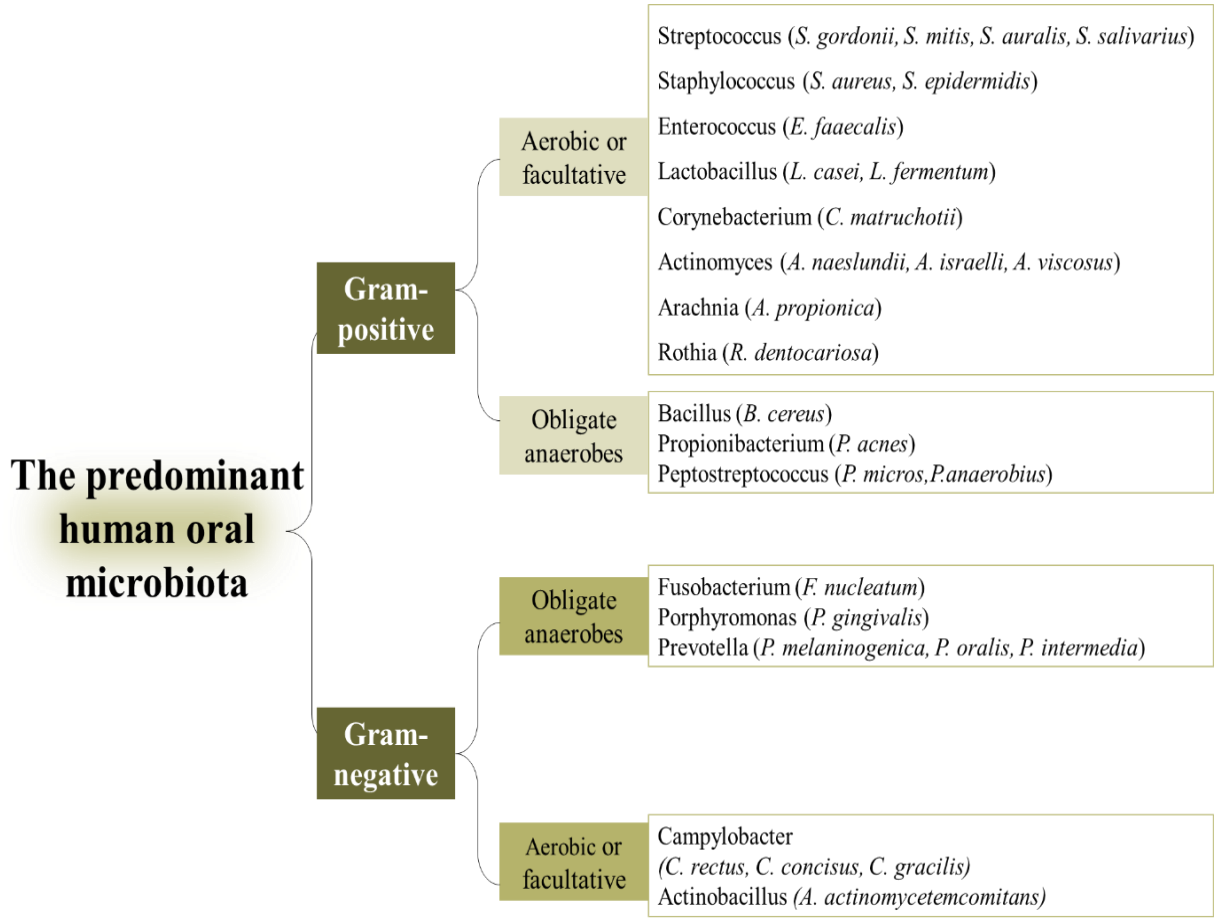

Fig.1.2 The predominant human oral microbiota.

1.3 *Porphyromonas gingivalis*

Although uncountable bacterial species usually exist in the human oral mucosa microbiota, *Porphyromonas gingivalis* appears to be the primary etiological agent in the pathogenesis and progression of the inflammatory events of periodontal disease (Hajishengallis et al., 2012). More than 85% of the subgingival plaques of patients with chronic periodontitis have been infected with this bacterium (Datta et al., 2008). *P. gingivalis* was firstly discovered in 1921 (Oliver and Wherry, 1921) and named *Bacterium melaninogenicum*, then reclassified in long series to be named as *Bacteroides gingivalis*, until its recent reclassification (Fig.1.3) as a new genus of *Porphyromonas* (Nisengard and Newman, 1994). This bacterium (Fig.1.4) is a non-motile, non-spore forming asaccharolytic, gram-negative bacterium, an obligately anaerobic rod, forms black-pigmented colonies on blood agar medium, and most of the strains require iron and vitamin K for growth (Olczak et al., 2005; Hunt et al., 1986). Iron uptake by this bacterium in the form of heme has been proven to play an essential role in its growth and virulence. Nonetheless, unlike many other microorganisms, *P. gingivalis* does not possess a siderophore scavenging system for iron uptake or the enzymes required for heme biosynthesis. Instead, it employs alternative mechanisms through degrading the iron-transporting plasma proteins albumin, hemopexin, haptoglobin and transferrin to obtain iron from host heme (Gao, et al., 2010; Smalley et al., 2011). Various investigations revealed that the infection is either iron-limited or severely restricted by the availability of iron. Therefore, the virulence of a microorganism is greatly increased with its ability to contest with the host for iron. A study conducted by McKee et al. (1986), revealed that the virulence of *P. gingivalis* could be controlled by the level of protoheme added to the culture medium. Moreover, iron regulation of the expression of outer membrane proteins since they observed the induction of 10 surface proteins when *P. gingivalis* was grown in hemin restricted

conditions hypothesized the involvement of the iron-regulated proteins in hemin uptake and virulence of *P. gingivalis*. (Bramanti and Holt, 1990).

Outer cell membrane receptors, proteases (mainly gingipains), lipoproteins, and other specific proteins are involved in iron/heme capturing (Olczak et al., 2005). Recent studies strongly hypothesized that *P. gingivalis* interact with other host-microbiota members by producing various pathogenic factors, leading to the disease progression. The initiation and development of periodontal tissue destruction are complex processes involving plaque accumulation, release of bacterial substances, and host inflammatory response. *P. gingivalis* produces several virulence factors that could penetrate the gingival and destruct the host tissue directly or indirectly by induction of inflammation (Pennisi, 2005).

Virulence factors are constituents or metabolites of an organism that are crucial in various stages of the life cycle and cause damage to the host and produce substances that could initiate tissue destruction. The most potent adhesins and virulence factors of *P. gingivalis* are fimbriae, lipopolysaccharides, proteases (gingipains), and potential adhesion molecules such as hemagglutinins (Connolly et al., 2017). This pathogenicity factors are playing a vital role in the infection process by allowing *P. gingivalis* to adhere to the surface of host cells. This process facilitates the acquisition of heme through erythrocyte binding. It causes lysis and aggregation of erythrocytes via several proteolytic enzymes known as gingipains, which release the heme moiety from the hemoglobin molecule as a nutrient (Lewis, 2006; Olczak, 2008).

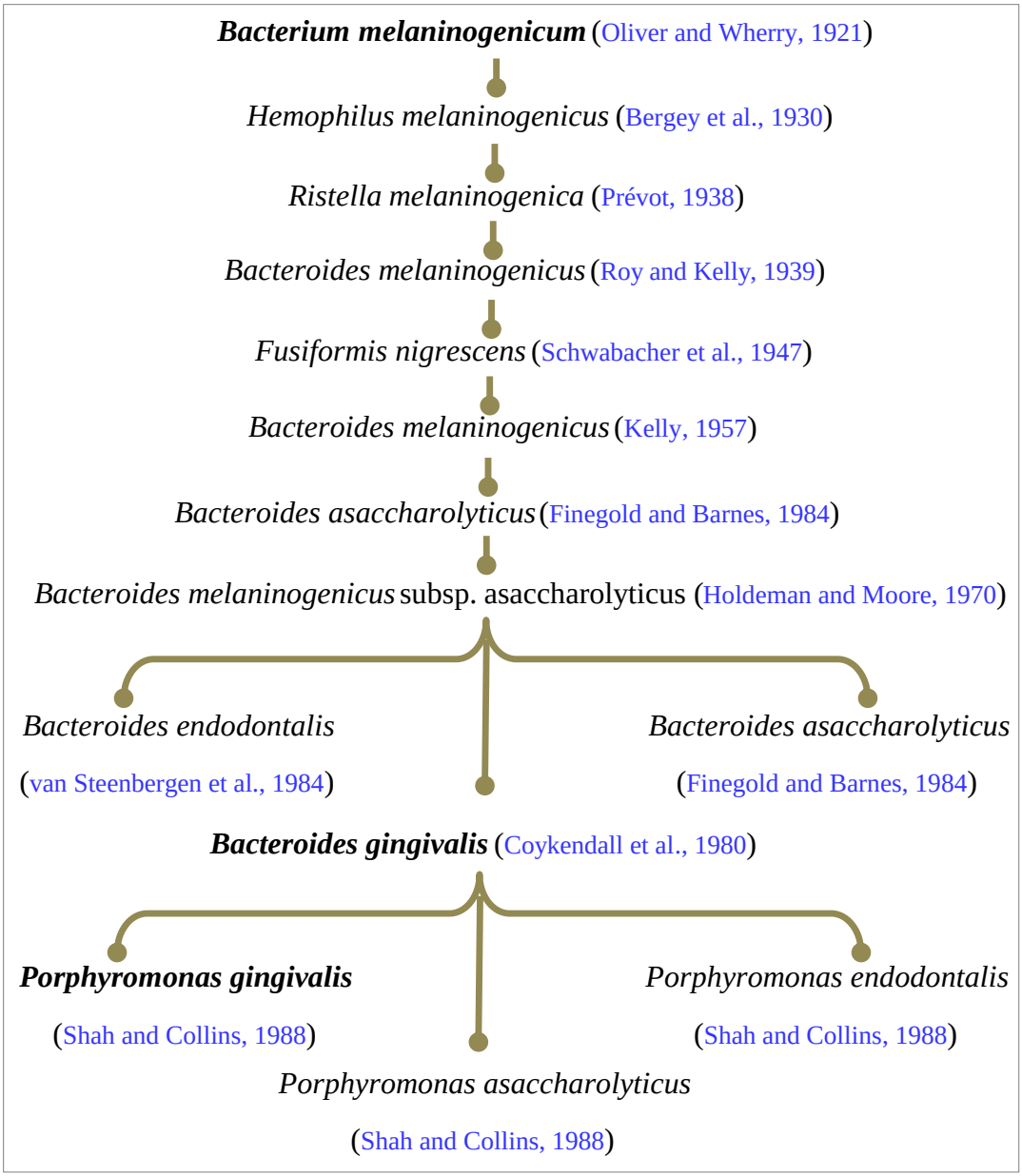

Fig.1.3 Evolution of *P. gingivalis* taxonomy

1.4 The virulence factors

P. gingivalis manifests numerous virulence factors associated with periodontal disease initiation, tissue destruction, and accumulation of dental plaque resulting in the infection. The

pathogenicity factors of this bacteria support the adhesion and colonization following by the maturation of other virulence.

1.4.1 Gingipains

Gingipains are the vital virulence factors secret by *P. gingivalis*. These proteases are responsible for several activities in the infection progress, including attachment and colonization, acquisition of nutrients, evasion of host defense, and tissue invasion and dissemination (Guo et al., 2010). Gingipains play a key role in the pathogenic functions of *P. gingivalis*, including fimbriae assembly and processing of extracellular proteins. Correspondingly gingipains completely digest several host proteins such as collagen, fibronectin, immunoglobulin G, and TNFα (Cutler et al.,1995) to provide peptides for *P. gingivalis* growth and metabolism. It also limits proteolysis, which leads to the dysregulation of host defensive inflammatory reactions and failure to eradicate *P. gingivalis*, as well as mediate nutrient acquisition through hemagglutination, hemolysis, and iron uptake from heme within sites of periodontal infection (Smalley et al., 2008). In addition, these proteins develop the attachment of *P. gingivalis* to host tissue cells and other bacteria, stimulate extracellular matrix protein degradation, increase vascular permeability, induce cleavage of host cellular receptors, and processing the fimbrillin subunit (Inomata et al., 2009, Lourbakos et al., 200; Tichy and Novak, 1998).

Gingipain family comprises three related cysteine proteases identify as arginine-specific gingipains (RgpA and RgpB) and Lys-specific gingipain (Kgp). The first two proteins are products of two related genes rgpA and rgpB, whereas the Lys-gingipain (Kgp) is encoded by the kgp gene (Fitzpatrick et al., 2009). The gingipains are accumulated on the surface of *P. gingivalis* from where subfractions are secreted into the extracellular fluid (Amano, 2003). On the bacterial

membrane surface, RgpA and Kgp form multifunctional complexes that engage in proteolysis, heme acquisition, platelet activation, red blood cell agglutination, hemolysis, and adhesion to the extracellular matrix. All gingipains, including RgpB, RgpA, and Kgp, have a large hemagglutinin/adhesion domain inserted between the protease and C-terminal domains. However, RgpB is distributed into the periplasm as a proprotein consisted of an N-terminal prodomain, a protease domain, and a C-terminal domain. During the translocation squarely outer membrane, the pro-gingipains subject to proteolytic process leads to cleavage of the N-terminal prodomain and a C-terminal domain. Nevertheless, the hemagglutinin domain in RgpA and Kgp is fragmented into subdomains bound to the protease domain via non-covalent interactions (Sztukowska et al., 2012).

1.4.2 Hemagglutinins

The adherence of *P. gingivalis* to host tissues and other bacterial pathogens is a vital step in periodontal disease initiation (Okuda et al., 1986). Fimbriae mediates adherence to other oral bacterial species and numerous host components such as hemoglobin, collagen, fibronectin, and periodontal cell surfaces (Amano et al., 2003; Cutler et al., 1995). Moreover, Hemagglutination activity is one of the major phenotypic characteristics that distinguish P. gingivalis from other oral and nonoral asaccharolytic black-pigmenting species (Holdman et al., 1986). Hemagglutinins are a large class of virulence factors that *P. gingivalis* produces (Han et al., 1996; Lee et al., 1996; Shi et al., 1999) to agglutinate erythrocytes of sixteen animal species, including human erythrocytes regardless of their blood type (Hanazawa et al., 1988). Furthermore, hemagglutinins associated with lipopolysaccharides and cell surface lipids, in hemagglutination and hemolysis activities along with other enzymes to bind erythrocytes and epithelial cells. Then indorse the colonization, maturation, and acquisition of hemin and iron from hemoglobin found in host erythrocytes to allow

the proteolytic gingipains releasing the heme moiety from the hemoglobin molecule through dual uptake system known as HmuY/HmuR (Lewis et al., 2006; Olczak et al., 2008; Okuda et al., 1986). *Porphyromonas gingivalis* possess more than eight hemagglutinins encoded by a group of five genes known commonly as the hag genes (hagA, hagB, hagC, hagD, and hagE). Subsequently, these genes are subdivided into two distinct families. The first consists of hagB and hagC that share 93% identity, whereas hagA, hagD, and hagE comprise the second family with more than 70% homology. HagA and hagB are major hemagglutinins of *P. gingivalis* (Bélanger et al., 2012; Song et al., 2005). HagA plays a vital role in co-aggregation of *P. gingivalis* with other microbial species such as *T. denticola* (Ito et al., 2010). Therefore, contributing to the ecology of the plaque biofilm. HagB accelerates the early infection process in experimental animal models (in vivo) (Lee et al., 1996). Additionally, they are necessary for the viability of *P. gingivalis* by assisting its adherence to oral epithelial cells (Lamont et al., 1995; Dorn et al., 2000) and biofilm formation (Connolly et al., 2015).

1.4.3 Fimbriae

In the outer surface membrane of *P. gingivalis,* several specific protrusions are produces called fimbriae. Fimbriae are responsible for mediation of several processes in inflammation events such as adherence and invasion of host cells (Nakagawa et al., 2002; Yilmaz et al., 2002), colonization within the oral environment (Maeda et al., 2004), auto-aggregation (Lin et al., 2006), and induction of the host immune response (Hajishengallis et al., 2009). Fimbriae are categorized into two groups: major fimbriae which are composed of the fimbrillin protein (FimA, a 41-kDa protein encoded by the fimA gene) (Dickinson et al., 1988) and minor fimbriae which are composed of the 67-kDa mfa1 protein encoded by the mfa1 gene (Hamada et al., 1996; Park et al., 2005). The

fimbrillin protein plays an essential role in the attachment to a variety of oral structures and subsequent invasion of the host cells. It has been shown to mediate adherence to human gingival fibroblasts and epithelial cells (Njoroge et al., 1997; Sugano et al., 2004; Weinberg et al., 1997) as well as to induce periodontal bone loss in rat models (Malek et al., 1994). Instead, the minor fimbrial protein, mfa1, has been shown to elicit an inflammatory response in a mouse model (Hiramine et al., 2003). Besides, the enabling of co-aggregation with other bacteria, such as *S. gordonii* (Lamont et al., 2002).

1.4.4 Lipopolysaccharides

Lipopolysaccharides (LPS) are components of the outer cell membrane of all gram-negative bacteria. They play a harmful role in chronic periodontitis (Park et al., 2010; Trent et al., 2006). It has been found that the LPS has influenced by multiple factors, such as its structure (Hajjar et al., 2002) and growth conditions (Lee and Baek, 2013) including temperature (Curtis et al., 2011) and the hemin level (Al-Qutub et al., 2006; Cutler et al., 1996). A high level of hemin in the environment results in a change of some lipids structure, resulting in immune suppression (Reife et al., 2006). Equally, low levels of hemin result in immune evasion. Accordingly, hemin concentration in the surrounding environments and the associated lipid structure might help protect other bacteria and support biofilm formation (Coats et al., 2009).

1.5 *P. gingivalis* strains

P. gingivalis has many strains classified as invasive or non-invasive strains based on the ability to cause the inflammation, at which they vary in their pathogenicity and ability to attack tissues and cells (Dorn et al., 2000; Lundberg et al., 2010; Dolgilevich et al., 2011; Olsen and Progulske-

Fox, 2015). Up to the present, about eight completed strains of nineteen *P. gingivalis* genome sequences have been published, including W83, ATCC 33277, TDC60, HG66, A7436, AJW4, 381, and A7A1-28. Besides eleven high-coverage draft sequences (JCVI SC001, F0185, F0566, F0568, F0569, F0570, SJD2, W4087, W50, Ando, and MP4-504).These strains were isolated from several sources using laboratory cultures through diversity in the degree of virulence, clinical samples from patients with different disease states, and an environmental strain isolated from a hospital bathroom sink drain (Tsute et al., 2017). The most aggressive strain has been isolated from a severe periodontal lesion at Tokyo Dental College in Japan named TDC60. This strain exhibited higher pathogenicity in causing abscesses in mice than strains W83 and ATCC 33277 and other strains (Watanabe et al., 2011). This strain is also found to be the most divergent sequence among all nineteen *P. gingivalis* genomes on the base of 16S rRNA gene sequences that are very similar and frequently have a single number of nucleotides mismatches between any two strains. Moreover, the TDC60 strain has several unique proteins compared with other strains based on the percentage of the unique proteins identified by Tsute et al. (2017). For its unique features and aggressiveness, *P. gingivalis* TDC60 was selected for investigation in this study.

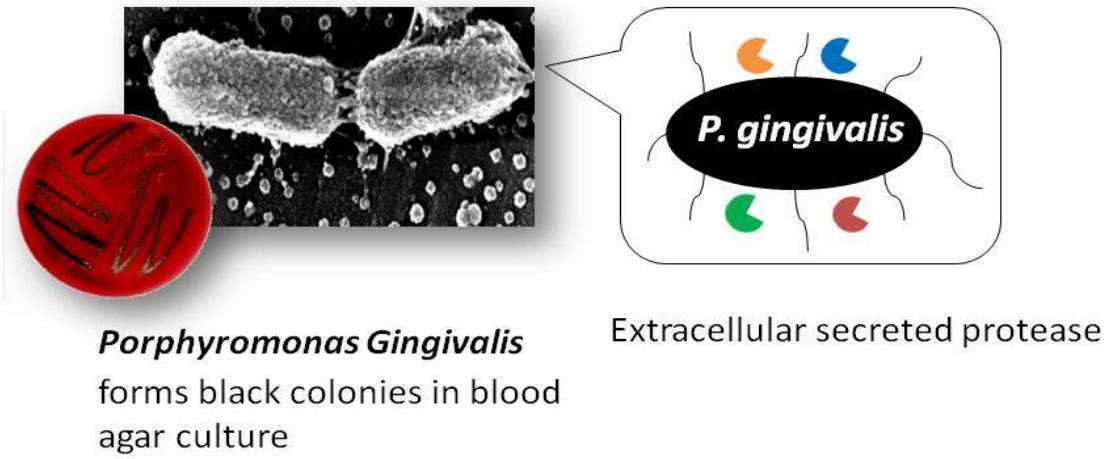

Fig.1.4 Cell morphology and growth of *Porphyromonas gingivalis*. The bacterium forms rod and black colonies growth anaerobically in blood agar culture. Several proteases secreted in the outer cell membrane, which are the virulence factors of this bacterium.

1.6 Treatment of periodontal diseases

The treatment of periodontal diseases depends on the stage and severity of the infection. There are differences between chronic and aggressive periodontitis. At the same time, the clinical appearance of both entities is similar, through periodontal loss of attachment, presence of local inflammation, loss of collagen, and then a loss of bone support. Thus, the periodontal treatment for both is similar and directed to achieving an adequate level of plaque control and reduction in pocket probing depth. Periodontal treatment can be performed in five steps, including mechanical therapy, antimicrobial therapy, host modulation therapy, tissue regeneration therapy, and behavioral and economic concerns (Chapple, 2009). The first phase of periodontal treatment involves mechanical debridement of supra and subgingival microbial plaque and calculus from teeth and surrounding structures. The removal of sub-gingival plaque from deep periodontal pockets is difficult. However, the re-colonization of these sites by pathogenic microbes is tackled by using antimicrobial agents and antiseptic substances. More novel approaches enhancing the

immune response might hold promise but are not in general use yet. This approach focus in the suppression of periodontal destruction (Chapple, 2009), vaccination against certain virulent microorganisms, and stimulation of T cells into the regulatory subtype (Choi and Seymour, 2010). Besides the use of certain pro-resolving lipid mediators of inflammation such as lipotoxin, resolvins, and protectines (Serhan, 2008; Van Dyke, 2008). Another approach adopted by some specialties is the replacement of lost soft and hard tissues (Chapple, 2009). Enamel matrix derivatives have been used in tissue regeneration therapy and act by promoting the process of periodontal regeneration (Bosshardt, 2008). Emdogain is one of the agents that has been shown, in vitro, to stimulate the proliferation of periodontal ligament cells (Gestrelius et al., 1997) and to increase the attachment of periodontal ligament fibroblasts to the diseased root surfaces (Davenport et al., 2003) with enhancement of matrix synthesis (Haase and Bartold, 2001). Therefore, it represents a possible alternative to open surgical debridement (Esposito et al., 2004, Venezia et al., 2004).

The Current strategies for developments of anti-pathogenicity agents work in the understanding of the mechanism of virulence factors processing and secretion, which recognized as beneficial targets for the therapeutic process. It is well known that *P. gingivalis* cell adhesion predominantly relies on the functions of hemagglutinins and gingipains. Therefore, using of antiadhesive compounds that influence the interaction between virulence factors and host proteins could hinder periodontitis in the early stages of the inflammation (Miyachi et al., 2007). The rising in disease incidence (particularly in developing countries) with increasing resistance by pathogenic bacteria to currently used antibiotics and chemotherapeutics, opportunistic infections in immune-compromised individuals, and financial considerations worldwide, specifically in developing countries. Consequently, the globe requires alternative prevention and treatment options and

products for oral diseases that are safe, low-priced, effective, and economically available (Badria and Zidan, 2004; Lachenmeier. 2008). The most recent strategy for the invention of a new therapeutic agent against *P. gingivalis* focuses on blocking the very early steps of the bacterial adhesion to the host cells through the inhibition of virulence factors. Over the past few years, plant-derived natural products, mainly polyphenols and polysaccharides (Alshafei et al., 2016), have been described as a putative adjunctive therapy that aims to reduce inflammation as well as interface with the adhesion agents of *P. gingivalis*. The naturally occurring products derived from medicinal plants have proven to be a rich source of biologically active compounds. Most of them have been the basis for the development of new lead chemicals for pharmaceutical applications. Sudan is the third-largest country in Africa with a diverse climate that contributed to its diverse and vibrant flora. Most of the Sudanese people in rural areas rely on traditional medicine to treat many infectious diseases. In this study, we select 38 plant organs from 25 plant species traditionally used in the Sudanese folkloric medicine to investigate their effect on *P. gingivalis* growth and virulent factors (Table 1.1).

1.7 Overview of selected medicinal plants and isolated biological compounds

Sudanese traditional medicine is characterized by a unique combination of Islamic, Arabic, and African cultures. Traditional medicines continue to be used for human illnesses treatment in many African countries and in various parts of the world. The useful components derived mainly from natural products such as herbs, plants, and animals. (Karar and Kuhnert, 2017). In Sudan, 90% of the population relies on traditional medicine (Elegami et al., 2002; Koko et al., 2000). Several plants have been used for oral disease treatment (El Ghazali et al., 2003; Khalid et al., 2012). Based on the ancient and traditional uses of plants and plant parts in Sudanese folk medicine, we have

selected 38 organ parts from 25 plant species (Table 1.1) to investigate the potential inhibitory activities of the plant aqueous extracts prepared following the traditional way. Most of the selected Sudanese plant aqueous extracts revealed an influence on either *P. gingivalis* proliferation and/or its virulence factors. However, among the plants chosen, *Origanum vulgare* leaves extract was non-specific on the inhibition activity. It showed a reliable suppression against *P. gingivalis* proliferation and virulence factors. In contrast, the aqueous extract of *Monechma ciliatum* had a potent inhibitory activity on *P. gingivalis* exo-hemaglutinins compared to the other tested plants. *Solenostemma argel* had a potent activity on the impedance of *P. gingivalis* growth and proliferation. The three plants extract were selected for isolation, purification, and identification of the inhibitory compounds.

1.7.1 *Origanum vulgare*

Origanum vulgare is known as oregano (Fig.1.5), which use as a spice all over the world (Lawrence, 1984). It is an annual herb widely distributed in Eurasia, North Africa, and North America (Ayoub et al., 1984). This plant belongs to the family of Lamiaceae. The family is divided into several subspecies as hirtum, vulgare, viridulum, glandulosum, gracile vulgare, virens and subspecies viride (Letswaart, 1980). It has light green to greenish-brown leaves, about 3 cm long with crenate to crenate-serrate margins and glandular punctate. It has spike-like clusters flowers which are bracts – broadly ovate, 2.5-3 × 2 mm with prominent hairs on the outer surface (Bagchi and Srivastava, 2003). Oregano has a robust camphoraceous odor and a warm, pungent, bitter, camphoraceous taste. The plant is collected when in flower and dried in the shade at less than 35 °C (Bagchi and Srivastava, 2003). It has been used traditionally to treat several illnesses because of its carminative, diaphoretic, expectorant, stimulant, stomachic, and tonic effects.

Furthermore, it is used against colic, coughs, headaches, nervousness, toothaches, and irregular menstrual cycles (Kintzios, 2002). *O. vulgare* oil has been used as an antimicrobial against bacteria, fungi and yeast species (Sahin et al., 2004). O. vulgare oil's chemical composition mainly contains the mono and sesquiterpenes (Skoula and Harborne, 2002; Stahl-Biskup, 2002), carvacrol, thymol, p-cymene, α and β-pinene, myrcene, limonene, linalool, and estragol (Sivropoulou et al., 1996; Milos et al., 2000, Aligiannis et al., 2001; Azizi et al., 2009). The phenolic components in the essential oil of oregano, such as carvacrol and thymol, have a strong antifungal power (Curtis et al., 1996). Adam et al. (1998) reported that carvacrol and thymol showed higher antifungal activities against human pathogens than p-cymene and γ-terpinene. Additionally, the aqueous and methanolic extract of *O. vulgare* has antioxidant activity and is applied in human health. Cervato et al. (2000) prove that the antioxidant activities of oregano leaf extract (both aqueous and methanolic extracts) can inhibit all phases of lipid peroxidative process. In this study, a wide range of inhibitor has been isolated from the aqueous extract of *O. vulgare* leaves against *P. gingivalis* and its pathogenicity factors. The inhibitor was identified as a hydrolyzable tannin-like compound.

Table 1.1: List of selected Sudanese plants and their traditional medical uses

Scientific name	Family	Local name	Traditional uses
Tamarindus indica L.	Fabaceae	Aradeb	The fruits are used to treat constipation, malaria, jaundice, and toothache.
Salvadora persica L.	Salvadoraceae	Arak	Mouth wash and to facilitate digestion. The fruits are used to treat hypertension, stomach pain, and wounds, and the leaf-fibers are used to treat eye infections.
Erythrina abyssinica L.	Fabaceae	Habalaroos	The bark is used to treat snake bites, malaria, sexually transmittable diseases such as syphilis and gonorrhea, amebiasis, cough, liver inflammation, stomachache, colic, and measles.
Origanum vulgare L.	Lamiaceae	Bardagosh	It is used as an antibacterial agent to reduce spoilage of fatty acids in food products such as meat and digestion aid.
Ambrosia maritima L.	Asteraceae	Dimsisa	The herbs are used as an anti-malaria treatment, to treat urinary tract infections, and to eliminate kidney stones. The leaves are used as an anti-diabetic and anti-hypertensive agent.
Acacia nilotica (L.) Willd. ex Del	Fabaceae	Garad	The fruits are burned, and the fumes are inhaled to treat colds and pharyngitis. Fruit macerates are used as an antiseptic.

Guiera senegalensis J.F.Gmel	Combretaceae	Gubesh	The leaves are used as an anti-hypertensive and anti-diabetic agent.
Grewia tenax (Forssk.) Fiori	Malvaceae	Gudeim	The fruits are used to treat malaria and iron deficiency anemia.
Solenostemma argel (Del.) Hayne	Ascepiadaceae	Harjal	The leaves are used as an antispasmodic, carminative, anti-diabetic agent and to treat malaria.
Trigonella foenum-graecum L.	Fabaceae	Hilba	The seeds are used as an antidiarrheal, antispasmodic, anti-amoeba, dysentery, and anti-diabetic agent. The seeds are also used as a food additive to increase milk secretion in lactating mothers and facilitate expulsion of the placenta.
Balanitesa egyptiaca (L.) Del.	Balanitaceae	Hijlij	Purgative, wound healing, anti-rheumatic, anti-diabetic, anti-anthelmintic. Fruits are used to treat dysentery and constipation. Seed oil is used to treat tumors and wounds.
Glycyrrhiza glabra L.	Fabaceae	Irgsoos	It use as anti-allergy, to treat HIV-1, Japanese encephalitis, yellow fever. Acts as an anti-carcinogenesis, anti-diabetic and anti-inflammatory agent. In the past, it was used for its anti-viral activities and its anti-ulcer, laxative, and antipyretic properties.

Ceratonia siliqua L.	Fabaceae	Kharoub	To relieve pain and to prevent and treat osteoclasis and osteoporosis.
Acacia oerfota (Forssk.) Schweinf. A. nubica Benth	Fabaceae	Laoot	It used as anti-rheumatic, to treat snake bites, swelling, and scorpion bites.
Khaya senegalensis (Desv) A. Juss	Meliaceae	Mahogani	The stem bark is used to treat malaria, hepatic inflammation, and enteriogasteritis. The leaves are used to treat skin diseases and trachoma.
Cymbopogon schoenanthus (L.) Spreng. Ssp. Proximus	Poaceae	Mahareb	The leaves are used to treat gout, renal colic, helminthiasis, and inflammation of the prostate.
Monechma ciliatum	Acanthaceae	Mahlab aswad	Effective laxative. It believed to ease general body pain, liver, bowel troubles (diarrhea), and sterility in women.
Salvia officinalis (Sage)	Lamiaceae	Merameya	Traditionally used to treat many disorders such as seizure, ulcers, gout, rheumatism, inflammation, dizziness, tremor, paralysis, diarrhea, and hyperglycemia
Azadira chtaindica	Meliaceae	Neem	The leaves are used to treat skin diseases, helminthiasis, and malaria.

Lepidium virginicum L.	Brassicaceae	Rashad	Used to treat madura foot, as an anti-asthmatic, and for treating diarrhea.
Cyperus rotundus L.	Cyperaceae	Sedaa	Diuretic, carminative, emmenagogue, anthelminthic, stomachic, stimulant, analgesic, hypotensive, anti-inflammatory, anti-dysenteric, and anti-rheumatic agent.
Leptadenia arborea (Forssk.) Schweinf	Asclepiadaceae	Shaaloub	Taken as a stem decoction to facilitate expulsion of the placenta after birth and is applied to stop bleeding after circumcision. It is also considered as a tonic and to treat diabetes. In Comoros, a stem decoction containing several other plant species is taken to treat fever and colic.
Ziziphus spina-christi (L.) Desf.	Rhamnaceae	Sidr	Antispasmodic used to treat gonorrhea and as anti-purgative.
Acacia seyal var. seyal Del.	Mimosaceae	Taleh	Anti-rheumatic, mouth wash, and the stem fumigant is used to treat rheumatic pain.
Capparis deciduas (Frossk) Edgew.	Capparidaceae	Tundub	Used to treat jaundice, swelling, headache, and as an anti-rheumatic agent.

1.7.2 Tannins

Tannins are extensively distributed in plants and food of plant origin, including fruits, legume seeds, and cereal grains besides different beverages (tea, cocoa, and cider). Tannins are divided into two groups. Hydrolysable tannins are esters of phenolic acids such as either gallic acid in gallotannins or other phenolic acids derived from the oxidation of galloyl residues in ellagitannins and a polyol with glucose (Clifford and Scalbert, 2000). The other tannins are proanthocyanidins, which are common in our food and produce anthocyanidins upon heating in acidic media. They are polymers made of elementary flavan-3-ol units. Structurally, tannins possess a huge number of phenolic groups with several aromatic rings per 1000 units of relative molecular mass (Haslam, 1998). That explains their high molecular weight, which makes the tannins and similar phenolic polymers found in processed food products such as red-black tea different in structure and properties from the low-molecular-weight phenolic acids and monomeric flavonoids. The phenolic polymers, formed by enzymatic and/or chemical transformation of simple flavanols, proanthocyanidins, and other phenolic compounds known as tannin-like compounds (Lewis et al., 1990). Tannins are Water-soluble phenolic compounds with molecular weights between 500 and 3000 *m/z*. These compounds have unique features, reactions, and unique properties, such as the ability to precipitate several proteins like gelatin and alkaloids (Haslam, 1989). Tannins precipitate salivary proteins in the oral cavity, which gives the feeling of an astringent known in tannin-rich food. This property is essential to explain their role in plant protection against pathogens (Scalbert, 1991) or to deter herbivores from feeding on tannin-rich plants (Feeny, 1970). Tannins have valuable benefits for health. Numerous studies have reported the anticarcinogenic, antioxidative, antimicrobial activities of tannins. As well as tannins inhibited the growth of many fungi, yeasts, bacteria, and viruses. (Chung et al., 2010).

1.7.3 *Monechma ciliatum*

Monechma ciliatum known as Black mahlab, it belongs to the Family Acanthaceae. This plant is an annual hispid scabrous or almost glabrous herb, 30-65 cm high, and woody below. It has a linear or narrowly linear-lanceolate leaves up to 10 cm long, and 1.25 cm broad (Massey, 1929). *M. ciliatum* flowers are cream-white with purple and orange strakes. Seeds are dark brown to black, with kidney shape (Fig.1.5), pods with a tuft of rigid thick hairs at the hilum, and similar tuft at the other end (Andrews, 1952). *M. ciliatum* is a native African plant in tropical areas and sometimes India. Distributed from Senegal to Cameroon and eastward to the central and southern provinces of the Sudan and south through East Africa, Zambia (Wickens, 1976) and Nigeria (Uguru, 1998). In Sudan, *M. ciliatum* grows widely in Kassala State (Galabat), Southern Blue Nile State (Jongol post), and Kordofan State (Elobeid). This plant has a crucial role in cosmetic and nutritional products in western Sudan. The oil is used to manufacture traditional fragrance and lotion. The plant has medicinal importance as a laxative medicine to remedy stomach troubles and used to control scale dandruff, which forms on the skin in case of oily hair. In Botswana, it believed that its useful for body pain, liver and bowel trouble (diarrhea), and sterility in women (Hedberg and Stengard, 1989). The seeds of this plant are rich with hydrocarbons and fatty acids such as palmitic, stearic, linoleic in addition to tocopherols and protein besides elements (K, Ca, Mg, Al, Pb, Ni, Mn, Cu, Cr, Co, and Fe) (Mariod et al., 2009) Furthermore, flavonoids, tannins, triterpens, and anthraquinones (Oshi, et al., 2010). In our study, we isolated and identified coumarin, oleic acid and its esters with inhibitory activity on *P. gingivalis* exo-hemagglutinins.

1.7.4 Fatty acids and its esters

The carboxylic acids resulted from the hydrolysis of triglycerides are known as fatty acids. Nutritional triglycerides represent the primary form of stored lipids at the small intestine of mammalian organisms. In addition to their biochemical role as a storage form of energy, fatty acids act as the bedrock of glycolipids and phospholipids, molecules that play essential structural and physiologic functions in the organism. Various fatty acids have been isolated from animals, plants, and microbial origins. Fatty acids might be formed in a single chain or branched chain. However, a few branched fatty acids have been isolated from plant and animal sources. The hydrocarbon chain may be a long or short chain of carbons with different saturation degree, unsaturated chain comprising of one or more double bonds, or saturated with no double bonds, by a terminal carboxyl group (Huang et al., 2010). The long-chain fatty acids are found to have antibacterial and anti-inflammatory activities by serving as precursors to produce eicosapentaenoic acid and docosahexaenoic acid, which can reduce inflammation (Raffaelli et al., 2008; Kesavalu et al., 2007). A study conducted by Huang et al. (2010) revealed three major polyunsaturated fatty acids (eicosapentaenoic acid, docosahexaenoic acid, α-linolenic acid) and their ester derivatives exhibited strong antimicrobial activity against various oral pathogens (Huang et al., 2010). A relationship has been found between the carbon chain length of n-6, n-7, and n-9 fatty acids and antimicrobial activities against oral microorganisms (Huang and Ebersole, 2010).

1.7.5 Coumarin

Coumarins are secondary metabolites from plants, bacteria, and fungi (Iranshahi et al., 2009). They were initially found in tonka bean (*Dipteryx odorata* Wild) and are reported in about 150 different species from 30 different families, of them, Rutaceae, Umbelliferae, Clusiaceae,

Guttiferae, Caprifoliaceae, Oleaceae, Nyctaginaceae, and Apiaceae. Chemically, coumarins (known as 2H-1-benzopyran-2-one) consist of a large class of phenolic substances found in plants and are made of fused benzene and α-pyrone rings (Aoyama et al., 1992). This compound displays anti-inflammatory properties and is used in the treatment of edema (Piller, 1975). It also exhibited anti-inflammatory activity in rat colitis induced by trinitrobenzene sulfonic acid (Witaicenis et al., 2010; Kwon et al., 2011). Additionally, coumarins have anticoagulant activity (Aoyama et al., 1992) as well as low antibacterial activity, but compounds having long chain hydrocarbon substitutions such as ammoresinol and ostruthin show activity against a wide spectrum of gram-positive bacteria such as *Bacillus megaterium*, *Micrococcus luteus*, *Micrococcus lysodeikticus*, and *Staphylococcus aureus* (Hod´ak et al., 1976). Another coumarin compound anthogenol from green fruits of *Aegle marmelos* (Wiebe et al., 2000) shows activity against *Enterococcus*. Imperatorin, a furanocoumarin isolated from *Angelica dahurica* and *Angelica archangelica* (Umbelliferae) (Baek et al., 2000), shows activity against *Shigella dysenteriae* (Raja et al., 2011). In addition, inophyllums and calanolides represent novel HIV inhibitory coumarin derivatives (Luo et al., 2011). Coumarins are had many pharmacological properties that lead to further study in their features.

1.7.6 *Solenostemma argel*

Solennostemma argel (Fig.1.5), which locally known as harjel, is a desert plant indigenous to Africa and belongs to the family Apocynaceae and subfamily Asclepiadaceae (Elkamali and Khalid, 1996). It is a herbaceous perennial plant that grows up to 60-100 cm tall, with several vigorous stems. It has oval, leathery leaves covered with fine hairs. The flowers are many with white petals and a strong aroma. The fruits about 5 cm pods long and 1.5-2 cm wide, green with

violet lines, contain pubescent seeds (Elkamali, 2001). This plant found in many countries and its leaves extract one of the popular drinks and traditionally used as medicine in African countries (Sudan, Libya, Chad, Egypt, and Algeria), Saudi Arabia, and Palestine (Elkamali and Khalid 1996; Ahmed, 2004; Shayoub et al., 2013). In Sudan, *S. argel* initially found in the northern region (Orange, 1982) and widely spread in the places between Dongola and Barber, particularly around Abu Hamad area (Elkamali and Khalid, 1996). The folkloric uses of *S. argel* leaves as an anti-inflammatory, anti-spasmodic, anti-rheumatic agent, carminative, and as an anti-diabetic (Shayoub et al., 2013; Kamel et al., 1982; Hassan et al., 2001; Idris, 2011). Moreover, Hanafi et al. (2010) and Plaza et al. (2005) reported anticancer activity from seeds extract of *S. argel.* The present study resulted in the isolation of pregnane glycoside with a very potent inhibitory activity on P. gingivalis proliferation as well as gingipains and hemagglutinins inactivation.

Fig.1.5 The selected Sudanese medicinal plants

1.7.7 Pregnane glycosides

Pregnane glycosides are a class of secondary metabolites compounds widely distributed in the plant kingdom. In traditional remedies, medicinal plants are generally used due to their secondary metabolites, which frequently found in glycosylated forms, such as steroidal, terpenoidal, cardiac, and pregnane glycosides (Williams et al., 1989). Pregnane glycosides are polar compounds that form esters of a linear oligosaccharide chain with steroidal compounds through acetal linkage (Panda et al., 2006). Recently, pregnane glycosides isolated from many plant organs and recognized to have a pharmaceutical property with a good impact on health such as a pregnane glycosides isolated from *Cynanchum atratum* roots with inhibitory activity against acetylcholinesterase, which associated with Alzheimer disease (Lee et al., 2003). Oshima et al. (1987), isolated pregnane glycosides from *Periploca sepium* roots, exhibited significant anti-complimentary activity. A study conducted by Liu et al. (2003) revealed that glycosides might either stimulate bone formation or have potential activity against osteoporosis. Moreover, anticancer activity was detected by three pregnane glycosides isolated from *Dioscorea collettii* rhizomes which inactive six cultured human tumor cell lines in the colon, prostate, and breast (Hu et al., 1999).

1.8 Aims of the study

The thesis work was performed to screen the potency of a group of Sudanese medicinal plants traditionally used in the Sudanese folkloric medicine against *P. gingivalis* proliferation and virulence factors.

The research was conducted with the following specific aims:

- Investigation of the inhibitory activity of 38 plant organs against *P. gingivalis* growth and its virulence factors.
- Isolation, purification, and structure elucidation of the biologically active constituents impeded *P. gingivalis* proliferation and its pathogenicity factors, including hemagglutinins and gingipains in the outer cell membrane.
- Understanding the structure-function relationship of the long-chain fatty acids on the inhibition and secretion of *P. gingivalis* exo-hemagglutinins.

CHAPTER TWO

Non-specific inhibitor from *Origanum vulgare* leaves restrains *Porphyromonas gingivalis* growth and virulence factors

2.1 Introduction

Oral diseases are important and common chronic ailments (Kadowaki et al., 2004; Peres et al., 2019). Of them, periodontal disease (periodontitis) is caused by bacterial inflammation of the tissues supporting the teeth and is a major oral health problem. Periodontal disease causes different symptoms and can result in the loss of teeth. Additionally, recent studies have demonstrated a strong association between periodontal disease and serious systemic diseases such as diabetes, atherosclerosis, stroke and coronary heart diseases (DeStefano et al., 1993; Beck et al., 1999; Beck et al., 2017).

Many different bacteria exist simultaneously in the oral cavities of patients with periodontal disease. In particular, *Porphyromonas gingivalis* is a gram-negative, asaccharolytic, obligate anaerobic rod bacterium and appears to be the prime etiological agent in the pathogenesis and progression of the inflammatory events underlying periodontal disease (Gron et al., 1997; Kan et al., 2019). *P. gingivalis* requires exogenous amino acids, porphyrin, and iron for growth and virulence through production of several virulence factors such as hemagglutinins and gingipains. Hemagglutinins attach to the host tissues and lyse erythrocytes to uptake Fe ions as an essential nutrient; peptidases degrade connective-tissue proteins into small peptides and amino acids for use in growth and metabolism. In addition, gingipains are cysteine proteinases in *P. gingivalis* that are believed to be major virulence factors (Snipas et al., 2001; Lamont et al., 2018). Compounds that

suppress the adhesion of *P. gingivalis* to teeth by suppression of these virulence factors represent a new strategy for preventing periodontitis (Cutler et al., 1995; Kan et al., 2019).

Pathogenic bacteria are increasingly resistant to currently used antibiotics and chemotherapeutic medications and thus medicinal plants are being investigated for alternative treatment options for oral diseases. The use of medicinal plants is generally safe, and they are abundant, inexpensive, and cost-effective, particularly in developing countries. Medicinal plants are a treasure trove of biologically active compounds, many of which have acted as lead compounds for the development of pharmaceuticals (Cheesman et al., 2017).

In Sudan, people have been using plant remedies to treat illnesses since ancient times. Many studies have verified the utility of Sudanese medicinal plants for treating various diseases, including bacterial and fungal infections. However, little has been reported to date regarding the inhibitory activities of Sudanese plants on the growth of *P. gingivalis* (Mohieldin et al., 2017). To the best of our knowledge, the inhibition activities of Sudanese medicinal plants against hemagglutination, hemolysis, and gingipain has not been investigated. Furthermore, several researchers have hypothesized that inhibition of gingipains is independent of cell growth suppression (Niehues et al., 2010; Messing et al., 2014; Feghali et al., 2012; Kushiyama et al., 2009). Therefore, in this study, we focused on blocking the primary phases of bacterial adhesion to the host by preventing hemolysis, specifically through the inhibition of hemagglutinins and proteases that provide nutrients to *P. gingivalis* for their growth and survival.The inhibitory activities of the aqueous extracts of 38 plants were evaluated against hemagglutinins, hemolysis, gingipain and bacterial growth. Among them, *Origanum vulgare* suppressed all virulence factors. Following phytochemical analysis, the inhibitory activity of isolated and related bioactive compounds was investigated.

2.2 Materials and methods

2.2.1 Preparation of plant aqueous extracts

Plant samples were collected from different areas in the state of Khartoum in Sudan. The voucher specimens were prepared and identified at the faculty of agriculture and the faculty of forest, university of Khartoum, Khartoum, Sudan. The list of selected plants and their folkloric uses are presented in Table 1.1. Cleaned and powdered plant materials were extracted three times with distilled water (ratio of 1 g sample to 10 mL water) for 30 min at room temperature. The supernatants were collected by centrifugation, and then filtered and lyophilized. The yields of crude extracts are listed in Table 2.1 The plant extracts were stored at −20°C and used for bioassays.

2.2.2 Cultivation of *P. gingivalis*

P. gingivalis strain TDC60 (Watanabe et al., 2011) was provided by the RIKEN BRC Bioresource Center, Microbial Materials Development Office (JCM), through the National Bio-Resource Project of MEXT, Japan. The strain was grown in tryptic soy blood agar medium Agar Base EH; Difco^{TM}, (Becton, Dickiton and Company, France), supplemented with hemin (5 mg.mL^{-1}), menadione (1 mg.mL^{-1}) and horse blood at 37°C under anaerobic conditions for 4–5 days (Gao et al., 2010; Rangarajan et al., 2017). The bacterial cells were streaked on the agar medium and grew for 4 days at 37°C. Cells were collected and suspended in 1 mL of 20 mM Tris-HCl buffer (pH 8.0) per plate. Subsequently, the supernatant was ultrasonically disrupted for 3 min with Pulse ON 3 sec, Pulse off 20 sec, 70 Ampl using a micro ultrasonic homogenizer QSONICA Q 125 (Wako Pure Chemical Industries, Ltd.). Sonicated cells were centrifuged at 10000 ×g for 15 min. The supernatant was designed as (Pg-A.sup) and the remaining precipitate was suspended with the same amount of Tris-HCl buffer as the supernatant as and named as (Pg-A.pre).

For the samples prepared from the liquid medium, a small amount of *P. gingivalis* from agar medium inoculated into 5 mL Gifu Anaerobic Medium (GAM broth medium) (Umemoto et al., 1996), and incubated anaerobically at 37°C for 2 to 3 days until the bacteria reached late stationary phase of bacteria growth as evaluated spectrophotometrically at 600 nm. The culture was centrifuged at 8000 ×g for 20 min at 4°C. harvested cells by centrifugation and the culture supernatant (Pg-sup). In this study, the sample (Pg-sup) was used as the source of *P. gingivalis* virulence factors in the outer cell membrane. All the samples were stored at -20°C for further investigation.

2.2.3 Hemagglutination and hemolytic inhibition assays

Erythrocyte aggregation (hemagglutination) and hemolytic assays were performed as described previously (Saiki and Konishi, 2007) with minor modifications. Horse defibrillated blood (Cosmo Bio Co., Ltd., Tokyo, Japan) was washed three times using 1× phosphate buffer saline (PBS), pH 7.4 (8g of NaCl, 0.2g of KCl, 1.44g of Na2HPO4, and 0.24g of KH2PO4 in 1 L of distilled water). The mixture was centrifuged (2000 ×g, 5 min, 4°C) and the supernatant was carefully removed. The washed erythrocytes were diluted to 5% (V/V) with PBS and stored at 4° C and then used within 6.0 h for the hemolysis and Hemaglutination assays.

For the hemagglutination test, 80 µL PBS, 10 µL Pg-sup, and 10 µL plant extract were mixed in a 96-well, round-bottom microtiter plate and shaken well. After incubation for 10 min at room temperature, 100 µL of the 5% washed horse erythrocyte suspension was added. Hemagglutination was evaluated visually after incubation at room temperature for 2 h.

The hemolysis of erythrocytes was observed visually. Briefly, 80 µL PBS, 10 µL Pg-sup, and 10 µL plant extract were mixed in a 96-well flat-bottom microtiter plate and shaken well. After

incubation for 10 min at room temperature, 100 µL of the 5% washed horse erythrocyte suspension was added. The plate was incubated at 37°C for 2 h and centrifuged at 2000 ×g for 5 min, after which 100 µL supernatant was transferred to a clean plate and liberated hemoglobin was observed. In both hemagglutination and hemolysis assays, PBS was used instead of Pg-sup and plant extract as the negative control. The minimum inhibitory concentration was calculated from the final concentration of the lowest dilution exhibiting complete inhibitory activity.

2.2.4 Protease inhibition

The protease inhibitory activity of plant extracts was determined as described previously (Kariu et al., 2017). Compounds exhibiting activity inhibitory to gingipains were identified using *t*-butyloxycarbonyl-L-valyl-L-prolyl-L-arginine-4-methylcoumaryl-7-amide (Boc-VPR-MCA) as a protease substrate. Ten microliters of Pg-sup and 10 µL of plant extracts were added into the mixture containing 40 µL of 0.5 M Tris-HCl buffer (pH 7.5) and 30 µL of distilled water contain 5 m*M* of L-cystein. After 5 min preincubation at 37°C, 10 µL of 500 µ*M* Boc-VPR-MCA was added to the mixture. The release of amino methyl-coumarin was measured with an excitation at 380 nm and emission at 440 nm using a fluorescence spectrophotometer (Infinite M 200 Pro, TECAN, Männedorf, Switzerland, Japan). The linear increase of amino methyl-coumarin release was recorded for 5 min in the presence or absence of aqueous crude extracts at different concentrations and the half maximal inhibitory concentration (IC_{50}) was calculated from the obtained dose-response curve.

2.2.5 *P. gingivalis* growth inhibition

The influence of the plants' aqueous extracts on *P. gingivalis* growth was investigated by measuring the turbidity of bacterial suspension in a 96-well microplate. Ten microliters of plant extracts (20 $mg.mL^{-1}$) was added to 200 µL of *P. gingivalis* suspension standardized at 2 $x10^7$ CFU (Kariu et al., 2017). The plates were incubated anaerobically at 37°C for 60 h. The turbidity was then measured at 600 nm via a microtiter plate reader (Model 680, Bio-Rad Laboratories Inc., Hercules, CA, USA).

2.2.6 Isolation of biologically active compounds

The extraction of the inhibitory active constituents from *O. vulagare*leaves (Fig.2.1) was performed according to the method described by Mendonça-Filho (2006). About 40 g of dried and powdered leaves was extracted three times with 400 mL distilled water for 30 min at room temperature. The soluble extracted compounds were collected by filtration followed by centrifugation. The extract was portioned between distilled water and ethyl acetate. The water phase was fractionated using methanol precipitation (30% methanol for 20 min). The supernatant of methanol which had the highest activity for inhibition of hemagglutination, hemolysis and the protease, was fractionated using an ODS column (Cosmosil 75C18-OPN; Nacalai Tesque, Kyoto, Japan) eluted first with MeOH in H_2O (0–80%, 20% increments) and then absolute methanol. The 40% methanol fraction exhibited the highest activity and was subjected to silica gel column chromatography (Daisogel IR-60-63/210; Daiso, Osaka, Japan) by eluting first with acetone/hexane (0–100%), then 100% methanol, and finally distilled water. The compounds in the water eluate were fractionated using a C18 Sep Pak column cartridge (Sep-Pak C18 20 cc Vac cartridge, 5 g sorbent per cartridge, 55–105 µm particle size; Waters, USA). The column was

conditioned with MeOH and equilibrated with distilled water, then eluted with H_2O/MeOH (0%, 20%, 40%, 60%, 80%, and 100% methanol) to give six subfractions. The 60% methanol fraction exhibited the greatest inhibitory activity and was injected into a high-performance liquid chromatograph (HPLC) equipped with an ODS column (Cosmosil 5C18-AR-II, 4.6 ID × 150 mm; Nacalai Tesque) and eluted using the following analytical conditions: gradient elution program, 5–80% B/(A + B) within 60 min; solvents, milli-Q water (A) and acetonitrile (B); flow rate, 0.8 $mL.min^{-1}$; column temperature, 40°C; detection, UV 280 nm.

2.2.7 Characterization of polymer-like compounds

The Prussian blue test was used to detect phenolic compounds as previously described (Price and Butler, 1977). Thin layer chromatography (TLC) on silica gel 60 F254 aluminum sheets (Merck, Darmstadt, Germany) was conducted to identify the types of large polyphenolic compounds isolated using 2-methyl-2propanol *tert*-butanol–acetic acid–water (3:1:1, v/v) as the mobile phase (Harborne, 1989). The compounds were detected using UV at 254 nm. Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) was used to analyze the tannin (I. Tannin) isolated from *O. vulagare* and standard tannic acid obtained from Sigma-Aldrich (St. Louis, MO) using 2,5-dihydroxybenzoic acid as the MALDI matrix. I. Tannin (10 μL) (1.0 $mg.mL^{-1}$) and tannic acid (1.0 $mg.mL^{-1}$) were separately mixed with the matrix solution, and 1.0 μL was dropped onto the target plate and dried. The samples were analyzed using an Auto-flex TOF instrument (Bruker Daltonics Inc., Billerica, MA).

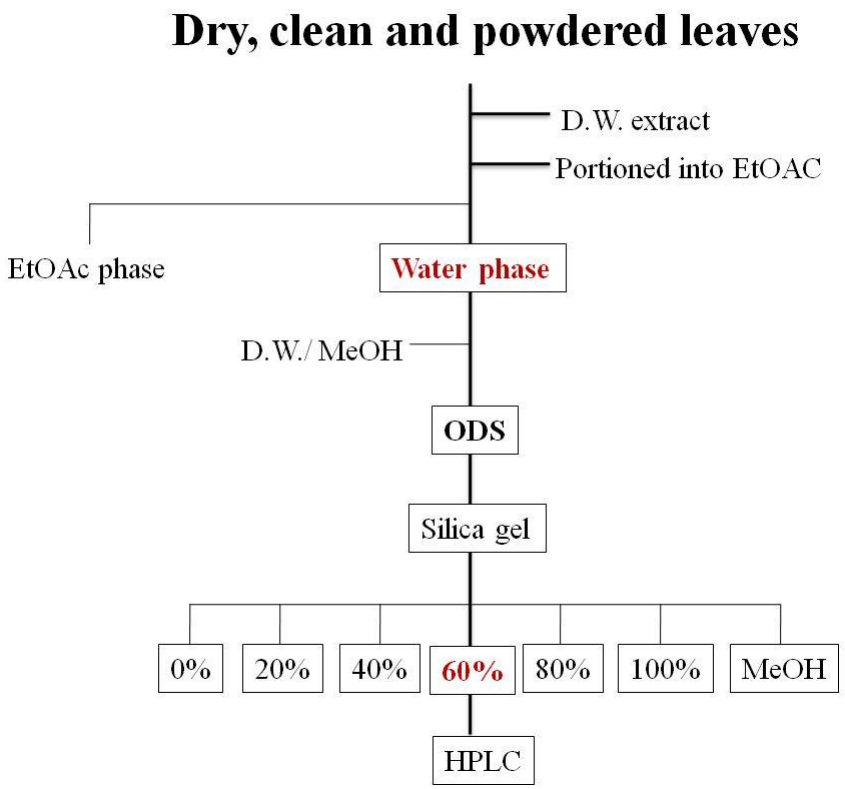

Fig.2.1 Scheme for isolating inhibitory compounds from *O. vulagare*

2.3 Structural study

Cysteamine degradation was assessed as previously reported (Zhang and Lin, 2008). I. Tannin (50 µL, 4.0 mg.mL^{-1}) was dissolved in 50 µL of 3.3% hydrochloric acid in methanol, added to 100 µL of cysteamine hydrochloride in methanol (50 mg.mL^{-1}), and heated at 40°C for 30 min. After cooling to room temperature, the solution was filtered (Φ13, 0.22 µm, Merck) and 10 µL of the sample solution was analyzed by HPLC. The remaining I. Tannin sample was partially acid hydrolyzed using a method described previously (Tanaka et al., 1986) by heating at 100°C in 5% sulfuric acid for 10 h. The solution was filtered and a 10µL aliquot was analyzed by HPLC using the following analysis conditions: elution solvents, 0.1% formic acid (A) and acetonitrile contain

0.1% formic acid (B); gradient elution (3–60%) over 10 min; column, Cosmosil C18, (ø 1.7 μm × 50 mm); temperature, 40°C.

2.3 Results

2.3.1 Inhibitory activities of plant extracts against virulence factors in *P. gingivalis*

In this study we investigated 38 organ parts from 25 plant species belonging to 17 families. Our selection was based on the historical and traditional uses of plants and plant parts in Sudanese folk medicine. The potential inhibitory activities of aqueous extracts of these samples against *P. gingivalis* activities associated with periodontal disease were evaluated in a 96 well plate. The minimum inhibitory concentration (MIC) of each sample as determined by hemagglutination and hemolysis assays, and the IC_{50} value for protease inhibition, are summarized in Table 2.1. Except for the aqueous extract of *Grewia tenax* and *Ziziphus spina-christi* fruits, all tested samples exhibited inhibitory activities toward at least one tested parameter. Among the plant extracts, 23 showed inhibitory activities against hemagglutination with MIC values of 0.06–4.0 $mg.mL^{-1}$, 32 inhibited hemolysis with MIC values of 0.03–4.0 $mg.mL^{-1}$ and 16 plant extracts inhibited protease activity with IC_{50} values of 0.11–1.70 $mg.mL^{-1}$.

All plant extracts were tested at 1.0 $mg.mL^{-1}$ for antibacterial activity against *P. gingivalis*. Ten of the 38 extracts exhibited inhibitory activity, with four extracts suppressing growth 81–100%, two suppressing growth 61–80%, and four extracts suppressing growth 50–60%. Although, several plant extracts showed potent inhibitory activities against virulence factors or cell growth independently, only, the leaf extracts of *O. vulgare* and aerial part extracts of *Glycyrrhiza glabra* and *Salvia offinalis* suppressed all the tested virulence factors and bacterial growth.

Table 2.1: The inhibitory activities of the aqueous extracts from selected Sudanese medicinal plants against *Porphyromonas gingivalis* virulence factors.

Scientific name	Used part	Yield (%)	MIC (mg.mL^{-1})		IC_{50} (mg.mL^{-1}) Gingipain	Growth inhibition at 1.0 mg.mL^{-1}
			Hemagglutination	Hemolysis		
Tamarindus indica	F	72.9	ND	4	ND	-
	L	22.2	ND	1	0.14	-
	S	19.7	ND	0.03	1.03	-
Salvadora persica	B	22.4	0.5	0.5	0.28	-
	L	38.3	4	0.5	ND	-
	M	12.6	4	0.5	ND	-
Erythrina abyssinica	S	9.06	ND	ND	0.13	-
Origanum vulgare	L	21.1	0.25	0.25	0.34	+
Ambrosia maritima	A	15.1	ND	4	0.25	+
Acacia nilotica	D	15.5	ND	0.03	0.28	-
Guiera senegalensis	L	10.3	0.5	0.5	ND	-
Grewia tenax	F	62.9	ND	ND	ND	-
	S	30.1	0.5	4	ND	-
Solenostema argel	L	45.5	4	1	ND	++
Trigonella foenum	S	27.1	1	4	0.35	-
Balanites aegyptiaca	F	79.6	1	0.5	ND	+-
	L	34.0	ND	ND	ND	+-
	P	34.8	1	1	ND	+-
Glycyrrhiza glabra	A	16.1	1	4	1.70	++
Ceratonia siliqua	F	52.7	ND	4	ND	-
	S	21.2	4	4	ND	-
Acacia oerfota	M	11.1	4	4	ND	-
Khaya senegalensis	L	20.7	ND	1	ND	-
Cymbopogon schoenanthus	L	8.1	ND	1	ND	-
	A	14.8	4	1	ND	++
Monechma ciliatum	S	10.8	0.06	4	0.11	-
Salvia offinalis	A	19.5	4	0.25	0.18	++
	L	19.7	4	4	ND	-
Azadirachta indica	L	29.4	ND	4	ND	-
Lepidium sativum	S	19.2	0.5	4	ND	-
Cyperus rotundus	A	81.3	4	0.5	0.53	-
	Z	6.1	4	1	ND	-
Leptadenia arborea	L	36.5	1	1	0.72	-
Ziziphus spina	F	59.5	ND	ND	ND	-
	L	19.4	0.06	ND	0.14	-
Acacia seyal	M	21.6	ND	0.03	0.96	-
	B	30.1	ND	0.5	0.35	-
Capparis deciduas	M	13.9	4	ND	ND	+-

A aerial part, B bark, D pod, L leaf, M stem, P peel, S seed, F fruit, Z rhizome.
Yield %: based on dry weight
ND: not detectable inhibition activity
++ 81–100%, + 61–80%, ±50–60%, - < 50% inhibition rate.

Among these three plants, the aqueous extract of *O. vulgare* leaves exhibited the most potent inhibitory activity against all tested virulence parameters, and thus this sample was selected for further investigation and purification.

2.3.2 Identification of inhibitory compound in the aqueous extract of *O. vulgare* leaves

The compounds in the *O. vulgare* leaf extract exhibiting inhibitory activity on virulence factors was extracted and identified as described above. HPLC analysis of purified aqueous extract of *O. vulgare* leaves provided a single, broad polymer-like peak (Fig.2.2A). Because of its heat resistance, high water solubility, and broadness of the single peak, we speculated that the active fraction harbors a polyphenolic or tannin-like polymeric compound. Colorimetric reactions of this active fraction and tannic acid with Prussian blue provided a dark blue to turquoise color, indicating that the isolated fraction contains a large polyphenolic or tannin-like compound (Fig.2.2B). We verified this possibility by TLC analysis (Fig.2.3) of the isolated fraction and standard tannin and found that the isolated fraction did not contain procyanidin oligomers and that the phenolic polymer provides a TLC profile similar to that of the tannic acid standard.

The isolated compound and tannin standard were analyzed using MALDI-TOF MS. The molecular mass of the isolated compound ranged from 509 to over 1000, and that of tannic acid from 530 to over 1500. The mass spectrum of the isolated fraction was similar but not the same as that of tannic acid: both showed peaks at similar intervals of 179 mass units for the isolated compound and 152 mass units (corresponding to gallic acid) for tannic acid (Fig.2.2C). The isolated tannin-like polymer was found to be stable against chemical degradation by cystamine (data not shown), confirming that the isolated compound is hydrolysable tannin.

Identification of the monomer of the isolated tannin-like compound (I. Tannin) was carried out by subjecting it to acid degradation, followed by HPLC and absorbance spectrum analysis. Comparison of the HPLC chromatograms of the degraded isolated tannin-like compound with those of ellagic and gallic acids showed that the single peak of I. Tannin at 5 min corresponded to that of ellagic acid (Fig.2.4A). The UV spectrum of I. Tannin was somewhat similar to that of ellagic acid (Fig.2.4B).

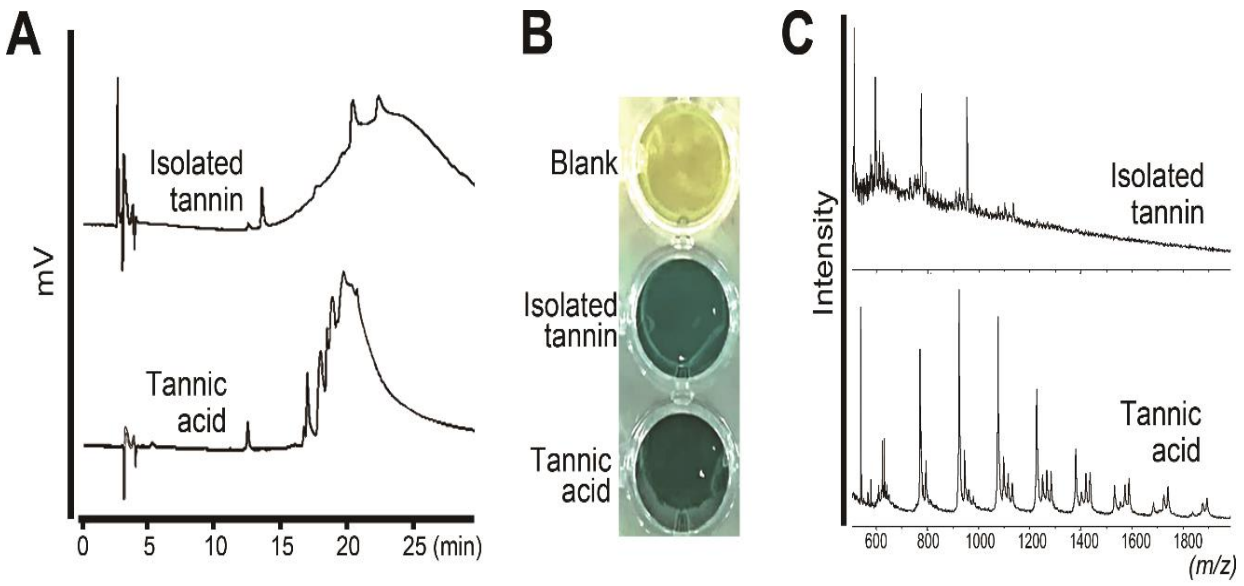

Fig.2.2 Characterization of polyphenolic compound isolated from *O. vulagare*. **(A)** HPLC chromatogram of the tannin-like compound and tannic acid. **(B)**The reaction of the isolated polyphenolic compound and tannic acid with Prussian blue. **(C)** MALDI-TOF MS analysis of tannin-like compound and tannic acid.

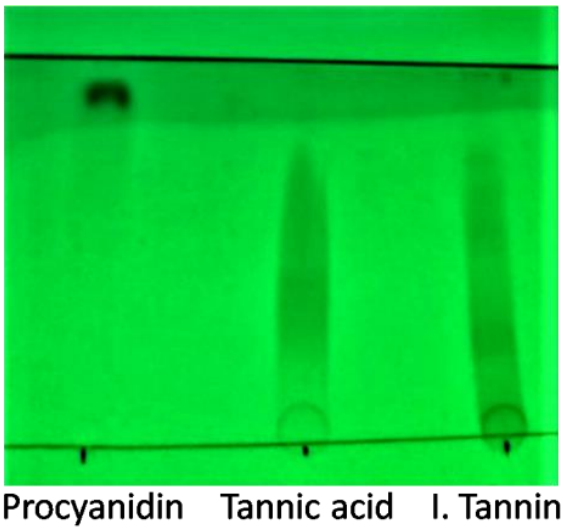

Fig.2.3 Discrimination of tannin-like compound by TLC analysis.

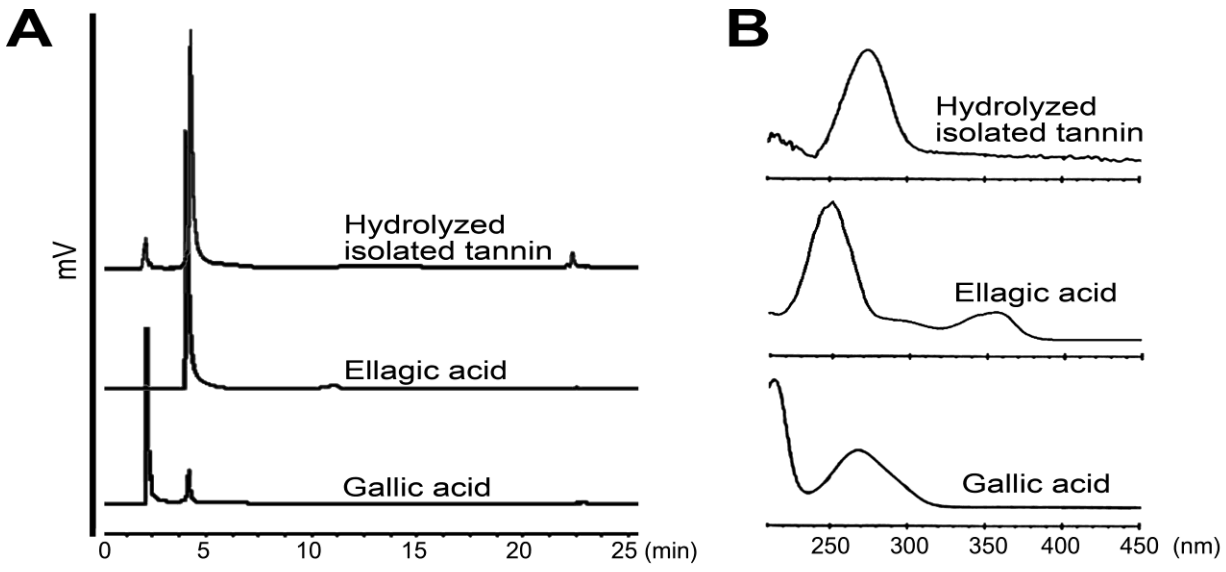

Fig.2.4 HPLC chromatogram **(A)** of the acid-hydrolyzed tannin-like compound and UV spectrum of the single peak **(B)** compared to that of ellagic and gallic acid.

2.3.3 Inhibitory activity of the isolated compound

The isolated tannin-like compound at 0.05 mg.mL^{-1} exhibited inhibitory activity against all tested periodontal factors, including *P. gingivalis* growth, hemagglutination (MIC: 0.0063 mg.mL^{-1}), hemolysis (MIC: 0.0063 mg.mL^{-1}), and protease (IC_{50}: 0.05 mg.mL^{-1}) (Table 2.2). Tannic acid also exhibited inhibitory activity against hemagglutination, hemolysis, and protease comparable to that of I. Tannin but the antibacterial activity of I. Tannin was 3-fold higher than that of tannic acid.

Table 2.2: Inhibitory activity of the isolated tannin-like polymer against *P. gingivalis* virulence agents

Compounds	**MIC (mg.mL^{-1})**			**IC_{50} (mg.mL^{-1})**
	Hemagglutination	**Hemolysis**	**Growth**	**Gingpain**
Isolated tannin	0.0063	0.0063	0.05	0.05
Tannic acid	0.0063	0.0032	0.15	0.05

2.4 Discussion and conclusion

The aqueous extracts from 38 plant organs from 25 plant species used in folkloric Sudanese medicine were evaluated for inhibition of virulence factors (hemagglutination, hemolysis, and protease activity) and for suppression of growth of *P. gingivalis*. Gingipains adhere to host cells to obtain the nutrients required for bacterial survival and the production of virulence factors. In the primary stages of inflammation, *P. gingivalis* secretes hemagglutinin and proteases which form large multifunctional complexes known as hemagglutinin/adhesion domains. These domains engage in proteolysis and are involved in the adhesion of host cells to red blood cells for acquisition

of heme through hemagglutination and hemolysis (Kadowaki et al., 2004; Tezuka et al., 2006; Guo et al., 2010; Kan et al., 2019). Several studies have, therefore, suggested targeting these virulent factors to block the primary stages of *P. gingivalis* pathogenicity, using natural, safe, and effective therapeutic agents (Nakayama et al., 1996; Kadowaki et al., 1998; Lu et al., 2019). Gingipains are obvious targets as their activity is independent of bacterial cellular programs and cell viability.

Most of the tested aqueous extracts inhibited one or more virulent proteins but only 10 plant extracts suppressed bacterial growth. This suggests that the inhibition of bacterial growth in a liquid medium and the outer cell membrane virulence of gingipains are achieved through different inhibition mechanisms. Similar findings were obtained using tea catechin, lactoferrin, cranberry proanthocyanidin, and prenyl flavonoids, all of which inactivated gingipains independent of bacterial growth suppression (Kushiyama et al., 2009; Niehues et al., 2010; Feghali et al., 2012; Messing et al., 2014). In contrast, leaf extracts of *O. vulgare* and aerial part extracts of *G. glabra* and *S. offinalis* suppressed gingipains and *P.gingivalis* growth, suggesting that they may harbor ideal inhibitors for all *P. gingivalis* virulence factors. No ideal periodontal inhibitor has been reported to date, thus further analysis to identify biologically active compounds in these plant extracts is essential.

Comparison of the MIC values of the three plant extracts (*O. vulgare*, *G. glabra*, and *S. offinalis*) showed that *O. vulgare* exhibited low MIC against all tested parameters especially for hemolysis and hemagglutination (Table 2.1). Hemagglutination is vital for *P. gingivalis* to adhere to host cells in gingival tissues and to uptake heme and iron by the aggregation and lysing of erythrocytes, resulting in the accumulation of dental plaque and the secretion of other virulent factors (Guo et al., 2010). Several natural and non-natural products have been reported to reduce *P. gingivalis* pathogenicity by blocking bacterial adhesion and decreasing proteolytic activity

(Löhr et al., 2011; Curtis et al., 2002; Yokoyama et al., 2007; Kan et al., 2019; Kariu et al., 2019). Since *O. vulgare* is used worldwide primarily as a food additive due to its pleasant aroma (Illias et al., 2018), we therefore, focused on aqueous extracts of *O. vulgare* leaves and succeeded in isolating a tannin-like compound as a non-specific inhibitor against all virulence agents.

Tannins are widespread in plants and in plant-based foods and are classified into two groups. The first group is hydrolysable tannins (HTs), which are esters of phenolic acids and a polyol, usually glucose. The phenolic acids are either gallic acid (in gallotannins) or phenolic acids derived from the oxidation of galloyl residues (in ellagitannins). The second group of tannins is proanthocyanidins (PAs), which are the most common type of tannin in our diet (Santos-Buelgaand Scalbert, 2000; Smeriglio et al., 2017). Treatment with cystamine does not affect isolated tannins, perhaps due to the C-C or C-H linkages in tannins and their degree of polymerization. However, in this study, sulfuric acid degradation followed by HPLC analysis of the isolated and standard tannins, provided a single peak with a retention time and UV spectrum parallel to that of ellagic acid with a slight difference. This result indicates that the isolated tannin might be a derivative of ellagic acid and may be a new type of very polar HTs.

The isolated tannin-like compound exhibited high inhibitory activity against hemagglutination (MIC 0.0063 $mg.mL^{-1}$), hemolysis (MIC 0.0063 $mg.mL^{-1}$), protease activity (IC_{50} 0.05 $mg.mL^{-1}$), and bacterial growth (MIC 0.05 $mg.mL^{-1}$). These findings are in agreement with reports of antimicrobial properties of HTs in extracts from edible and non-edible plants (Santos-Buelga and Scalbert, 2000; Buzzini et al., 2008; Pinelli et al., 2015; Smeriglio et al., 2017). Several studies have reported an association between a reduction in polyphenolic compounds from green tea and cranberry polyphenols and an increase in dental diseases (Feghali et al., 2012; Kushiyama et al., 2009). These polyphenolic compounds prevent biofilm formation by *P. gingivalis* and

Fusobacterium nucleatum and reduce the activity of several *P. gingivalis* proteases. Epicatechin-3-O-gallate-(4β,8)-epicatechin-3′-O-gallate isolated from the aerial parts of *Rumexa cetosa* L. interacts with the active side of Arg-gingipain and hemagglutinin from *P. gingivalis* (Schmuch et al., 2015). Furthermore, the antimicrobial activity of flavogalonic acid dilactone and terchebulin from a methanolic extract of *C. hartmannianum* that inhibited metalloproteinase-9 matrix of *P. gingivalis* growth (Mohieldin et al., 2017).

The non-specific inhibition of HTs might be connected to their ability to neutralize free radicals (–R) by donating a hydrogen atom (–RH) or an electron (–R–), chelating metal ions in aqueous solutions, and binding or precipitation of proteins due to extensive coating of hydrophobic surfaces of peptides. To identify the real reasons and inhibition mechanism, more investigation is required of the chemical formula of HTs (Smith et al., 2005; Aaby et al., 2007). We conclude that the water extracts of the selected plants can play a potent role in blocking the very early stages of *P. gingvalis* virulence-related factors. Thus, targeting those virulent factors might hinder pathogenicity and improve the expansion of new, safe, effective therapeutic and/or preventative agents. It is a challenging and complicated task to impede all the virulence-related factors (Ingar and Potempa, 2014). The HTs from *O. vulgare* leaves show a wide spectrum, impeding most of the targeted virulence-related factors in this study. The polarity and water solubility of this compound might be suitable for further applications to support oral hygiene.

CHAPTER THREE

Isolates from *Monechma ciliatum* seeds' extract hampered *Porphyromonas gingivalis* hemagglutinins

3.1 Introduction

Periodontal diseases are major oral health problems caused by bacteria that induce inflammation in the tissues supporting the teeth. The primary stage of bacterial pathogenesis begins with the adhesion of bacterial cells to host tissues followed by colonization, which provides nutrients and allows bacteria to survive, grow, and produce further virulence factors (Beck et al., 1999; DeStefano et al., 1993). *Porphyromonas gingivalis*, a gram-negative anaerobic bacterium, is considered to be a major pathogen for periodontitis (Holt et al., 1999; Slot and Genco, 1984). This bacterium produces a number of molecules associated with adherence and colonization including fimbriae, lipopolysaccharides, proteases like gingipains, and potential adhesion molecules such as hemagglutinins, all of which are known as virulence factors (Connolly et al., 2017). The most important virulence factor that *P. gingivalis* produces is the hemagglutinins (Han et al., 1996; Lee et al., 1996; Shi et al., 1999). This group of proteins plays a vital role in the infection process by allowing *P. gingivalis* to adhere to the surface of host cells. This, in turn, facilitates the acquisition of heme through erythrocyte binding and causes lysis and aggregation of erythrocytes via several proteolytic enzymes known as gingipains, which release the heme moiety from the hemoglobin molecule as a nutrient (Lewis et al., 2006; Olczak et al., 2008). It is well known that iron is an essential growth factor for most bacteria (Olczak et al., 2005). Unlike many other microorganisms, *P. gingivalis* does not possess a siderophore scavenging system for iron uptake or the enzymes required for heme biosynthesis. Instead, it employs alternative mechanisms to obtain iron from host heme (Gao et al., 2010; Smalley et al., 2011). For this process, *P. gingivalis*

possesses five different hemagglutinins located on the cell surface, in addition to secreting exohemagglutinins, which are responsible for effective binding to erythrocytes and epithelial cells (Okuda et al., 1986). The development of anti-adhesive compounds against *P. gingivalis* hemagglutinins could be a promising cytoprotective strategy to prevent the harmful effects of long-term bacterial infection (Cutler et al., 1995). Over the past few years, plant-derived natural products have been described as adjuvant remedies that aim to reduce inflammation through interaction with the adhesion of *P. gingivalis* to host cells (Kariu et al., 2017; Palaska et al., 2013).

Recently, we screened a number of medicinal plants used in Sudanese folk medicine to determine their inhibitory activity against virulence factors including hemagglutination of *P. gingivalis* (Eltigani et al., 2019). Among them, *Monechma ciliatum* exhibited potent inhibitory activity against hemagglutinins. *M. ciliatum* or black mahlab, belonging to the family Acanthaceae, is a famous medicinal plant that grows in some parts of arid and semi-arid lands in tropical Africa including western Sudan, especially in the Nuba Mountains and Gabel Mara area (Oshi and Abdelkarim, 2013; Sharief, 2002). The seeds contain aromatic components and a high amount of oil. Thus, this plant is used mainly as a flavoring agent in traditional fermented bread, kisra, to give a sweet aroma to the final product. In addition, it is used in the production of traditional Sudanese fragrances, lotions, and other cosmetics used for wedding preparations and childbirth (Sharief, 2002). Black mahlab is also utilized as an effective laxative in several African countries. In Botswana, it is believed to diminish general body pain, liver problems, and bowel problems (diarrhea), as well as sterility in women (Hedberg and Stangard, 1989). In Nigeria, it is used to induce labor and facilitate menses. Furthermore, the dried leaves are powdered and burnt as an inhalation for colds (Uguru and Evans, 2000). In Sudan, the entire dried plant is used for diarrhea, vomiting, and as a perfume preparation (Sharief, 2002). However, despite its various traditional

uses, few studies have authenticated its use in folk medicine and determined the bioactive compounds responsible for its potential effects. Therefore, in the present study, we investigated the role of dried seed components against *P. gingivalis* hemagglutinins following aqueous extraction. The inhibitory activities of the four compounds isolated and purified for the first time from the aqueous extract of *M. ciliatum* seeds are discussed.

3.2 Materials and methods

3.2.1 Preparation of aqueous extract of *M. ciliatum* seeds

M. ciliatum seeds were obtained from Khartoum State, Khartoum, Sudan. The seeds were authenticated at the Faculty of Agriculture, University of Khartoum. Soluble compounds were extracted using distilled water (three times) from cleaned and powdered seeds (1 g sample: 10 ml water) for 30 min at room temperature under slow shaking. The supernatants were collected by filtration followed by centrifugation and lyophilization. The extract was stored at −20°C until analysis.

3.2.2 Cultivation of *P. gingivalis*

P. gingivalis TDC60 (Watanabe et al., 2011) was cultivated and prepared as mentioned in chapter two. The bacterium was grown in Tryptic Soy Blood Agar medium anaerobically for 4–5 days. Subsequently, the cells were then inoculated into 5 ml of GAM broth medium and incubated under anaerobic conditions at 37°C for 2–3 days then the growth rate was evaluated by a spectrophotometer at 600 nm and the cells were removed by centrifugation. The collected culture supernatant (Pg-sup) was used for hemagglutination inhibition assay.

3.2.3 Inhibition of hemagglutination

Erythrocyte aggregation (hemagglutination) was performed as described in chapter two. Briefly, the hemagglutination test was performed in a 96-well microtiter plate (round bottom) using a reaction mixture consisting of 80 µL of PBS, 10 µL of Pg-sup, and 10 µL of plant extract. The mixture was shaken thoroughly and incubated for 10 min at room temperature. Then 100 µL of 5% blood was added to the assay mixture. Hemagglutination was evaluated visually after 2 h of incubation at room temperature. PBS was used instead of Pg-sup and aqueous extract of *M. ciliatum* as a negative control. The minimum inhibitory concentration was calculated from the final concentration of the lowest dilution with complete inhibitory activity.

3.2.4 Isolation of biologically active compounds

Extraction of the active inhibitory constituents from *M. ciliatum* seeds (Fig.3.1) was performed according to the method described by Mendonça-Filho (2006). About 100 g of dried and powdered seeds was used for the extraction of soluble compounds by soaking in 1.0 L of distilled water three times for 30 min at room temperature under slow shaking. The soluble extracted compounds were collected by filtration followed by centrifugation. The clarified extract was portioned between distilled water and ethyl acetate (EtOAc), yielding a water fraction (6.0 g) and EtOAc fraction (1.3 g). The EtOAc portion underwent further fractionation by normal-phase chromatography. The column was packed with 130 g of silica gel powder (Wakogel C-300, Silica Gel, Wako Pure Chemical Industries, Osaka, Japan) and eluted with acetone/hexane (0%–100%). Fraction of 20% acetone was the active fraction (0.650 g), next subjected to the column chromatography of silica gel, which eluted with EtOAc/hexane (0%–100%) solvent. The active compounds (0.544 g; 20% EtOAc fraction) were subjected to silica gel column chromatography with elution solvents of

EtOAc/ hexane. The ratio of each solvent was adjusted to 3%, 5%, 8%, 11%, and 50% EtOAc in hexane. The volume of the eluent was set at 300 ml and fractions were collected into five subfractions (A, B, C, D, and E; all subfractions were set at 60 ml) for each eluent (Fig.3.1). The collected fractions were analyzed using thin-layer chromatography (TLC silica gel 60 F254, EMD Millipore Corporation, Darmstadt, Germany) to detect the isolated compounds with anisaldehyde at 254 nm. Fractions that showed similar constituent spots were combined.

3.2.5 Identification of the isolated compounds

All isolated compounds of the silica gel column chromatography with elution solvents of EtOAc/hexane were analyzed via 1H and 13C NMR spectra and recorded on a Bruker Avance (Bruker, Billerica, MA, USA) with CDCl3 using a 600 MHz spectrometer. Among them, fractions 8A, 8C, and 11B contained a single compound. Fraction 8C was an aromatic compound; thus, further analysis was performed using GC/MS to obtain the molecular mass. Compounds 8A and 11B were hypothesized to be fatty acids. To obtain more information about their molecular masses and formulas, GC/MS analysis was performed for both compounds after derivatization (methylation) with trimethylsilyl (TMS) diazomethane according to the method described by Presser and Hüfner (2004), with some modifications. One milligram of each compound was dissolved in a mixture of toluene– MeOH (1:2 v/v, 1 ml) and 100 µl of TMS diazomethane was added to the solution. The mixture was heated for 10 min at 80°C and subsequently dried in a nitrogen stream. Hexane (1 ml) was added to the residue, followed by extraction of 10 µl, which was diluted 100-fold with the same solvent and analyzed by GC/MS (Shimadzu GCMSQP2010C, Kyoto, Japan). GC/MS analysis was performed using a DB-23 column (thickness, 0.25 µm; length, 30 m; and inner diameter, 0.25 mm). The oven temperature was 50°C and the mass range was m/z

40–800. The injection volume was 1 µl with 220°C as the injector port temperature. The following oven temperature program was carried out with helium (at a constant flow rate of 1 ml/min) as the carrier gas: 2 min at 50°C, increasing to 160°C at 15°C/min, and then to 220°C at a rate of 4°C/min. The interface and ion source were kept at 220°C and 200°C, respectively. All mass spectra were acquired in the electron impact mode. Ionization was off during the first 5 min to avoid solvent overloading and the mass range was m/z 40–800. Methylation followed by the GC/MS analysis was not suitable for the identification of compound 11B.

Therefore, further purification was performed using preparative TLC. About 5 mg of 11B was spotted onto TLC (Silica Gel 60 F254, EMD Millipore Corporation) and developed via 20% ethyl acetate in hexane. Subsequently, the edges of the plate were sprayed with anisaldehyde. Thereafter, the silica gel corresponding to the detected spots was collected by scratching. The compounds were extracted from the silica gel by the same solvent mixture.

Two subfractions were obtained and named B4 and B5 (Fig.3.2). B4 and B5 were analyzed by NMR and ESI mass spectroscopy. The ESI mass spectra were recorded using a Waters Quattro Micro mass spectrometer combined with a Waters Photodiode Array Ultra-Performance Liquid Chromatography (UPLC) system in a positive mode. The mass range was m/z 200–800 and the column was an ACQUITY UPLC BEH C18 column (2.1 × 50 mm, 1.7 µm, Waters). The solvents were acetonitrile containing 0.1% formic acid and Milli-Q containing 0.1% formic acid with gradient elution from 5% to 100% for 10 min. The flow rate was 0.2 ml/min and the column temperature was 40°C. The HRMS was recorded using an Exactive Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA).

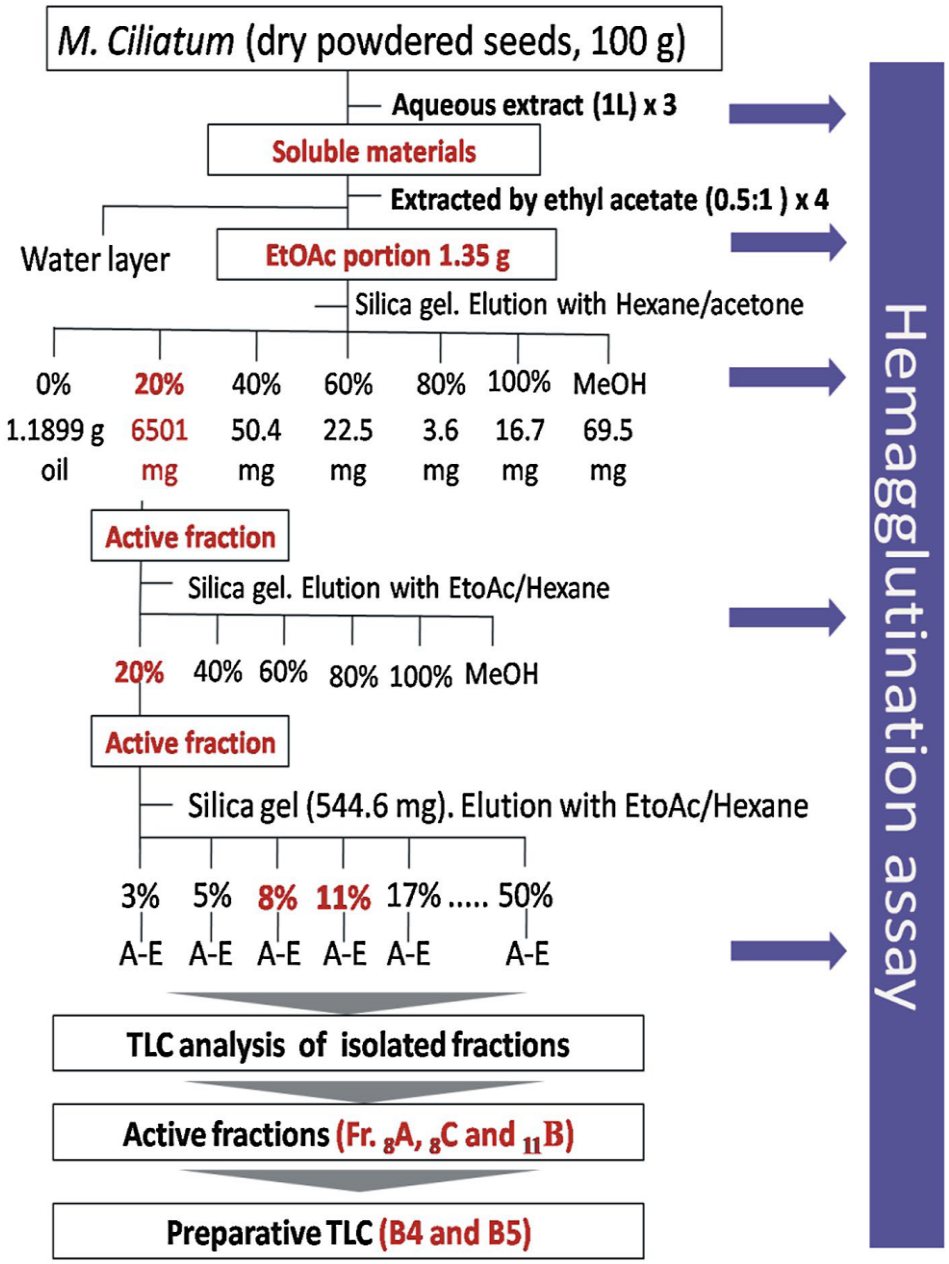

Fig.3.1 Scheme of sample preparation and isolation of inhibitors

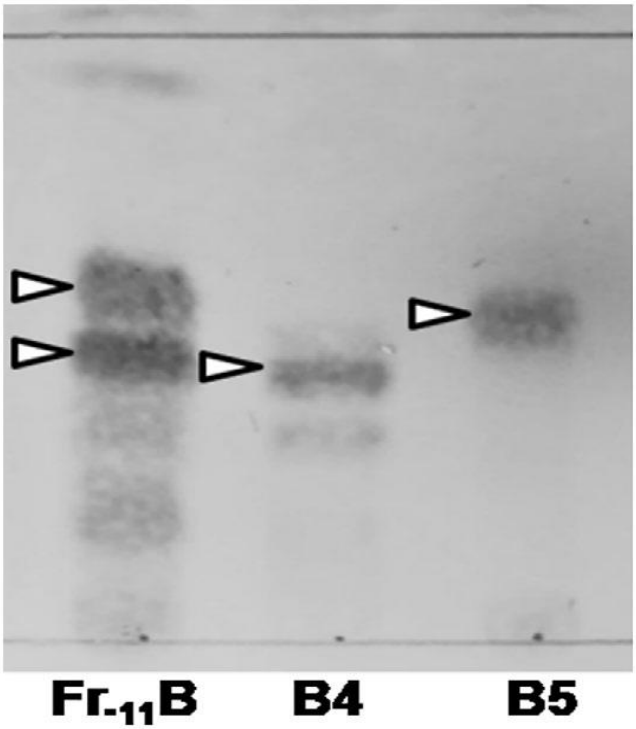

Fig.3.2 TLC detection of compounds B4 and B5

3.2.6 Minimum inhibitory concentration of the isolates

The dried fractions were used to prepare a stock solution of 1 $mg.mL^{-1}$. Each fraction was diluted directly in a microplate with a round bottom containing the hemagglutination assay reaction mixture to obtain a final concentration ranging from 100 to 1.6 µg/ml. The MIC was calculated from the lowest concentration of the fraction that completely inhibited the hemagglutination activity. The experiment was conducted in triplicate.

3.3 Results

The inhibitory activity against hemagglutination of the crude aqueous extract of *M. ciliatum* seeds was evaluated at different concentrations (4.00–0.035 $mg.mL^{-1}$). The crude extract of *M. ciliatum* revealed high anti-hemagglutination activity with a MIC value of 0.07 $mg.mL^{-1}$ (Fig.3.3).

To identify the compounds responsible for hemagglutination inhibition, several purification steps were performed as illustrated in Fig.3.1. Four compounds were isolated and identified as anti-hemagglutination agents (Fig.3.4). The physical and chemical characteristics of these compounds were as follows: Compound 8A (34 mg) was an amorphous colorless oily compound. The molecular weight of 8A was determined to be 282 on the basis of ESI-MS (m/z 282.4 [M + H]+). The pure compound was methylated using TMS diazomethane and subjected to GC/MS analysis. We detected a single peak at Rt 16.2 min, and the peak showed the following spectrum: m/z (relative intensity %) 264 (25), 222 (8), 180 (10), 123 (10), 97 (50), 96 (49), 83 (51), 74 (52), 96 (75), 55 (100), and 41 (70). A database search with NIST08 mass spectral library indicated that the methylation product from compound 8A was 9-octadecenoic acid (Z)-methyl ester, which is an oleic acid methyl ester. Thus, the original compound prior to methylation was thought to be oleic acid. To confirm this, the 1H NMR data (appendices figures S1-S4) of the isolated compound before methylation (Table 3.1) were compared with the data of authentic oleic acid (Wako Pure Chemical Industries, Osaka, Japan). The isolated compound 8A and oleic acid showed the same 1H NMR spectrum proving that the isolated compound 8A was oleic acid (Fig.3.4B). Compound 8C (100 mg) was a white powder with a pleasant aroma. We analyzed the compound by GC/MS. The analysis showed a single peak at Rt. 21.5 min. The mass spectrum of this peak was as follows: m/z (relative intensity %) 146 (100), 118 (89), 90 (36), 89 (35), and 63 (20). A database search with NIST08 Mass Spectral Library indicated that this compound is 5,6-benzo-α-pyrone, otherwise known as coumarin (Fig.3.4A). The retention time of authentic coumarin (Wako Pure Chemical Industries) is the same as that of compound 8C, and the mass spectra are identical. The 1H and 13C NMR data are shown in Table 3.1 and are identical to those of the authentic compound.

Compounds B4 and B5 (2.4 and 1.7 mg) were colorless and amorphous. As shown in Table 3.2, the 1H NMR spectra of these compounds showed signals corresponding to oleic acid, but the 1H NMR signal corresponding to the carboxyl group was not detected. In the spectrum of B4, additional signals at δH4.20–4.19 ppm were detected, while in the spectrum of B5, signals at δH4.30–4.16 ppm were detected. The ESI mass spectra of compounds B4 and B5 were identical to each other and showed a sodium adduct ion at m/z 643.8 ([M + Na]+) (Fig.3.5), indicating that their molecular weights were 620. On the basis of these data, these compounds were considered to be 1,2-dioleylglycerol and 1,3-dioleylglycerol (Fig.3.4C, D). Fig.3.5 and Fig.3.6 show the comparisons of the ESI mass spectra and retention times of B4 and B5 on LC/MS analysis, respectively. The authentic 1,2-dioleylglycerol and 1,3-dioleylglycerol were used as standards. The results of 1H NMR spectra in Table 3.2 approved that the compounds B4 and B5 were 1,2-diolein and 1,3-diolein.

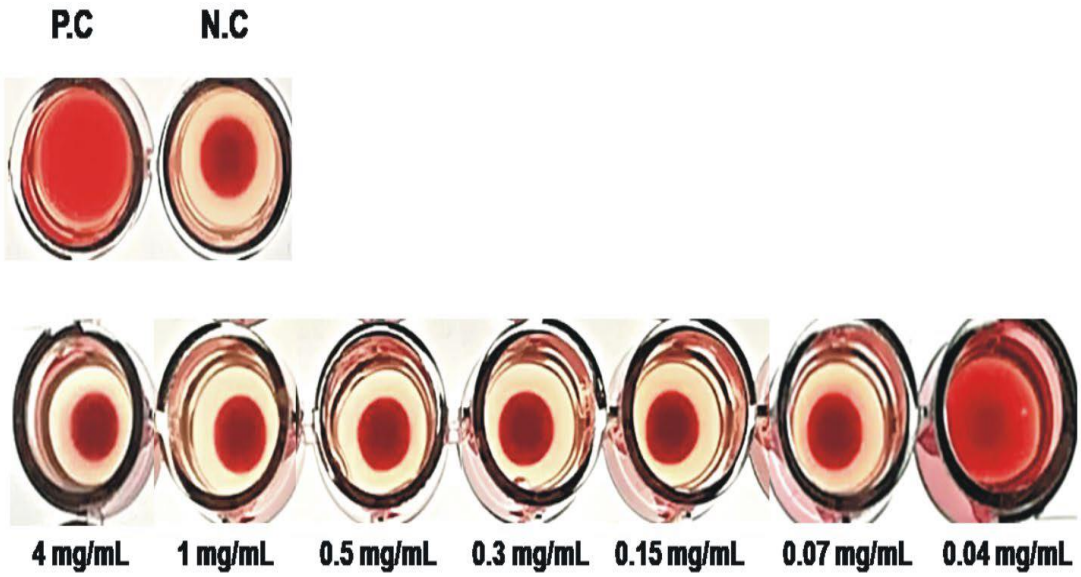

Fig.3.3 Inhibitory activity of *M. ciliatum* seeds' aqueous extract on *P. gingivalis* hemagglutinins. P.C and N.C are positive control and negative control, respectively.

3.3.1 The inhibitory activity of *M. ciliatum* and identified compounds

The purified and identified compounds (8A, 8C, B4, and B5) were first tested for the hemagglutination inhibitory activity using a reaction mixture containing the purified compounds at a final concentration of 100 μg/ml followed by assays with different dilutions (Table 3.3). The MIC was calculated from the last concentration that led to complete inhibition toward exohemagglutinin from *P. gingivalis* formed in planktonic cells from a broth culture. Oleic acid (8A) had higher inhibitory activity among the isolated compounds with a MIC value of 15 μg/ml followed by coumarin (8C), which showed inhibitory activity with a MIC value of 50 μg/ml. The diacylglycerols (B4 and B5) were less effective on *P. gingivalis* hemagglutinins than oleic acid and coumarin, and their MIC value was 100 μg/ml. However, 1,2-dioleylgycerol (B4) showed slightly stronger anti-hemagglutination activity than 1,3-dioleylglycerol (B5).

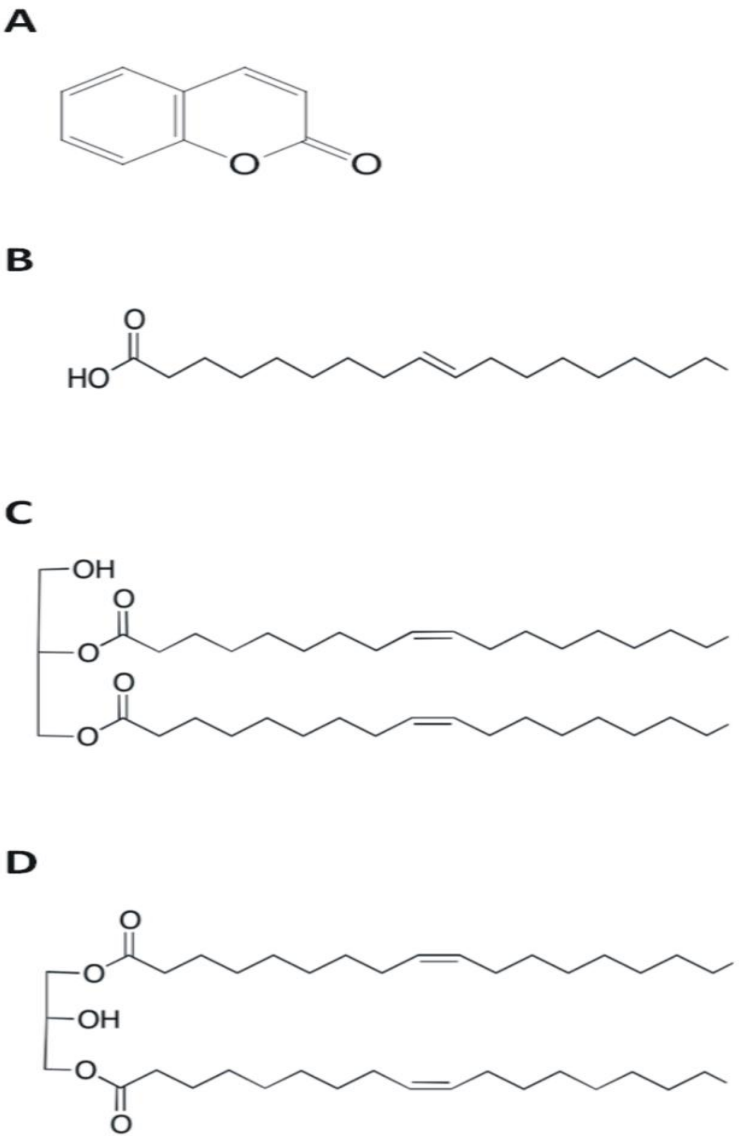

Fig.3.4 Chemical structures of the isolated compounds: (**A**) Coumarin, (**B**) oleic acid, (**C**) 1,2-dioleylglycerol, and (d) 1,3-dioleylglycerol

Table 3.1: ^{13}C (150 MHz) and ^{1}H (600 MHz) NMR data of compound $_8A$ and $_8C$ ($CDCl_3$)

Compound $_8$A		**Compound $_8$C**	
δ_C	δ_H (*J*=Hz)	δ_C	δ_H (*J*=Hz)
179.54	1H, 10.69 m	160.68	1H, 7.71 d (9.54)
130.01	2H, 5.34 m	154.03	1H, 7.52 ddd (8.6, 7.5,1.5)
129.71	2H, 2.34 t (7.8)	143.35	1H, 7.48 dd (7.5, 1.5)
34.05	4H, 2.00 m	131.79	1H, 7.32 d (8.6, 1.0)
31.89	2H, 1.63 m	127.81	1H, 7.27 ddd (7.5, 7.5,1.0)
29.67	20H, 1.34-1.26 m	124.36	1H, 6.42 d (9.54)
29.42	3H, 0.88 t (7.2)	118.7	
29.16		116.83	
27.7		116.65	
24.8			
22.5			
14.05			

Table 3.2: 1H NMR data of compounds B4 and B5 (CDCl3, 600MHz)

Compound B4	**Compound B5**
δ_H (*J=Hz*)	δ_H (*J=Hz*)
4H, 5.38-5.31 m	4H, 5.35 m
1H, 5.08 m	4H, 4.30 m
2H, 4.20 m	1H, 4.16 m
2H, 3.73 m	4H, 2.34 t (7.8)
4H, 2.34 t (7.8)	8H, 2.04 m
8H, 2.00 m	4H, 1.61-1.59 m
40H, 1.38-1.25 m	40H, 1.38- 1.28 m
1H, 1.2- 1.00 m	6H, 0.88 t (7.2)
6H, 0.88 t (7.2)	

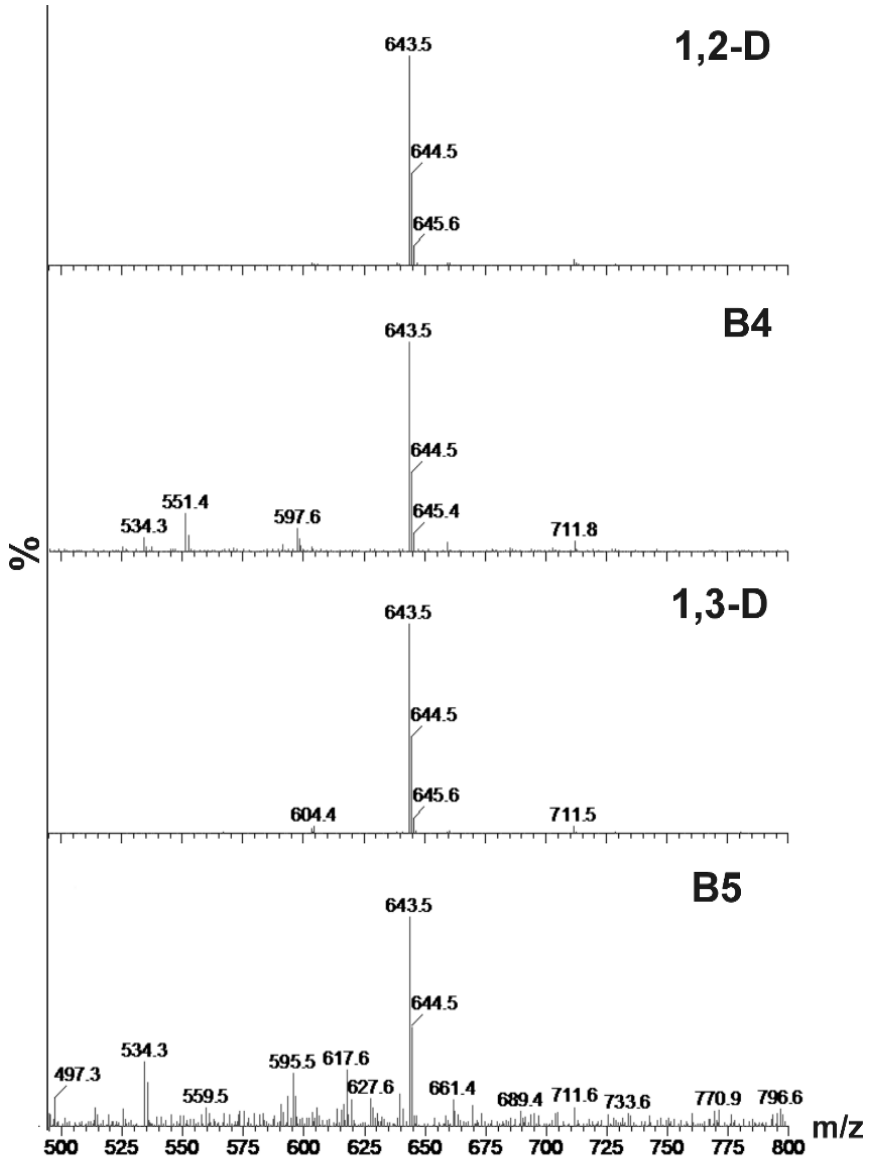

Fig.3.5 Mass spectra of B4 and B5 compounds versus commercial 1,2-dioleylglycerol (1,2-D) and 1,3-dioleylglycerol (1,3-D)

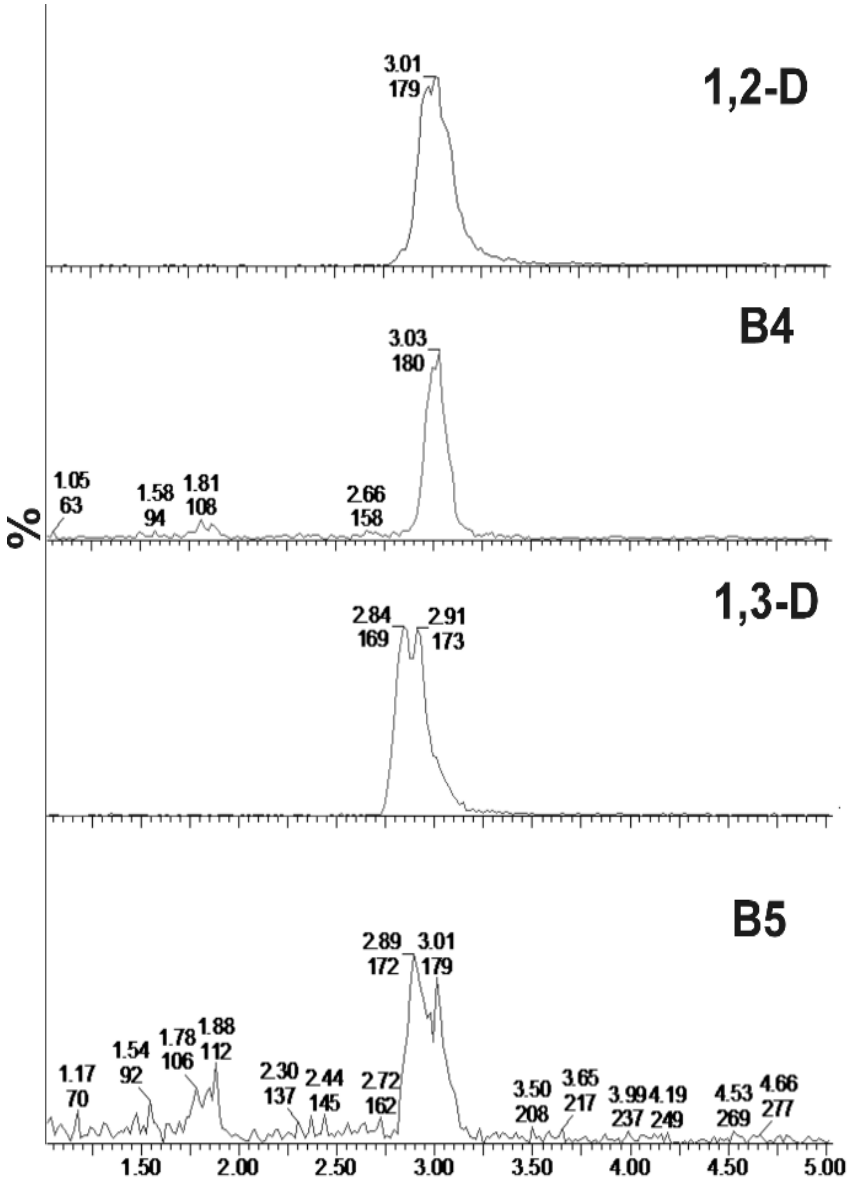

Fig.3.6 Comparison of retention times of B4 and B5 compounds versus commercial 1,2- and 1,3-dioleylglycerols

Table 3.3: Hemagglutination inhibition activities of isolated and standard compounds

Isolated compounds	**MIC (µg.mL^{-1})**	**Standard compounds**	**MIC (µg.mL^{-1})**
$_{8}$C	50	Coumarin	100
$_{8}$A	15	Oleic acid	≤15
B4	≤100	1,2-Diolein	≤100
B5	100	1,3-Diolein	100

3.1 Discussion and conclusion

Adhesion to the host's surface cells is a very important stage for mucosal virulent pathogens and enables them to survive and grow. Thus, prevention and remediation of their adherence might decrease and/ or impede bacterial colonization and subsequent tissue inflammation (Labrecque et al., 2006; Wittschier et al., 2009; Wittschier et al., 2007). Over the past decades, natural compounds with antibacterial and anti-inflammatory properties have been identified as alternative and new therapeutic agents that might cure periodontal infections. For instance, several natural products and synthetic protease inhibitors have been used to block bacterial proteases; they have been proven to be effective by weakening bacterial adhesion and reducing proteolytic efficiency (Curtis et al., 2002; Kadowaki et al., 2004). It is well known that *P. gingivalis* cell adhesion predominantly relies on the functions of hemagglutinins; therefore, the use of antiadhesive compounds that influence the interaction between hemagglutinins and erythrocyte proteins could hinder periodontitis in the early stages (Miyachi et al., 2007; Yokoyama et al., 2007). Recently,

we evaluated a group of Sudanese medicinal plants against virulence factors. The aqueous extract of *M. ciliatum* seeds revealed potent anti-hemagglutination activity (Eltigani et al., 2019). This plant has been reported by western Sudanese people as a natural pain killer for liver and stomach pains (Mariod et al., 2009). In addition, constituents of this plant have been shown to have anticytotoxic properties (Uguru and Evans, 2000). In our study, the main aim was to investigate the inhibitory activity of the aqueous extract of *M. ciliatum* seeds on hemagglutination by *P. gingivalis* and the related bioactive compounds. The crude aqueous extract of the seeds revealed high inhibitory activity against all exohemagglutinins existing in the broth culture, with a low MIC value of 0.07 $mg.mL^{-1}$ (Fig.3.3). This result confirmed that the aqueous extract of *M. ciliatum* seeds has a significant influence on the exohemagglutinin function. The isolated compounds were identified as oleic acid, coumarin, 1,2-dioleylglycerol, and 1,3-dioleylglycerol (Fig.3.4). Though the presence of oleic acid and coumarins in several plant species has been reported by several researchers (Mariod et al., 2009; Rosselli et al., 2009; Spino et al., 1998), none so far have been isolated or purified, and these compounds have not been chemically identified in *M. ciliatum*. Furthermore, we found other two compounds claasified into diacylglycerols (1,2-dioleylglycerol, and 1,3-dioleylglycerol) from *M. ciliatum*. These compounds, which exist in several vegetable and seed oil, are well known for their functional roles in medicinal fields (AlAttas et al., 2016; Huang et al., 2011; Lopes et al., 2014). However, and to the best of our knowledge, these compounds are very rare in higher plants. Therefore, the identification and isolation of 1,2- and 1,3-dioleylglycerols from *M. ciliatum* will be a convenient and cost-effective source of these bioactive compounds for therapeutic use, especially for people in arid and semi-arid lands. The fraction 11B, which was isolated from the silica gel column with 11% EtOAc/hexane, was analyzed by 1H NMR, and it was thought that it might contain more than one compound. Further purification using TLC

revealed two main compounds as illustrated in Fig.3.2. The mass spectrum of both purified compounds was 643.8 m/z [M + H]+, with the chemical formula C39H72O5 (Fig.3.4), which hinted at the similarity between the two purified compounds. The δH 3.72 ppm, 5.08 ppm and the multiple shapes of the chemical shift between 4.20 and 4.19 ppm (Table 3.3) described the corresponding esterified glycerol with the 1,2-diglyceride position. Moreover, the signal 4.08–4.21 ppm of another compound revealed 1,3-diglycerol (Nieva-Echevarría et al., 2015). Further confirmation was obtained using a mass chromatogram of isolated and purchase standard compounds (Fig.3.5 and Fig.3.6). The retention times were similar between B4 and 1,2-dioleylglycerol and B5 and 1,3-dioleylglycerol.

The current study showed that isolated oleic acid has inhibitory activity with a MIC value of 15 µg/ml, while 1,2- and 1,3-dioleylglycerols showed weaker inhibitory activities with a MIC value of 100 µg/ml (Table 3.3). These results agree with the findings of Huang et al. (2011). Their group found some correlation between the antimicrobial activities of fatty acids and their structure, and that the modification of the fatty acid carboxyl group might significantly influence the inhibitory activities. Commonly, fatty acids strongly inhibits oral bacterial growth compared with their esters. One hypothesis suggests that fatty acids and their esters might penetrate the cell wall of the bacterial membrane because they are bipolar and both have a hydrophilic head and hydrophobic tail (Huang et al., 2011). The n-6, n-7, and n-9 fatty acids were reported to have antimicrobial activity higher than the esters against periodontopathogens Actinobacillus actinomycetemcomitans and *P. gingivalis*. Moreover, the inhibitory activity of isolated compounds might influence protein structures through oxidation and the formation of ester bonds between aggregating proteins and inhibitor compounds, or by covalent binding and protein aggregation (Inoshita et al., 1986). Coumarins are phenolic compounds that are renowned as

potential therapeutic agents for treating periodontal disease. These compounds have been reported for their ability to inhibit *P. gingivalis* growth and MMP-9 collagenase (Marquis et al., 2012). In this study, the isolated coumarin inhibited *P. gingivalis* hemagglutinins, which are vital adherence molecules for iron and heme uptake via attachment, aggregation, and lysis of erythrocytes (Gao et al., 2010; Smalley et al., 2011). Some reports have shown that coumarins possess iron-chelating properties (Mladenka et al., 2010). Therefore, the inhibition activity of the isolated coumarin might be related to the chelation of iron in the reaction mixture, which then made iron unavailable for virulence factors. In conclusion, in recent decades, plants with a pleasant aroma and pharmaceutically bioactive compounds have received increasing attention as potent sources for the reformation of different foods and pharmaceutical products. The inhibition of *P. gingivalis* hemagglutinin using the aqueous extract of *M. ciliatum* seeds, which has a very pleasant aroma and contains valuable compounds associated with oral hygiene, supports the further therapeutic use of *M. ciliatum*.

CHAPTER FOUR

Long-chain fatty acids a biological defense against *Porphyromonas gingivalis* TDC60 pathogenicity

4.1 Introduction

Porphyromonas gingivalis is an anaerobic gram-negative, asaccharolytic bacterium associated with periodontitis (Isogai et al., 1988). This bacterium grows faster in medium supplemented with hemin, via adherence and agglutination of human erythrocytes to get heme molecules and amino acids as nutrients (Sakai et al., 2007). *P. gingivalis* produces putative virulence-associated with adherence and colonization, including fimbriae, lipopolysaccharides, proteases like gingipains, and potential adhesion molecules such as hemagglutinins, all of which are known as virulence factors (Connolly et al., 2017). The most important virulence factors that *P. gingivalis* produces are the hemagglutinins (Han et al., 1996; Lee et al., 1996; Shi et al., 1999). This group of proteins is considered to be the important protein of this bacteria, facilitating the adherence to host cells and accelerates the growth of this bacterium (Okuda et al., 1986). Following the adhesion process, gingipains proteins act mainly to aquacise the heme from erythrocytes to feed *P. gingivalis* and maturate other virulence. Gingipains are cysteine proteinases which encoded by three genes gingipain R1 (RgpA), gingipain R2 (RgpB), and gingipain K (Kgp). These proteins are consisting of propeptide, catalytic and C-terminal hemagglutinin/ adhesion domains (Genco et al., 1999). Therefore, the development of anti-adhesive compounds against *P. gingivalis* hemagglutinins could be a promising cytoprotective strategy to prevent the harmful effects of long-term bacterial infection (Cutler et al., 1995).

Numerous fatty acids have been isolated from animals, plants, and microorganisms with several biological functions such as antifungal, antibacterial, and anticancer (Yoon et al., 2018; Jóźwiak et al., 2020; Engelking, 2010). Furthermore, several studies approved the antibacterial and antiadhesion properties of the fatty acids from natural or artificial sources against oral pathogens, including *P. gingivalis* (Huang et al., 2011; Pan et al., 2019; Sulijaya et al., 2019; Johnson et al., 2014). Nonetheless, to date, there is no study investigating the influence of the fatty acids on the most aggressive *P. gingivalis* strain TDC60 and its exo-hemagglutinins and the other related protein involved in the pathogenicity event. Recently, we isolated oleic acid and its esters from Sudanese folkloric medicinal plant with inhibitory activity to *P. gingivalis* exo-hemagglutinins (Eltigani et al., 2019). Therefore, in this study, we investigated the influence of different fatty acids with various carbon chain-lengths and saturation degrees on hemagglutination and gingipains of *P. gingivalis* TDC60 using the sub-minimum inhibitory concentration model.

4.2 Materials and methods

4.2.1 Preparation of fatty acids stock solutions

Arachidic acid, arachidonic acid, decenoic acid, neo decanoic acid, oleic acid, linoleic acid, linolenic acid, and stearic acid were obtained from Sigma-Aldrich Japan (Tokyo, Japan). 2-Hexadecenoic acid was purchased from TCI (Tokyo Chemical Industry Co. Ltd.). Palmitic acid was obtained from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). A stock solution of all fatty acids was prepared with a concentration of 10 $mg.mL^{-1}$ in methanol.

4.2.2 Cultivation of *P. gingivalis*

As described previously in chapter two, the bacterium was grown in Tryptic Soy Blood Agar medium supplemented with hemin (5 $mg.L^{-1}$), menadione (1 $mg.L^{-1}$), and horse blood at 37°C under anaerobic conditions for five days (Gao et al., 2010; Rangarajan et al., 2017). Followed by inoculation of the cells into 5 mL of Gifu Anaerobic Medium (GAM broth medium) and incubated under anaerobic conditions at 37°C for three days. The growth rate was evaluated using a spectrophotometer at 600 nm, and the cells were removed by centrifugation. The collected culture supernatant (Pg-sup) was used for the inhibition assays.

4.2.3 *P. gingivalis* growth inhibition

The influence of the long-chain fatty acids solution on *P. gingivalis* growth was investigated by measuring the turbidity of bacterial suspension in a 96-well microplate. Two microliters of fatty acids (1 $mg.mL^{-1}$) were added to 200 µL of *P. gingivalis* suspension standardized at 2 $x10^7$ CFU (Kariu et al., 2017). The plates were incubated anaerobically at 37°C for 60 h. Then the turbidity was measured at 600 nm using a microtiter plate reader (Model 680, Bio-Rad Laboratories Inc., Hercules, CA, USA). The positive control was designed as *P. gingivalis*, and negative control was the GAM broth medium, without fatty acids solutions.

4.2.4 Inhibition of hemagglutination

Hemagglutination or erythrocyte aggregation was performed as described previously in chapter two. In a 96-well microtiter plate (round bottom) using a reaction mixture consisting of 80 µL of PBS, 10 µL of Pg-sup, and 10 µL of fatty acids solutions. The mixture was shaken carefully then incubated for 10 min at room temperature. Next, 100 µL of 5% blood was added to the assay

mixture. Hemagglutination was evaluated visually after 2 h of incubation at room temperature. PBS was used instead of Pg-sup and fatty acids solutions for a negative control. The minimum inhibitory concentration was calculated from the final concentration of the lowest dilution with complete inhibitory activity.

4.2.5 Identification of exo-hemagglutinins secreted by *P. gingivalis*

Proteins secreted into *P. gingivalis* culture supernatants were sampled after 30h of anaerobic cultivation. Hemagglutinins were partially purified using ammonium sulfate precipitation at a concentration of 80%. The partially purified proteins were analyzed using a 15% SDS-PAGE under denaturing conditions or by zymography and stained with Coomassie Brilliant Blue (CBB). The N-terminals of partially purified proteins were identified by blotting the proteins onto a polyvinylidene fluoride membrane. The membrane was stained with CBB stain, and the bands were excised, then the N-terminal amino acid sequences were determined by automated Edman's degradation method using Shimadzu PPSQ-31A Protein Sequencer (Shimadzu 190 Corporation, Kyoto, Japan). The N-terminal sequences of individual protein were searched against the non-redundant NCBI database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) to identify hemagglutinins proteins.

4.2.6 Influence of fatty acids sub-MICs on hemagglutinins secretion

Effect of fatty acids sub-MICs on hemagglutinins induction was investigated according to the method mentioned by Kan et al. (2019), by minor amendments. Antibacterial activities of the unsaturated fatty acids on *P. gingivalis* were determined using final concentration equivalent to $^1/_2$, $^1/_4$, and $^1/_8$ of identified MIC value for each fatty acid. The sub-MICs for 2- hexadecenoic and

linolenic acids were prepared in the concentrations of 2.5 µg. mL^{-1} ($^1/_2$), 1.3 µg. mL^{-1} ($^1/_4$) and 0.6 µg. mL^{-1} ($^1/_8$). Oleic, linoleic and arachidonic sub-MICs were prepared with concentrations of 1.3 µg.mL^{-1} ($^1/_2$), 0.6 µg.mL^{-1} ($^1/_4$) and 0.3 µg.mL^{-1} ($^1/_8$). The samples anaerobically incubated at 37°C in GAM broth media. After 30 hrs, the culture supernatant and cells of *P. gingivalis* were separated by centrifugation at 8000xg for 20 min at 4°C. Supernatants were partially purified using ammonium sulfate at a concentration of 80%. Hemagglutination activity of partially purified proteins was evaluated, and the induction of hemagglutinins was analyzed SDS-PAGE using 8 µg protein. The collected cells were weighted, and identical weight was suspended in 0.5 M Tris-HCl buffer (pH 7.6), then sonicated for 3 min on ice to be used for protease activity analysis.

4.2.7 Protease inhibition

Gingipains activities of *P. gingivalis* cells preincubated with unsaturated fatty acids at different concentration of sub-MICs was determined as stated previously (chapter two) with some changes. Gingipains activities were assayed using *t*-butyloxycarbonyl-L-valyl-L-prolyl-L-arginine-4-methylcoumaryl-7-amide (Boc-VPR-MCA), and *t*-butyloxycarbonyl-l-valyl-l-leucyl-l-lysine-4-methylcoumaryl-7-amide (Boc-VLK-MCA) was adopted as a protease substrate for Rgp and Kgp, respectively. Ten microliters of *P. gingivalis* cell-free extract were added into the mixture containing 40 µL of 0.5 M Tris-HCl buffer (pH 7.6) and 40 µL of distilled water containing 5 mM of L-cystein. After 5 min preincubation at 37°C, 10 µL of 500 µM Boc-VPR-MCA or Boc-VLK-MCA was added to the mixture. The release of amino methyl-coumarin was measured with an excitation at 380 nm and emission at 440 nm using a fluorescence spectrophotometer (Infinite M 200 Pro, TECAN, Männedorf, Switzerland, Japan). The linear increase of amino methyl-coumarin

release was recorded for 10 min in the presence or absence of unsaturated fatty acids at various sub-MICs concentrations.

4.3 Results

4.3.1 The in-vitro inhibition of *P. gingivalis* and exo-hemagglutinins

In this study, ten fatty acids were selected with a varied carbon chain length, between ten to twenty, and different in its saturation degrees, to compare its inhibitory activity against *P.gingivalis* TDC60 planktonic cells and its exo-hemagglutinins exists in the culture supernatant using an *in vitro* experiment model. The results of *P. gingivalis* growth inhibition and inactivation of hemagglutinins of fatty acids are presented in Table 4.1. The fatty acids can be separated into four groups based on their chain length. The first group contains neodecanoic acid and decenoic acid with 10 carbons saturated and unsaturated, respectively. Both fatty acids could not inhibit the growth of *P. gingivalis*, while decenoic acid had a very weak inhibitory activity to the hemagglutinins with MIC at >100 $\mu g.mL^{-1}$. Palmitic and 2- hexadecenoic acids, which represented the second group with 16 carbons, suppressed the bacterial growth and hemagglutinins. However, the unsaturated fatty acid, 2- hexadecenoic acid, exhibited low MIC values for both tested parameters compared to that of saturated fatty acid. A similar trend was observed in the third group, which comprises four fatty acids with different degrees of saturation named stearic, oleic, linoleic, and linolenic acids with 18 carbon atoms. The unsaturated fatty acids showed very potent inhibitory activity against bacterial and hemagglutination with MICs values ranged from 2.5 – 6.3 $\mu g.mL^{-1}$, while the stearic acid, a saturated fatty acid, displayed a moderate influence against the hemagglutination with no antibacterial activity on *P. gingivalis*. The last group of selected fatty acids covering the 20 carbons fatty acids, a clear variation between saturated and unsaturated fatty

acids was observed. Only arachidonic acid, unsaturated fatty acid, exhibited an effective inhibitory activity against *P. gingivalis* growth and hemagglutination. Based on the accumulated data of MIC values for saturated and unsaturated fatty acids with different carbon chain lengths, unsaturated fatty acids were found to have a potent inhibitory activity compared to saturated fatty acids. Therefore, unsaturated fatty acids were selected to investigate its influence on the production of the virulence factors at sub-MICs concentrations.

4.3.1 Effects of sub-MICs of unsaturated fatty acids on *P. gingivalis* pathogenicity

The impact of sub-MICs concentrations of unsaturated fatty acids on *P. gingivalis* cell proliferation and production of exo-hemagglutinins (Fig.4.1A and B)., Except linolenic acid, all unsaturated fatty acids powerfully impeded the cell proliferation (<5%) and hindered hemagglutinin production at a sub-MIC concentration equivalent to ½ of MIC. The reduction of unsaturated fatty acids concentration to ¼ of MIC revealed fluctuated effects on bacterial growth and hemagglutinin secretion. Linoleic and arachidonic acid retained its ability to inhibit bacterial growth to <15% and totally inhibited hemagglutinins. A moderate bacterial growth was observed in the existence of oleic and linolenic acid compared to that of 2- hexadecenoic acid, in which no inhibition activity was observed at a concentration equal to ¼ of its MIC value. However, oleic acid and 2- hexadecenoic acid were inhibited hemagglutinins at a concentration corresponding to ¼ MIC but not linolenic acid. In the lowest concertation ($^1/_8$ of MIC), there was no influence on neither cell proliferation nor hemagglutinin in the addition of oleic acid, linolenic acid, and 2-hexadecenoic acid. However, linoleic acid and arachidonic acid were inhibited hemagglutinin production despite the high growth of *P. gingivalis* (Fig.4.1A and B).

Table 4.1: the invitro inhibitory activity of the selected fatty acids on *P. gingivalis* growth and hemagglutination

Fatty acid	Degree of saturation	Growth inhibition	Hemagglutinin inhibition
		MIC (µg. mL^{-1})	
Neo decanoic acid	10:0	ND	ND
Decenoic acid	10:1	ND	>100
Palmitic acid	16:0	50	13
2- hexadecenoic acid	16:1	>5	≤ 6.3
Stearic acid	18:0	ND	25
Oleic acid	18:1	2.5	>6.3
Linoleic acid	18:2	2.5	> 6.3
Linolenic acid	18:3	5	6.3
Arachidic acid	20:0	ND	ND
Arachidonic acid	20:4	2.5	6.3

The correlation between the cell proliferation and hemagglutinin production was confirmed by SDS-PAGE (Fig.4.1C) after partial purification using 80% ammonium sulfate of the exo-hemagglutinins in the culture supernatant. There were several bands detected in the positive control sample (*P. gingivallis* only), and similar bands were observed in the samples pre-incubated with the lowest concentration of unsaturated fatty acids or that of active hemagglutinin. These bands were disappeared or weaken in the samples exposed active hemagglutinin. The main bands

that might be important for the erythrocyte aggregation and detected in all samples with high hemagglutination activity were around 72.3 kDa, 53.8 kDa, 46.4 kDa, and 43.5 kDa proteins, in addition to 19.1 and 18.5 kDa.

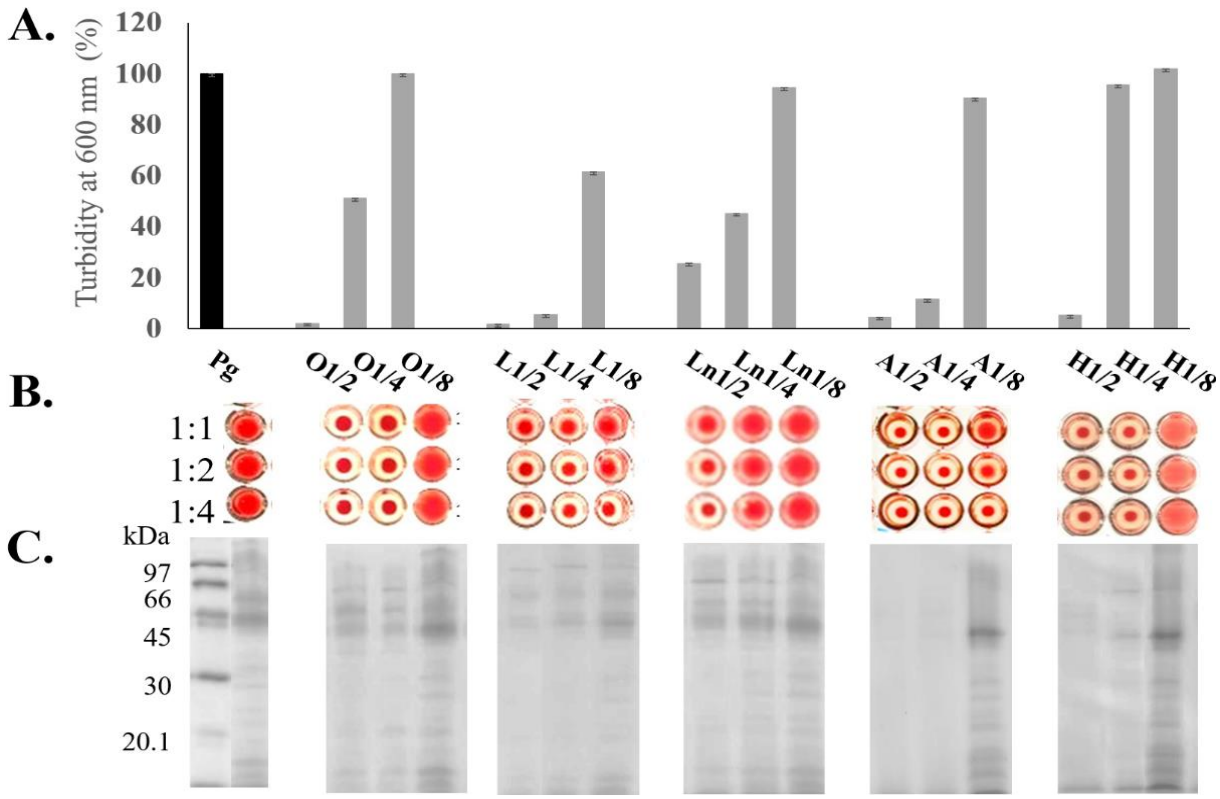

Fig.4.1 The influence of unsaturated fatty acids sub-MICs on *P. gingivalis* and its hemagglutination. **A:** inhibition of *P. gingivalis* proliferation. **B:** hemagglutination inactivation by unsaturated fatty acids sub-MICs. **C:** SDS-PAGE of hemagglutinin related proteins

4.3.3 Identification and molecular morphology of proteins with hemagglutination activity

To analyze partially purified hemagglutinations, N-terminal amino acid sequence analysis was performed for the entire bands appear in the SDS-PAGE of partially purified protein following 80% ammonium sulfate precipitation (Fig.4.2). The resulting N-terminal sequences were compared with those in the protein database using NCBI-BLAST program via NCBI webservers.

As presented in Fig.4.3, three protein sequences have multiple hemagglutinin related domains, and many repetitive sequences were observed as closest proteins. The 72.3 kDa and 53.8 kDa proteins showed homology to the middle sequence of the hemagglutinin domain in gingipain R1 hemagglutinin and Lys-gingipain protease. Meanwhile, the 46.4 kDa and 43.5 kDa proteins showed similar homology to the middle sequence of gingipain R1 hemagglutinin. The 19.1 kDa and 18.5 kDa proteins showed high homology to the middle sequence of the hemagglutinin domain, which is common to the three proteins gingipain R1 hemagglutinin, Lys-gingipain protease, and hemagglutinin.

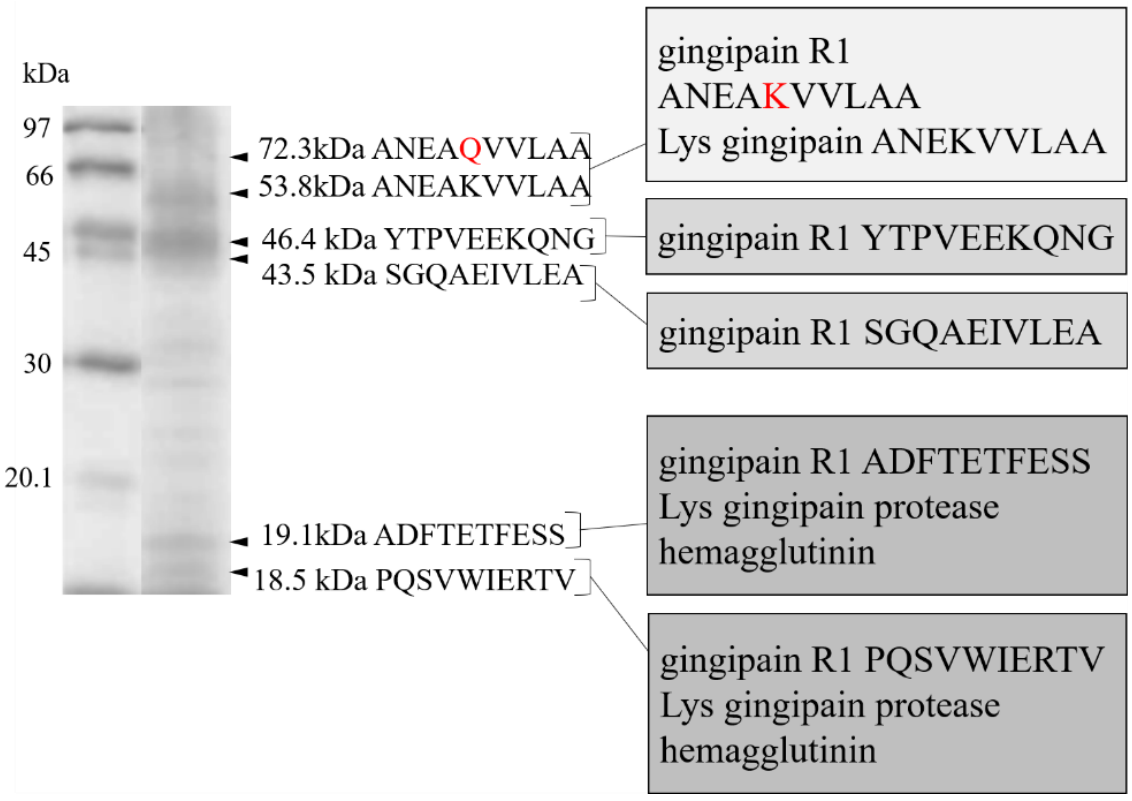

Fig.4.2 N-terminal amino acid sequence of the partially purified protein using 80% ammonium sulfate, bands appear in the SDS-PAGE were compared with those in the protein database using the NCBI-BLAST program.

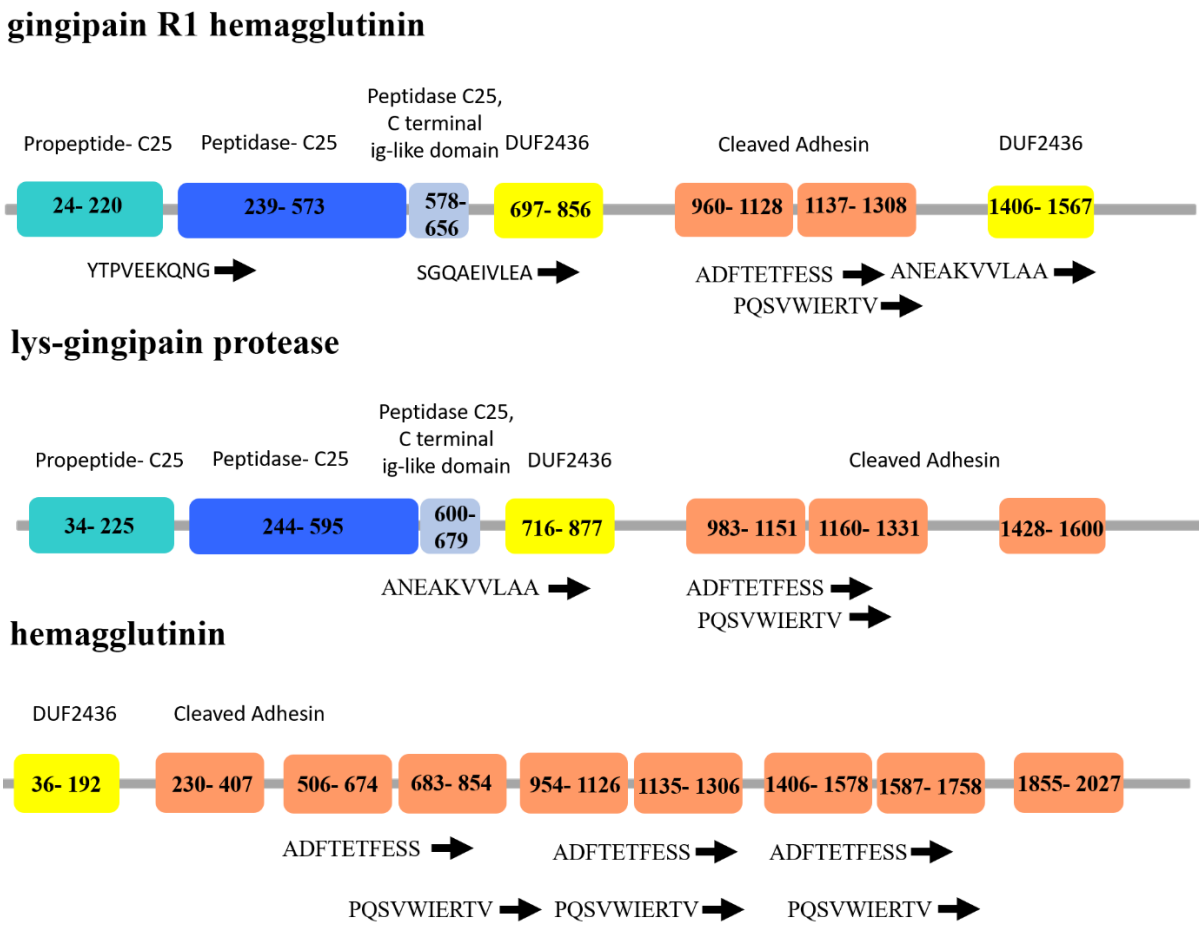

Fig.4.3 Sequence diagram of three proteins deduced from the N-terminal amino acid analysis of hemagglutinins related proteins.

4.3.4 Effects of sub-MICs of unsaturated fatty acids on *P. gingivalis* gingipains

The expression of gingipain R (Rgp) and gingipain K (Kgp) of *P. gingivalis* cell preincubated with sub-MICs concentrations of unsaturated fatty acid was determined and displays in Fig.4.4. Oleic and arachidonic acids blocked both Rgp and Kgp at entirely sub-MICs concentrations. A similar effect was obtained with linolenic and 2- hexadecenoic acids, which revealed a significant effect on gingipain R and K expression at $^{1}/_{2}$ and $^{1}/_{4}$ sub-MIC concentrations. In contrast, at $^{1}/_{8}$ sub-MIC concentration, linolenic acid suppressed 50% of Kgp and 80% of Rgp, while the same

level of 2- hexadecenoic acids was not impeded the gingipains of *P. gingivalis*. The unsaturated fatty acid linoleic acids showed a moderate influence on the gingipains activity at all sub-MIC concentrations with inhibition activity in the range of 65–61% and 25–50% for Kgp and Rgp, respectively.

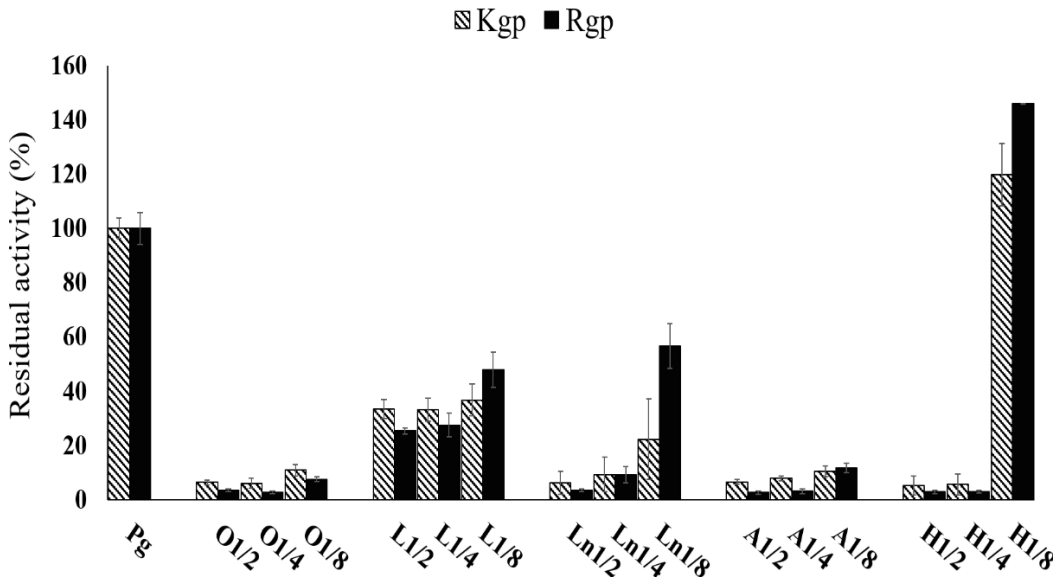

Fig.4.4 Impact of unsaturated fatty acids sub-MICs on *P. gingivalis* on gingipain R (Rgp) and gingipain K (Kgp), after the pre-incubation with unsaturated fatty acids sub-MICs

4.1 Discussion and conclusion

Hemagglutinins and gingipains protease are the main virulence factors of *P. gingivalis* for proliferation, colonization, and adherence to the host cells (Lewis et al., 2006; Olczak et al., 2008). Hemagglutinins are a large class of proteins that play a crucial role in nutrient acquisition and facilitation of erythrocyte binding following by absorption of heme, which has special significance because heme accelerates the growth of this bacterium (Sakai et al., 2007). Then allows the proteolytic gingipains to release the heme moiety from the hemoglobin molecule via the dual-

component uptake system HmuY/HmuR (Lewis et al., 2006; Olczak et al., 2008). Consequently, blocking these virulence factors might reduce and obstruct bacterial colonization and subsequent tissue inflammation (Labrecque et al., 2006; Wittschier et al., 2009; Wittschier et al., 2007). The antibacterial activity of the fatty acids has been reported against several oral pathogens including *P. gingivalis* (Sulijaya et al., 2019; Choi et al., 2013; Huang et al., 2011), but no study has been conducted to evaluate the impact of fatty acids on hemagglutinins and other virulence factors. Recently, we isolated oleic acid and its esters glycerols with inhibitory activity on *P. gingivalis* exo-hemagglutinins (Eltigani et al., 2019). Thus, in this study, we aimed to explore the antimicrobial and anti-hemagglutination of various long-chain fatty acids and saturation degrees and the expression of the proteins involved in hemagglutinating action by *P. gingivalis*. The in vitro impact of selected long-chain saturated and unsaturated fatty acids, with different carbon chain length, on *P. gingivalis* TDC60 strain and its outer cell membrane hemagglutinins illustrated in Table 4.1. The results revealed that unsaturated fatty acids exhibited high suppression of *P. gingivalis* proliferation in GAM broth media with MIC range from 5 to 2.5 $\mu g.mL^{-1}$ compared to that of saturated fatty acids. However, palmitic acid, a saturated fatty acid, showed moderate antibacterial activity at MIC 50 $\mu g.mL^{-1}$. This result agrees with the previously reported finding of Huang et al. (2011) and Choi et al. (2013), who found antibacterial activity from palmitic, oleic, linoleic, and arachidonic acids on *P. gingivalis* ATCC 33277 and 381. *P. gingivalis* TDC60 strain was found to be more aggressive in the pathogenicity than the other periodontal pathogenic bacteria such as *P. gingivalis* ATCC 33277 and 381 strains (Watanabe et al., 2011). Therefore, the current results will expand the knowledge on the function of long-chain fatty acid, particularly against the most aggressive strain that has been identified so far. The antibacterial activity of long-chain unsaturated fatty acids has been well-known as one of its properties for many years,

including oleic acid, linoleic acid, and linolenic acid, while long-chain saturated fatty acids, including palmitic acid and stearic acid, are reported as showing less antibacterial activity (Sun et al., 2003; Seidel and Taylor, 2004). In addition to cell growth and proliferation, *P. gingivalis* pathogen has several vital virulences such as hemagglutinins, which support the adhesion of bacterium to the host erythrocytes to obtain the nutrients like heme and amino acids. Hence, the use of antiadhesive materials to block the interaction between hemagglutinins and erythrocyte proteins could be one of the potent strategies to hamper periodontitis progression in the early stages (Miyachi et al., 2007; Yokoyama et al., 2007).

Concerning hemagglutinins inhibition, both saturated and unsaturated fatty acids revealed anti-aggregation of the erythrocytes in the *in-vitro* assay. Nonetheless, the longer unsaturated fatty acids had lower MIC values. Lately, unsaturated long-chain fatty acids gain important characteristics as anti-adhesive and antibiofilm formation beside their antimicrobial activity. This action is supposed to be related to the unsaturated form of the fatty acids and their physicochemical properties, which might reduce the adhesive work of *P. gingivalis* and control the progression of periodontitis (Sulijaya et al., 2019; Pan et al., 2019). On the other hand, many studies mentioned that outer cell membrane virulence factors have independent action in the inflammation process from cellular activities of *P. gingivalis* and suppression of bacterial growth does not require inhibition of other virulence factors productivity (Olsen and Potempa, 2014; Blankenvoorde et al., 1998). However, *P. gingivalis* secretes all these virulence factors to acquire nutrients from the surrounding environment. On those bases, the sub-MICs of unsaturated fatty acids suppressed bacterial growth has been studied for inactivation of hemagglutinins and gingipain secretion in different dosage manner equivalent to $^{1}/_{2}$, $^{1}/_{4}$ and $^{1}/_{8}$ of MICs values. The sub-MICs of unsaturated fatty acids decreased *P. gingivalis* growth (Fig.4.1A) through the inactivation of hemagglutinating related

proteins (Fig.4.1B). The influence of the unsaturated fatty acids on hemagglutination inhibition was confirmed by SDS page of the partially purified hemagglutinin containing sample (Fig.4.1C). Comprehensive analysis of the N-terminal amino acids of all bands of partially purified hemagglutinin secreted outside the cells revealed that six bands were proteins with a hemagglutinin domain. These proteins were derived from the translation products of three protein genes named gingipain R1 hemagglutinin, Lys gingipain protease, and hemagglutinin. The structure of these genes contains, in general, a propeptidase C25 sequence followed by a peptidase family C25 and several cleaved adhesin domains (Fig.4.3), which are well known through their function in bacterial cell adhesion (Li et al., 2010). The analyzed proteins equivalent to the band at, 46, 43, 72, 53, 18, and 19 kDa proteins were detected in activated and non-inactivated *P. gingivalis* hemagglutinin, confirming the influence of sub-MICs of unsaturated fatty acids on the synthesis process of hemagglutinins related proteins.

Interestingly, no band was detected on the native page of partially purified hemagglutinins (data not shown). The result was indicating that the hemagglutinin related proteins might construct a complex to perform aggregation functions. Since the N-terminal analysis revealed three genes possess proteases in addition to several cleaved parts.

This result pointed out the crucial role of gingipains and hemagglutinin for hemagglutination process. A similar finding was reported by Genco et al. (1999), who found that inactivation of gingipains R in the initial phase of the infection resulted in a weakening of *P. gingivalis* virulence in a mice experimental model. As well as Lys-gingipain protease, which implicated with Arg-gingipain protease and hemagglutinin/ adhesion domain in targeting host biological substrates for adhesive functions (Li et al., 2011). The expression of these proteases was influenced by the Sub-MICs of unsaturated fatty acids (Fig.4.2). Almost all unsaturated fatty acids inactivate both Kgp

and Rgp in the same means with antibacterial, and hemagglutination inhibition except linoleic acid showed similar reduction to Kgp and Rgp at all concentration dosages.

Inhibition of gingipains in the cellular protein of *P. gingivalis* supports our primary hypothesis that sub MIC of fatty acid significantly reduced the expression of related proteins. The current finding proposed that double-bond structure or double-bond position and length of the carbon chain are keys for fatty acids interaction with *P. gingivalis* proteins. However, several hypotheses suggested more than one theory for protein-fatty acids interaction, such as, that the hydrocarbon chain interacts primarily with hydrophobic residues of the protein. Additional view hypothesized that influence initiated through the interaction of the fatty acid carboxyl with basic amino acid residues of the bacterium proteins (Hamilton, 2002). Accordingly, we attempted to compare the same carbon number of the fatty acids (C10, C16, C18, and C20), and different in the presence of double-bond structure to inactivate hemagglutination action. Nevertheless, the mechanism of long-chain fatty acid interaction and inhibition on *P. gingivalis* hemagglutinins is still not clear and additional studies are required. However, the fatty acid affinity or to the multiple heterogeneous binding sites for each specific fatty acid; by close interaction of carboxylic group with the basic amino acid residue of the proteins through the H-bonded in their side chain (Hamilton, 2002).

In conclusion, the current study investigated the influence of saturated and non-saturated fatty acids with middle and long carbon chains against *P. gingivalis* growth and hemagglutinins. The long-chain unsaturated fatty acids exhibited high inhibitory activity compared to that of saturated fatty acids. Furthermore, the sub-MIC dosages of unsaturated fatty acids surprised secretion of hemagglutinin and gingipains of *P. gingivalis* TDC60. Therefore, the results presented here will expand the uses of fatty acids to impede the undesired microbes. Moreover, most of the selected fatty acids found abundantly in the natural products. Therefore, it is safe additive agents, especially

with the low MIC values level required for the inhibition process. The mode of action of these fatty acids against virulence factors, is not clear yet, more studies will be done to analyze the structure-function of these fatty acids and *P. gingivalis* virulence.

CHAPTER FIVE

Argeloside I a candidate hostile to pathogenicity of *Porphyromonas gingivalis* TDC60

5.1 Introduction

Periodontal diseases are chronic inflammatory diseases of tooth-supporting tissues which weaken these tissues and lead to tooth loss (Huq et al., 2013; Haffajee et al., 2013). Among bacterial pathogens, *Porphyromonas gingivalis* has recently proposed to be a major pathogen that causes destructive periodontal diseases (Haffajee et al., 2013). This bacterium adheres to host tissues to obtain nutrients for growth. The degradation of host proteins and accretion of dental plaque enables this bacterium to survive and produce virulence factors (Beck et al., 2013; DeStefano et al., 2013). The main virulence factors in the primary stage of pathogenicity are hemagglutinins and gingipains located on cell surfaces and on vesicles (Holt and Bramanti, 2013; O'Brien-Simpson et al., 2001). Hemagglutinins are a group of proteins that allow *P. gingivalis* to adhere to the surfaces of host cells to facilitate the acquisition of heme through erythrocyte binding, causing the lysis and aggregation of erythrocytes via several cysteine proteinases known collectively as gingipains (Curtis et al., 1999; Bhogal et al., 1997). Gingipains are mainly Arg–specific gingipains (Rgp) and Lys–specific gingipains (Kgp) and are also located in the outer cell membrane (Holt and Bramanti, 2013; O'Brien-Simpson et al., 2001). They play important roles in the acquisition and storage of the essential micronutrient iron and release the heme moiety from hemoglobin as a nutrient (Lewis et al., 2006; Olczak et al., 2008). Several strains of *P. gingivalis* have been identified (Watanabe et al., 2011; Naito et al., 2001; Nelson et al., 2003). We focused on the TDC60 strain to screen for compounds capable of inhibiting the pathogenicity of *P. gingivalis* as this strain was isolated from a severe periodontal lesion and its complete genomic

sequence is available (Watanabe et al., 2011). Strain TDC60 is more highly pathogenicity than other strains and cause severe abscesses in mice. We hypothesized that inhibition of the growth of TDC60 and/or its virulence factors might inhibit the growth of all *P. gingivalis* strains, thereby providing a cure for periodontal disease. The pathogenicity of the TDC60 strain has not been thoroughly investigated, despite its importance.

The therapeutic effects of a wide range of plant secondary metabolites, such as alkaloids, polysaccharides, glycosides, phenolic compounds, and sterols (Rahul et al., 2019), have been reported. Several studies have reported the specific inhibitory activities of polyphenolic compounds from green tea and cranberry against *P. gingivalis* and its secreted virulence factors (Feghali et al., 2012; Kushiyama et al., 2009). Moreover, oleic acid and its esters, together with coumarins from *Monechma ciliatum* seeds, have been reported as hemagglutinin inhibitors (Eltigani et al., 2019). We previously attempted to inhibit the growth of *P. gingivalis* TDC60 strain using aqueous extracts of Sudanese medicinal plants and found that *Solenostemma argel* leaf extracts potently inhibit *P. gingivalis* TDC60 (Eltigani et al., 2019).

Solenostemma argel, belongs to the family Apocynaceae and is widely grown in northern Africa, including Sudan, Egypt, Libya, and Algeria (Orange, 1982; Ahmed, 2004). In Sudan, *S. argel* is an important traditional medicine and is locally known as 'hargel' (Orange, 1982). The plant is widely distributed between Dongola and Barber, mainly around the Abu-Hamad area (Sulieman et al., 2009; Teia, 2018). *S. argel* leaf extract is used to make popular drink in Sudan. It is also used in traditional medicine to treat several diseases, including liver and kidney pain, bronchitis, neuralgia, gastrointestinal cramps, and stomach colic. The dry leaves are used as incense to treat measles and aid wound healing (Orange, 1982; Sulieman et al., 2009; Kamel et al., 1982; Hassan

et al., 2001). Several studies have reported that *S. argel* leaves contain various compounds such as quercetin, rutin, flavanones, alkaloids, flavonoids, and kaempferol (Tigani. and Ahmed, 2009; Shafek and Michael, 2012). In the present study, we isolated a compound that exhibits inhibitory activity against *P. gingivalis* TDC60 and its virulence factors from aqueous extracts of *S. argel*.

5.2 Materials and methods

5.2.1 Aqueous extraction of leaves

Solenostemma argel leaves were obtained in Khartoum, Sudan. The leaves were authenticated at the Faculty of Agriculture, University of Khartoum. Soluble compounds were extracted twice with distilled water from cleaned and powdered leaves (1 g sample: 10 mL water) for 30 min at room temperature with slow shaking. The supernatants were collected by filtration followed by centrifugation and lyophilization. The stock solution was prepared from the dried extract in distilled water at a concentration of 100 mg·mL^{-1}, followed by filtration using sterilization filters (MILLEX® GV 0.22 μm; Merk Millipore Ltd., Burlington, MA). The filtered stock was stored at –20 °C until analysis.

5.2.2 Cultivation of *P. gingivalis*

Porphyromonas gingivalis TDC60 (Watanabe et al., 2011) was provided by the RIKEN BioResource Center through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology, Japan. The bacterium was grown in tryptic soy blood agar medium (Agar Base EH) supplemented with hemin (5 mg·L^{-1}), menadione (1 mg·L^{-1}), and horse blood at 37°C under anaerobic conditions for 4–5 days (Gao et al., 2010; Rangarajan et al., 2017). The cells were then inoculated into 5 mL of Gifu anaerobic medium (GAM broth medium)

(Umemoto et al., 1996) and incubated under anaerobic conditions at 37°C for 2–3 days until the middle stationary phase of bacterial growth. The growth rate was evaluated at 600 nm using spectrophotometer. The cells were harvested by centrifugation and the culture supernatant (Pg-sup) was used for inhibition assays.

5.2.3 *P. gingivalis* growth inhibition

The influence of *S. argel* leaf aqueous extract on *P. gingivalis* growth was investigated by measuring the turbidities of bacterial suspensions in a 96-well microplate. Ten microliters of plant extract (10 mg·mL^{-1}) was added to 200 µL of *P. gingivalis* suspension standardized at 2 x10^{7} CFU (Kariu et al., 2017). The plates were incubated anaerobically at 37°C for 60 h. The turbidity was then measured at 600 nm using a microtiter plate reader (Model 680; Bio-Rad Laboratories Inc., Hercules, CA).

5.2.4 Hemagglutination assay

Erythrocyte aggregation was performed as described by Saiki and Konishi (Saiki and Konishi, 2007) with slight modifications. Horse defibrinated blood (Cosmo Bio Co., Ltd., Tokyo, Japan) was rinsed three times with phosphate buffer saline (PBS) using a pipette prior to centrifugation for 5 min at 2,000 ×g. The washed erythrocytes were diluted to 5% (v/v) with PBS and used for the hemagglutination assay. The hemagglutination test was performed in a 96 well microtiter plate (round bottom) using a reaction mixture consisting of 80 µL PBS, 10 µL Pg-sup, and 10 µL inhibitory compound. The mixture was shaken thoroughly and incubated for 10 min at room temperature, after which 100 µL 5% blood was added to the assay mixture. Hemagglutination was

evaluated visually after incubation at room temperature for 2 h. PBS was used instead of Pg-sup as a negative control.

5.2.5 Protease inhibition assay

The protease inhibitory activity of the purified compound (compound **2**) was determined as described previously (Kariu et al., 2017). Compounds exhibiting inhibitory activity to gingipains were identified by enzyme assays with *t*-butyloxycarbonyl-L-valyl-L-prolyl-L-arginine-4-methylcoumaryl-7-amide (Boc-VPR-MCA) and *t*-butyloxycarbonyl-L-valyl-L-leucyl-L-lysine (Boc-VLK-MCA) as substrates for Rgp and Kgp, respectively. Ten microliters each of Pg-sup and compound **2** were added to mixtures containing 40 μL of 0.5 M Tris-HCl buffer (pH 7.6) and 30 μL of distilled water containing 5 mM of L-cysteine. After 5-min preincubation at 37°C, 10 μL of 500 μM Boc-VPR-MCA or Boc-VLK-MCA was added to the mixture. The release of aminomethyl-coumarin was measured by excitation at 380 nm and emission at 440 nm using a fluorescence spectrophotometer (Infinite M 200 Pro; Tecan Group, Ltd., Männedorf, Switzerland). The linear increase in aminomethyl-coumarin release was recorded for 10 min in the presence or absence of purified compound at different concentrations, and the half maximal inhibitory concentration (IC_{50}) was calculated from the obtained dose-response curve.

5.2.6 Minimum inhibitory concentration and half inhibitory concentration

The minimum inhibitory concentration (MIC) was calculated from the final concentration of the lowest dilution with complete inhibitory activity. For the minimum inhibitory concentration of the purified fractions, a 10 $mg \cdot mL^{-1}$ stock solution was prepared in methanol. For the hemagglutination assay, each fraction was diluted directly in a microplate with a round bottom

containing the hemagglutination assay reaction mixture to obtain a final concentration ranging from 0.5 to 0.016 mg·mL^{-1}. The MIC was calculated from the lowest concentration of the fraction that completely inhibited hemagglutination activity. Each test was conducted in triplicate and the concentration required for 50% inhibition of viability (IC_{50}) was determined. For the growth inhibition assay, the fractions were diluted in separate vials, then used in the reaction mixture.

5.2.7 Isolation of biologically active compounds

Soluble compounds were extracted from about 200 g of dried and powdered leaves by soaking in 2.0 L distilled water twice for 30 min at room temperature with slow shaking. The soluble extracted compounds were collected by filtration, followed by centrifugation. The entire extract was partitioned between distilled water and ethyl acetate three times, yielding 3.24 g of ethyl acetate extract. The ethyl acetate extract was further fractionated by normal-phase chromatography. The column was packed with 162 g of silica gel powder (Wakogel C-300; Wako Pure Chemical Industries, Osaka, Japan) and eluted with acetone/hexane (0%–100%, v/v, 20% increments). The fraction eluted with 40% acetone (active fraction) yielded 1.65 g. This fraction was subjected to reversed phase chromatography with ODS gel (Cosmosil 75C18-OPN; Nacalai Tesque, Kyoto, Japan) eluted with methanol in water (0%–100%, v/v, 20% increments). The 80% methanol fraction (0.904 g) exhibited the highest inhibitory activity. HPLC fractionation of the 80% methanol fraction was performed under the following conditions: elution solvents, Milli-Q (A) and acetonitrile (B); gradient elution, 25%–100% B over 60 min; column, Cosmosil C18, (ø 20 ID × 250 mm); flow rate, 7.0 mL·min^{-1}; temperature, 40°C. The fractionation resulted in five sub-fractions (Fr **1**, **2**, **3**, **4** and **5**).

5.2.8 Identification of biologically active compounds

All isolated compounds were tested to evaluate their inhibitory activity and the major compound was analyzed by ^{1}H- and ^{13}C NMR spectroscopy (Avance II; Bruker Billerica, MA). ESI MS analysis was performed with a mass spectrometer (Micromass Quattro; Waters, Milford, MA) combined with an ultra-performance liquid chromatography system in positive mode. The mass range was *m/z* 200–800 and the column was an ACQUITY UPLC BEH C18 column (2.1 × 50 mm, 1.7 μm; Waters). The solvents were acetonitrile containing 0.1% formic acid and Milli-Q containing 0.1% formic acid with gradient elution from 5% to 100% for 10 min. The flow rate was 0.2 mL·min^{-1} and the column temperature was 40°C. HRMS was recorded using an Exactive Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA).

5.3 Results

5.3.1 Isolation of compound with inhibitory activity against the growth of *P. gingivalis* TDC60

We recently screened the aqueous extracts of a group of Sudanese medicinal plants for growth inhibition activity against *P. gingivalis* TDC60 strain by adding the extracts to GAM broth culture medium at a concentration of 1 mg·mL^{-1}. The aqueous extract of dry leaves of *S. argel* totally inhibited the growth of this bacterial strain (Fig.5.1). Furthermore, analysis of inhibitory activity at different concentrations revealed that the aqueous extract of *S. argel* suppressed bacterial growth with a MIC of 0.5 mg·mL^{-1}. We isolated the inhibitory compounds by extracting *S. argel* leaves with distilled water, followed by several purification steps, including liquid-liquid separation and column chromatography. Compounds in the active fraction were purified using HPLC. Five peaks were isolated, and the fractions were dried and weighed. The compounds were serially coded as

compounds **1**, **2**, **3**, **4**, and **5** (Fig.5.2). Compound **2** corresponded to the major peak (Fig.5.2) and was obtained in higher yield (70.6 mg) and inhibitory activity than the other fractions (Table 5.1).

5.3.2 Structure determination

Compound **2** was a white amorphous powder. The ^{1}H- and ^{13}C-NMR data of this compound showed the presence of aglycone and sugars. The ^{1}H NMR spectrum of the aglycone part is shown in Fig.5.3A and the assignments are given in Table 5.2. Signals were observed for three methyl groups at 1.03 (3H, s), 1.16 (3H, s), and 1.62 (3H, s), one olefinic proton at d 5.39 (1H, d, *J* = 3.0 Hz), and one signal at 4.53 (1H, s) equivalent to a proton binding to a oxygenated carbon. Furthermore, the ^{13}C NMR spectrum (Table 5.3 and Fig.5.3B) of the aglycone moiety proposed the presence of a pregnane skeleton providing twenty-one signals.

The ^{1}H NMR spectrum for the sugar part showed four doublet signals corresponding to methyl groups at δ_H 1.39 (3H, d, *J* = 6.1 Hz), 1.29 (3H, d, *J* = 6.1 Hz), 1.25 (3H, d, *J* = 6.2 Hz) and 1.25 (3H, d, *J* = 6.1 Hz), as well as three methoxy groups at δ_H 3.63 (3H, s), 3.45 (3H, s) and 3.42 (3H, s). In addition, four signals for anomeric protons were observed at δ_H 4.77 (1H, d, *J* = 9.6 Hz), 4.69 (1H, d, *J* = 9.6 Hz), 4.61 (1H, d, *J* = 8.1 Hz) and 4.44 (1H, d, *J* = 7.8 Hz). These data demonstrated the presence of four sugars in compound **2**. HR-ESI-MS of compound **2** (Fig.5.3C) (*m/z* 947.4954 $[M + Na]^+$, calcd for $C_{48}H_{76}O_{17}Na$, 947.4980) supported a molecular formula of $C_{48}H_{76}O_{17}$. On the basis of the ^{1}H- and ^{13}C-NMR and HR-ESI-MS data, we performed database search, and found that the NMR spectra of compound **2** was identical to those of argeloside I, (14*S*,16*S*,20*R*)-14,16-14,20-15,20-triepoxy-14,15-secopregn-5-en-3-ol-3-*O*-β-D-thevetopyranosyl-(1→4)-β-D-oleandropyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-

canaropyranoside (Fig.5.2), which was previously isolated and identified from *S. argel* hairy seeds (Plaza et al., 2005).

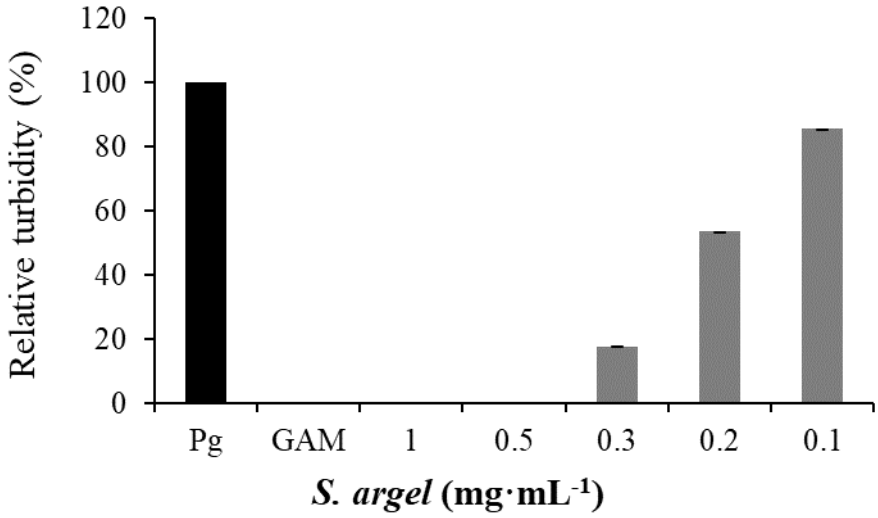

Fig.5.1 The inhibitory activity of *S. argel* aqueous extract on *P. gingivalis* proliferation

Table 5.1: Inhibition activities and yields of respective peaks on Fig.5.2

Peak	Yield (mg)	Antibacterial MIC (μg·mL^{-1})	Hemagglutination inhibition MIC (μg·mL^{-1})	Gingipain inhibition IC_{50} (μg·mL^{-1})	
				Rgp	Kgp
1	46.6	25	100	-	-
2	70.6	15	60	400	500
3	24.1	100	-	-	-
4	18.5	100	-	-	-
5	17.3	5	-	-	-

Where: -: no inhibition activity was detected up to 500 μg·mL^{-1}

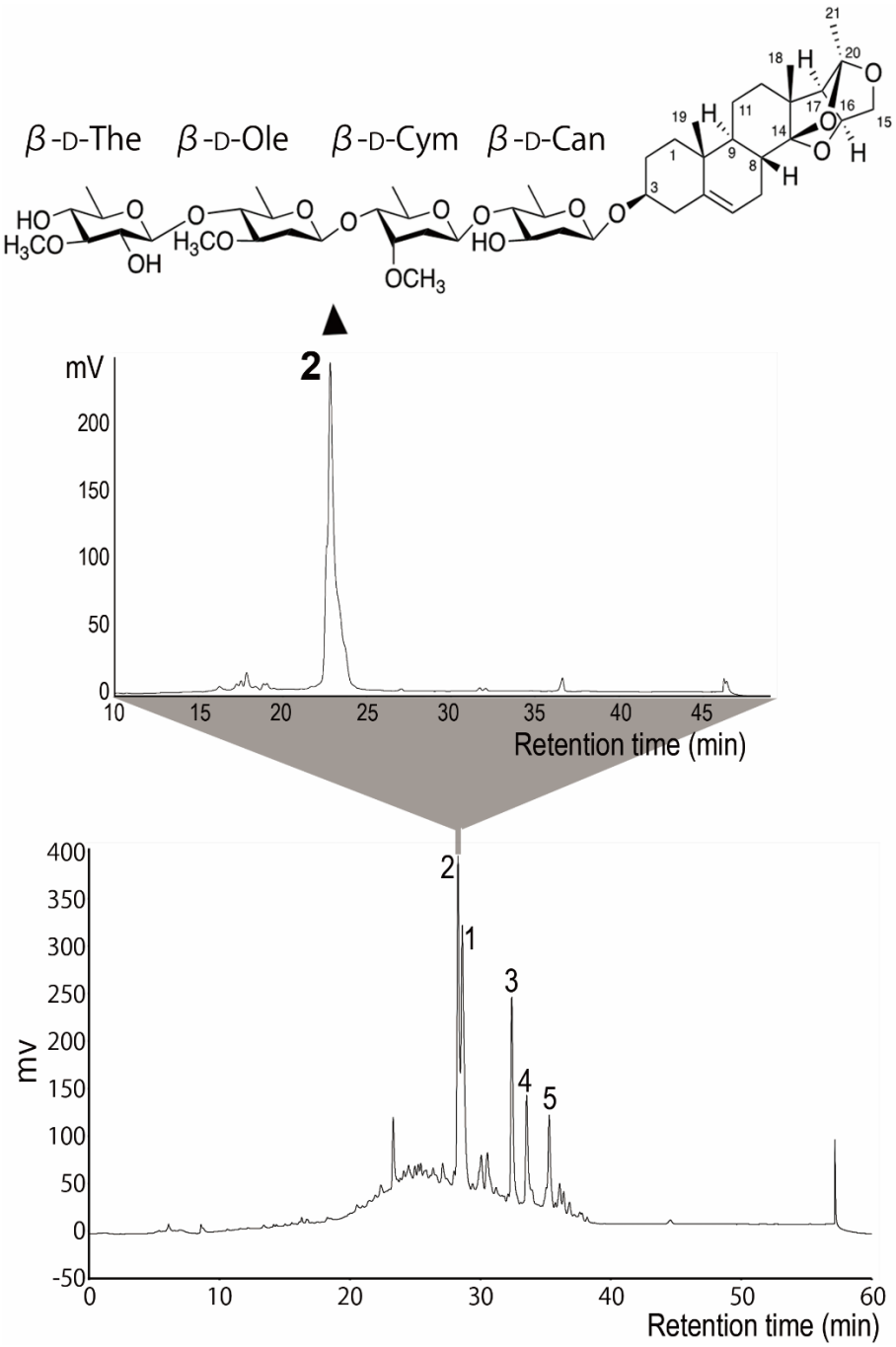

Fig.5.2. Isolation and purification of argeloside I. The bottom panel shows the HPLC chromatogram of the 80% methanol fraction obtained by ODS column chromatography during the purification of argeloside I. The middle and top panels show the HPLC chromatogram of the isolated compound and the proposed structure, respectively.

Table 5.2: ^{1}H NMR data (600 MHz, CD_3OD) of **2** and argeloside I

Position	Compound **2** δ_H, multi, *J* in Hz	Argeloside I[a)] δ_H, multi, *J* in Hz
Sugars		
β-D-Can		
1	4.69, d, *J* = 9.6 Hz	4.72 d, *J* = 9.2, 1.5 Hz
6	1.25, d, *J* = 6.0 Hz	1.27, d, *J* = 6.1 Hz
β-D-Cym		
1	4.77, d, *J* = 9.6 Hz	4.80, dd, *J* = 9.6, 2.0 Hz
6	1.25, d, *J* = 6.0 Hz	1.27, d, *J* = 6.1 Hz
MeO	3.45 s	3.47, s
β-D-Ole		
1	4.61, d, *J* = 8.4	4.63, dd, *J* = 9.6, 2.0 Hz
6	1.39, d, *J* = 6.1 Hz	1.40, d, *J* = 6.1 Hz
MeO	3.42, s	3.45, s
β-D-The		
1	4.44, d, *J* = 7.8 Hz	4.46, d, *J* = 7.9 Hz
6	1.29, d, *J* = 7.2 Hz	1.31, d, *J* = 6.1 Hz
MeO	3.63, s	3.65, s
Aglycone		
6	5.39, d, *J* = 3.0 Hz	5.43, dd, *J* = 3.3, 2.7 Hz
16	4.53, s	4.56, br m
17	2.49, d, *J* = 2.4 Hz	2.52, d, *J* = 2.2 Hz
18	1.16, s	1.20, s
19	1.03, s	1.06, s
21	1.62, s	1.65, s

Table 5.3: ^{13}C NMR data of **2** (150 MHz, CD_3OD) and argeloside I

δ_C of glycone part		δ_C of aglycone part	
2	Argeloside I (Plaza et al., 2005)	**2**	Argeloside I (Plaza et al., 2005)
104.3	104.3	141.1	141.3
102.3	102.8	122.5	122.4
100.6	100.7	114.9	114.8
98.9	98.9	110.3	110.6
88.8	89.1	79.3	79.0
87.5	87.9	78.2	78.0
83.9	84.3	71.7	71.9
83.9	83.8	58.6	58.9
80.2	80.2	50.0	49.9
78.3	78.3	46.9	46.9
76.7	76.5	39.8	39.6
75.6	75..7	38.5	38.1
73.2	72.9	37.1	37.3
72.5	72.6	32.2	32.1
71.7	71.8	30.9	30.9
70.7	70.9	30.4	30.4
70.4	70.5	25.8	25.6
61.02	61.1	23.8	23.5
58.8	58.7	20.8	20.4
57.6	57.6	19.6	19.3
40.0	40.2	15.5	15.5
37.7	37.7		
36.4	36.4		
18.9	18.8		
18.2	18.3		
18.1	18.2		
18.1	18.1		

5.3.3 Inhibitory characterization of argeloside I

The inhibitory activity of argeloside I against *P. gingivalis* growth was evaluated by measuring turbidity at 600 nm. The compounds greatly reduced cell proliferation and growth (Table 5.1) at a concentration of 10 $\mu g \cdot mL^{-1}$, and totally blocked cell growth and proliferation at 15 $\mu g \cdot mL^{-1}$ (Fig.5.4A) with an IC_{50} value of 8.8 $\mu g \cdot mL^{-1}$. Furthermore, we examined the inhibitory activity of argeloside I against *P. gingivalis* virulence factors in broth culture, and against exo-hemagglutinins and gingipains. This compound completely inhibited *P. gingivalis* exo-hemagglutinins, with a MIC value of 60 $\mu g \cdot mL^{-1}$ (Fig.5.4B). In addition, argeloside I suppressed Rgp activity, with an IC_{50} value of 400 $\mu g \cdot mL^{-1}$, and Kgp gingipains, with a slightly higher IC_{50} value of 500 $\mu g \cdot mL^{-1}$ (Fig.5.4C).

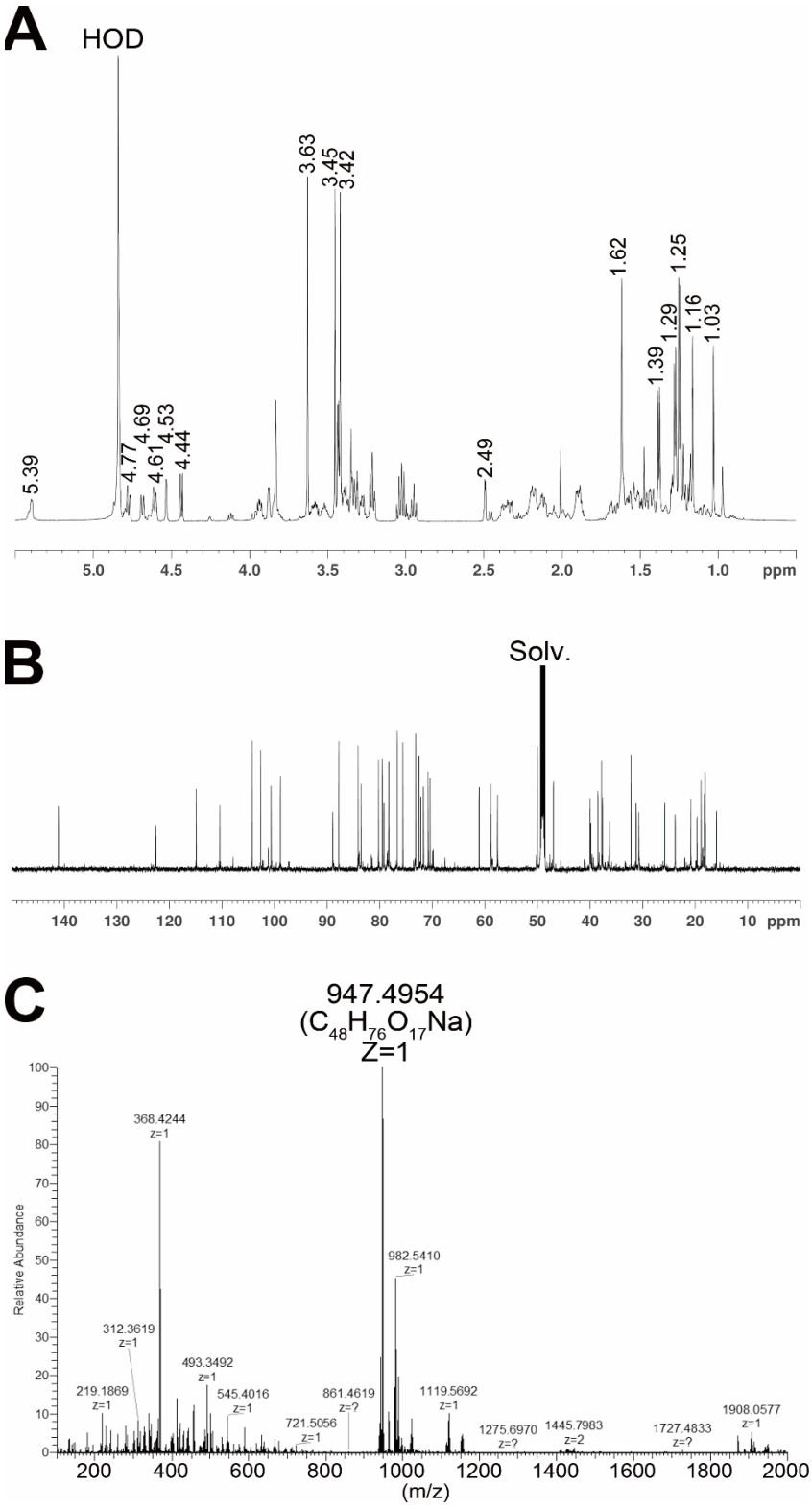

Fig.5.3 Spectroscopic analyses of compound **2,** which was subjected to ^{1}H NMR (A), ^{13}C NMR (B), and high resolution ESI mass spectroscopy (C).

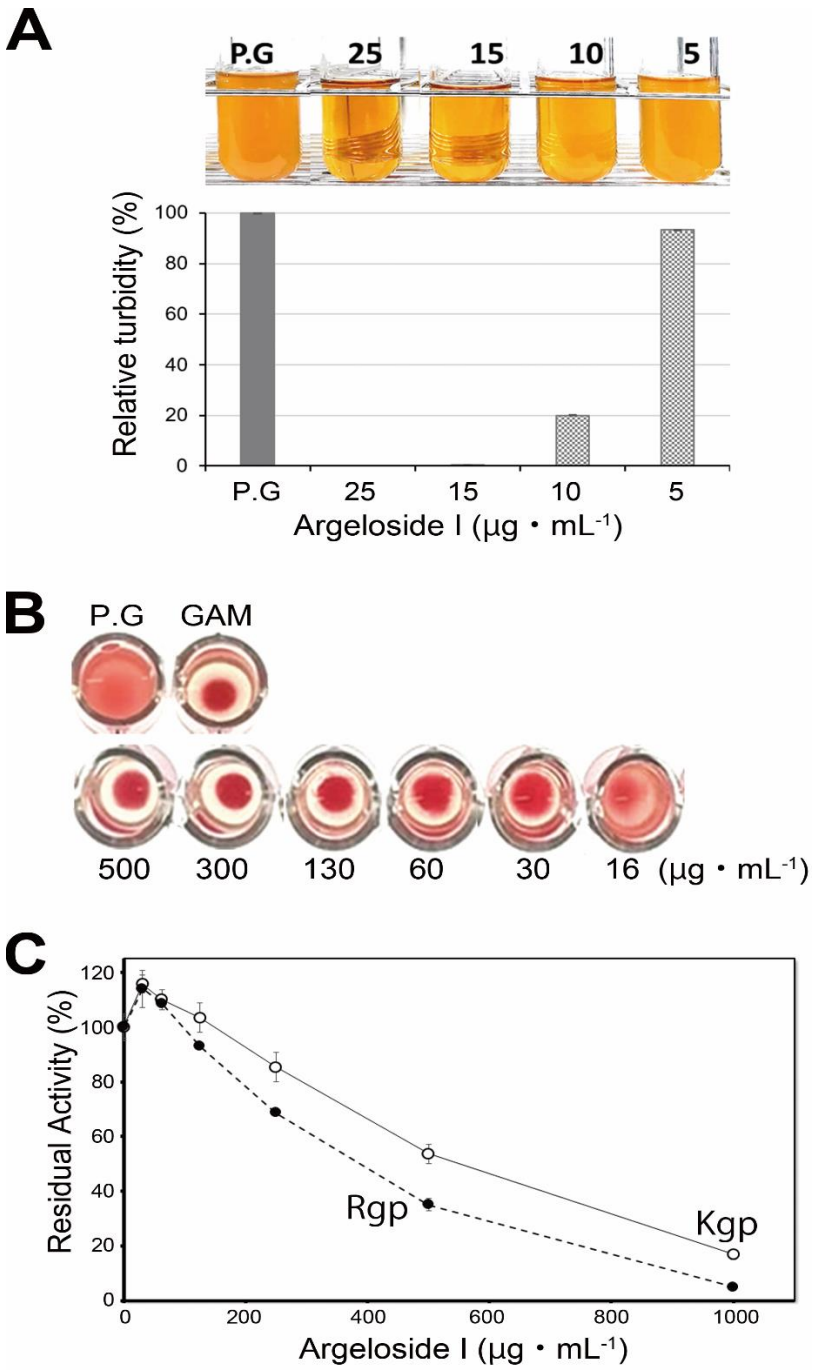

Fig.5.4 Inhibitory activity of argeloside I on *P. gingivalis* growth (A), hemagglutinins (B), and gingipains (C). A and B, P.G indicates *P. gingivalis* samples without argeloside I (negative control). C, each value represents the average of four independent experiments ± the standard deviation.

5.4 Discussion and conclusion

Porphyromonas gingivalis is an asaccharolytic bacterium that obtain nutrients and energy from the surrounding environment by adhering to host cells and degrading erythrocytes to extract iron as an essential nutrient in the very early stages of inflammation (Snipas et al., 2001; Shi et al., 1999; Dashper et al., 2004). Hemagglutinins and gingipains are the main virulence factors of this pathogen and their activities are independent of bacterial cellular programs and cell viability (Snipas et al., 2001). Therefore, inhibition of these virulence factors and/or bacterial growth will obstruct bacterial adhesion to the host cells and might enable manipulation of the interaction between hemagglutinins and gingipains with erythrocyte proteins to hinder periodontitis development in the early stages (Miyachi et al., 2007; Yokoyama et al., 2007).

Numerous studies over the past several decades have targeted these virulent factors to inhibit the primary stages of periodontitis caused by *P. gingivalis* strains such as W83 and ATCC33277 using natural therapeutic agents (Nakayama et al., 1996; Kadowaki et al., 1998). TDC60 strain was isolated from a patient with severe periodontal disease and displayed aggressive pathogenicity compared with W83, ATCC 33277, and other *P. gingivalis* strains (Watanabe et al., 2011). However, few studies have focused on the suppression of growth and virulence factors in TDC60. We therefore identified compounds in Sudanese medicinal plants that inhibit the growth of *P. gingivalis* TDC60 strain and its virulence factors (Eltigani et al., 2019). Of the tested plant extracts, only *S. argel* aqueous extract completely inhibited the growth of *P. gingivalis* TDC60 strain at a concentration of 1 $mg \cdot mL^{-1}$, and so in the present study, we aimed to isolate the agent responsible for this inhibiting activity.

Solenostemma argel aqueous extract is reported to have antimicrobial, antifungal, and antibacterial properties against different pathogens and suppresses the growth of *Aspergillus niger*, *Pennicilium italicum*, *Escherichia coli*, and *Salmonella typhi* (Sulieman et al., 2009; Teia, 2018). However, to our knowledge, the present study is the first attempt to identify an inhibitor of the periodontal pathogen *P. gingivalis* and its virulence factors in an aqueous extract from *S. argel* leaves. We focused on water extracts since they are regularly consumed as herbal teas in Sudan and other African countries and are also used as folk remedies (Tharib et al., 2008; Taj Al- Deen et al., 2014). Compared with organic extracts, water extracts will have the potential to expand the conventional applications of this plant to functional foods, particularly in the rural communities.

The ^{1}H NMR spectra of isolated compounds **1**, **2**, **3**, **4** and **5** showed that they are pregnane glycosides although only the structure of **2** was determined. The isolated pregnane glycosides showed different levels of antibacterial activity against the periodontal pathogen *P. gingivalis* TDC60 and its pathogenicity factors (Table 5.1). Compound **2** exhibited inhibition activity against the tested hemagglutinin and gingipain virulence factors. We therefore focused on the inhibitory activity of **2,** previously identified and named argeloside I (Plaza et al., 2005).

Argeloside I belongs to a large group of secondary metabolites known as pregnane glycosides. Pregnane glycosides are polar compounds consisting of combinations of steroidal aglycones and glycone parts of oligosaccharide chains arranged in a linear manner. The sugar chain is linked to the aglycon part through an acetal linkage to an aglycon hydroxy group (Panda et al., 2006). Recent studies have indicated a tremendous potential for medicinal applications of pregnanes because they exhibit anticancer activity and inhibit the proliferation of Kaposi's sarcoma cells (Plaza et al., 2005; Panda et al., 2006).

The isolated pregnane glycosides exhibited inhibitory activity against *P. gingivalis* cells, with low MIC as shown in Fig.5.4. The inhibition by this type of compounds may be due to the aglycone part. A structure-activity relationship analysis by Liu (Liu et al., 2003) with different analogs suggested that the aglycone part of such compounds strongly influences activity. Another study reported that the degree of inhibition depends on the aglycone type of pregnane glycosides and the sequences of sugar moieties (Panda et al., 2006). However, the relationship between pregnane glycosides and the inhibition of *P. gingivalis* cell growth remains unclear. Further investigation using various pregnane glycosides with different sugar lengths is required to identify the importance of each part on the inhibition mechanism.

Porphyromonas gingivalis produces several extracellular virulence factors (Gharbia and Shah, 1993; Okuda et al., 1986). These virulence factors include hemagglutinin and gingipains which are inhibited by argeloside I (Fig.5.4B and 5.4C). Hemagglutinin domains enable *P. gingivalis* to adhere to and colonize the tooth surface and/or the periodontal pocket to obtain heme by aggregating host erythrocytes, while gingipains provide nutrients for this bacterium to survive and proliferate. A recent study reported a strong correlation between Alzheimer's disease and *P. gingivalis* via gingipains, which is promotes neuronal damage (Dominy et al., 2019). Dominy and colleagues found that gingipain immunoreactivity in the brain of Alzheimer's disease patients was significantly greater than in non- Alzheimer's disease control individuals, suggesting that gingipains are involved in Alzheimer's disease in the early stage of brain infection by *P. gingivalis* (Dominy et al., 2019). Moreover, hemagglutinin is involved in *P. gingivalis* adhering to coronary artery endothelial cells, leading to cardiovascular disease (Lepine, and Progulske-Fox, 1996). The suppression of gingipain and hemagglutinin activity might also reduce the concentrations of peptide substrates and micro-nutrients available to *P. gingivalis*, hampering periodontitis

progression and potentially suppressing the development of other related diseases (Dominy et al., 2019; Ingar and Potempa, 2014). Several studies have shown importance of inhibiting virulence factors for oral health but the total suppression of *P. gingivalis* growth has not been demonstrated. Thus, it is reasonable to assume the involvement of other proteins in the growth strategy of this bacterium (Blankenvoorde et al., 1998). Accordingly, the suppression of cell growth, hemagglutination, and gingipain activity by argeloside I should offer an opportunity to develop effective new inhibitors. Such combined inhibition might be due to the effect of argeloside I on cellular programs as well as reducing or influencing the production of virulence factors.

In conclusion, this report demonstrates that *S. argel* aqueous extract diminishes the proliferation of the TDC60, the most aggressive strain of *P. gingivalis*. Argeloside I was purified from the aqueous extract and shown to have anti-periodontitis activity by decreasing the growth of the bacterial pathogen *P. gingivalis* and the activity of its virulence factors.

General conclusions

Periodontal diseases are a complex illness situation result from multi-microbial infections. *P. gingivalis* is among the pathogenic bacteria associated with the initiation of the gingival tissue inflammation, leading to the formulation of dental pockets, which form a suitable atmosphere for bacteria colonization, then the destruction of tooth-supporting tissues and finally loss of teeth. The primary stage of *P. gingivalis* inflammation initiates by adhesions to the host cells following by producing numerous virulence factors such as lipopolysaccharide, fimbriae, hemagglutinins, and several proteolytic enzymes. The proteolytic enzymes, which are known as gingipains and hemagglutinins, play a major role in *P. gingivalis* virulence since they are adherence factors that promote colonization and degrade host tissues. In addition, these enzymes may be crucial for providing heme and amino acids essential for the growth and proliferation of this asaccharolytic organism. The most recent strategies to inhibit periodontal disease by targeting the virulence factors of *P. gingivalis* in the early stages of the inflammation might hinder pathogenicity and improve the expansion of new, safe, effective therapeutic and preventative agents.

In the last decades, numerous plants and their natural products have been used around the world to treat several health disorders, including oral diseases. In Africa and particularly in Sudan, more than 80% of the population relies on traditional medicine to treat health problems for their abundance, safety, and fewer side effects. In this study, thirty-eight water extracts of selected plants used in Sudanese folkloric remedy were evaluated against *P. gingivalis* proliferation and its pathogenicity mediators. Several aqueous extracts exhibited a powerful role in blocking the very early stages of *P. gingvalis* virulence-related factors. Many studies reported that it is a challenging and complicated to impede all the virulence-related factors of *P. gingivalis.*

Nonetheless, the extract of *O. vulgare* leaves revealed multi-inhibition effects on the tested assays. The hydrolysable tannin from *O. vulgare* leaves showed a broad spectrum, impeding the targeted virulence-related factors. On the other hand, the isolated compounds from *M. ciliatum* seeds had a specific effect on the hemagglutinins of *P. gingivalis*. Coumarin, oleic acid, and its esters were the leading compounds against hemagglutinins. Since oleic acid revealed the lowest MIC value compared with other isolates, we subsequently compared the influence of saturated and non-saturated fatty acids with middle and long carbon chains against *P. gingivalis* and hemagglutinins. The long-chain unsaturated fatty acids exhibited high inhibitory activity compared to that of saturated fatty acids. Additionally, sub-MIC dosages of unsaturated fatty acids suppressed secretion of hemagglutinin and gingipains of *P. gingivalis* TDC60. In contrast, *S. argel* leaves aqueous extract had the most substantial influence on *P. gingivalis* growth since it diminished the proliferation of the most aggressive strain of *P. gingivalis* (TDC60 strain). Argeloside I purified from the aqueous extract of leaves were found to have anti- hemagglutinins and anti- gingipains activities besides its capability to suppress the growth of *P. gingivalis*.

Most of the selected medicinal plants in this study have been continuously used in folkloric medicine and as spices in daily cooking, which reflect their safety consumption. The pleasant aroma and pharmaceutically bioactive compounds of *O. vulgare* leaves and *M. ciliatum* seeds have received increasing attention as potent sources for the reformation of different foods and pharmaceutical products. Additionally, isolated hydrolysable tannins from *O. vulgare* leaves have a high polarity and water solubility, this feature might provide a suitable condition in the manufacturing applications as oral hygiene agents or functional food additives. Unsaturated fatty acids revealed a good influence on *P. gingivalis* and its virulence besides its nutritional roles. These

fatty acids found abundantly in natural products. Therefore, it is safe additive agents, especially with the low MIC values level required for the inhibition process.

Moreover, *S. argel* leaves aqueous extract is one of the popular drinks in many Arabic and African countries, including Sudan. It's believed to have superior effects against different health problems. However, anti- *p. gingivalis* pathogenicity function of the isolated compound (argeloside I) seems to be new. This function believed to be a starting point for expanding the use of *S. argel* extract and argeloside I. Overall, several compounds with potent influences on *P. gingivalis* pathogenicity were isolated and identified in the current dissertation, but their mode of action in the inhibition process still not clear and more studies are needed.

Appendices

^{1}H NMR of compound $_{8}$**A**

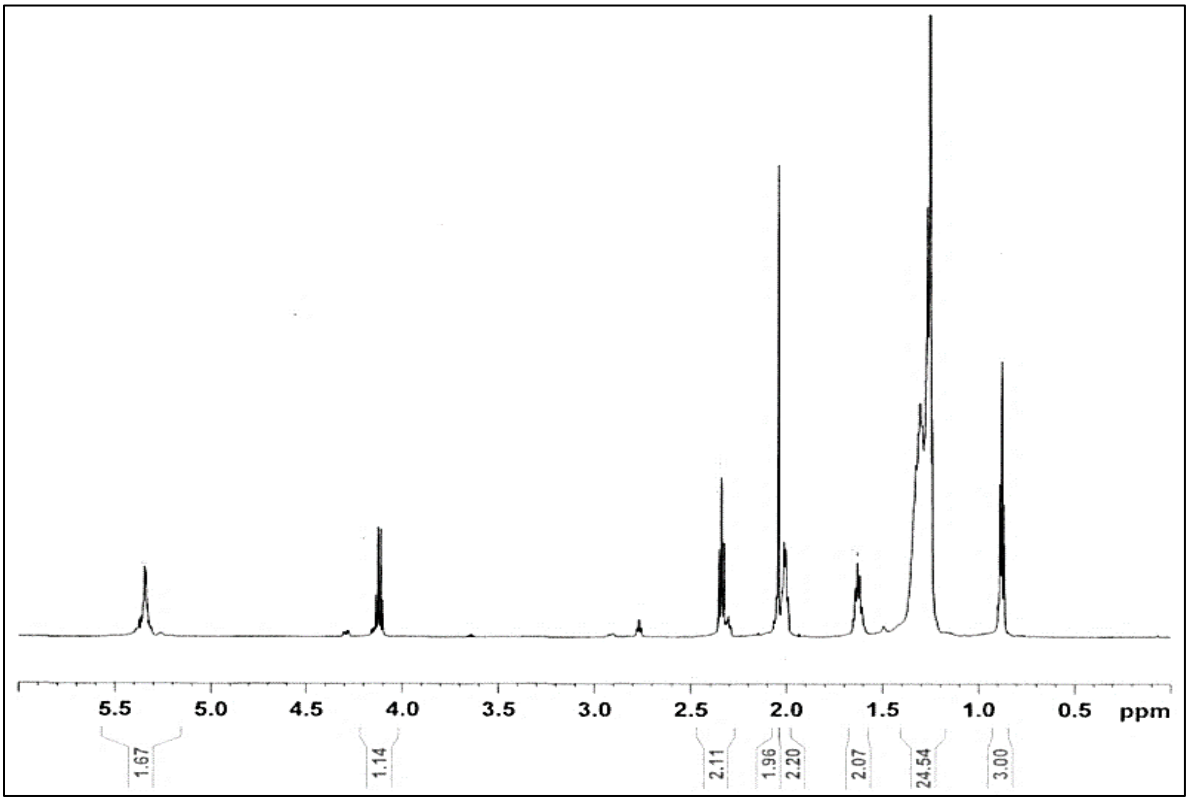

Fig.S1 the ^{1}H NMR spectrum of compound $_{8}$A (600 MHz, $CDCl_3$), which isolated from the aqueous extract of *Monechma ciliatum* seeds.

^{13}C NMR of compound $_{8}$**A**

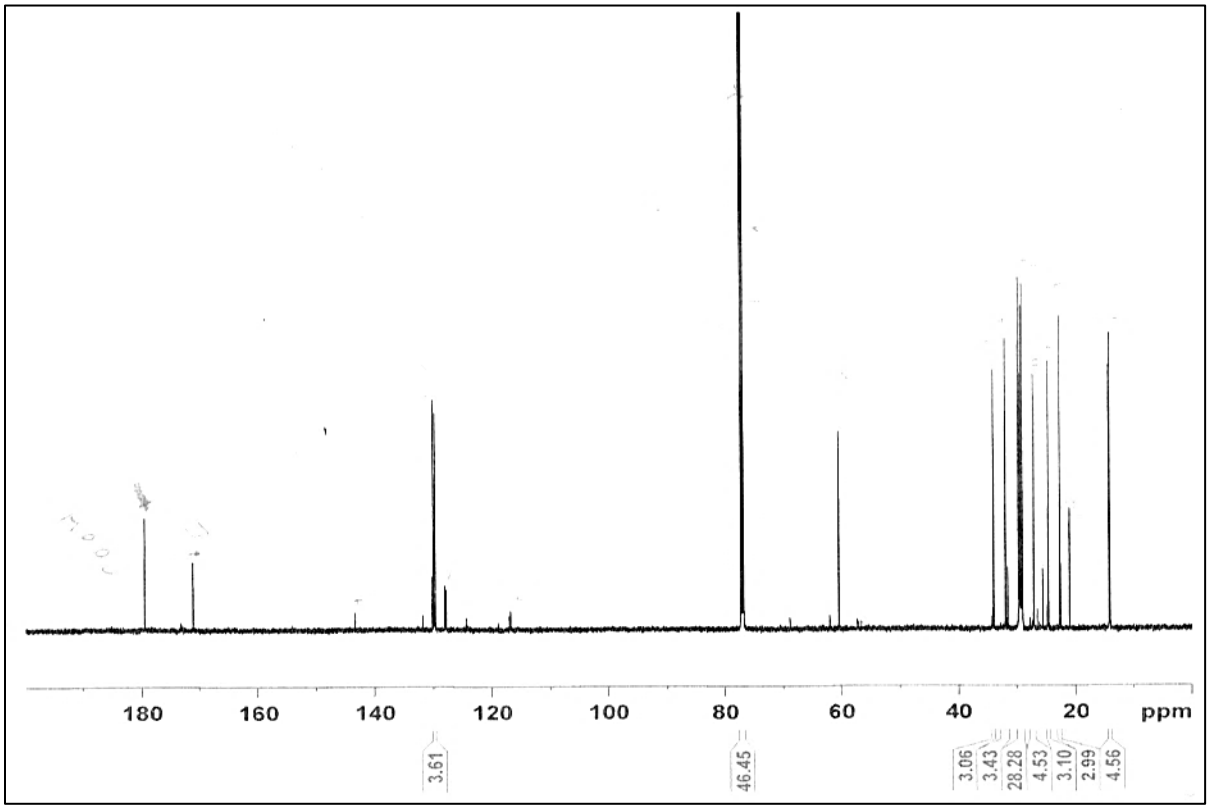

Fig.S2 the ^{13}C NMR spectrum of compound $_{8}$A (150 MHz, $CDCl_3$), isolated from the aqueous extract of *Monechma ciliatum* seeds.

^{1}H NMR of compound **$_{8}$C**

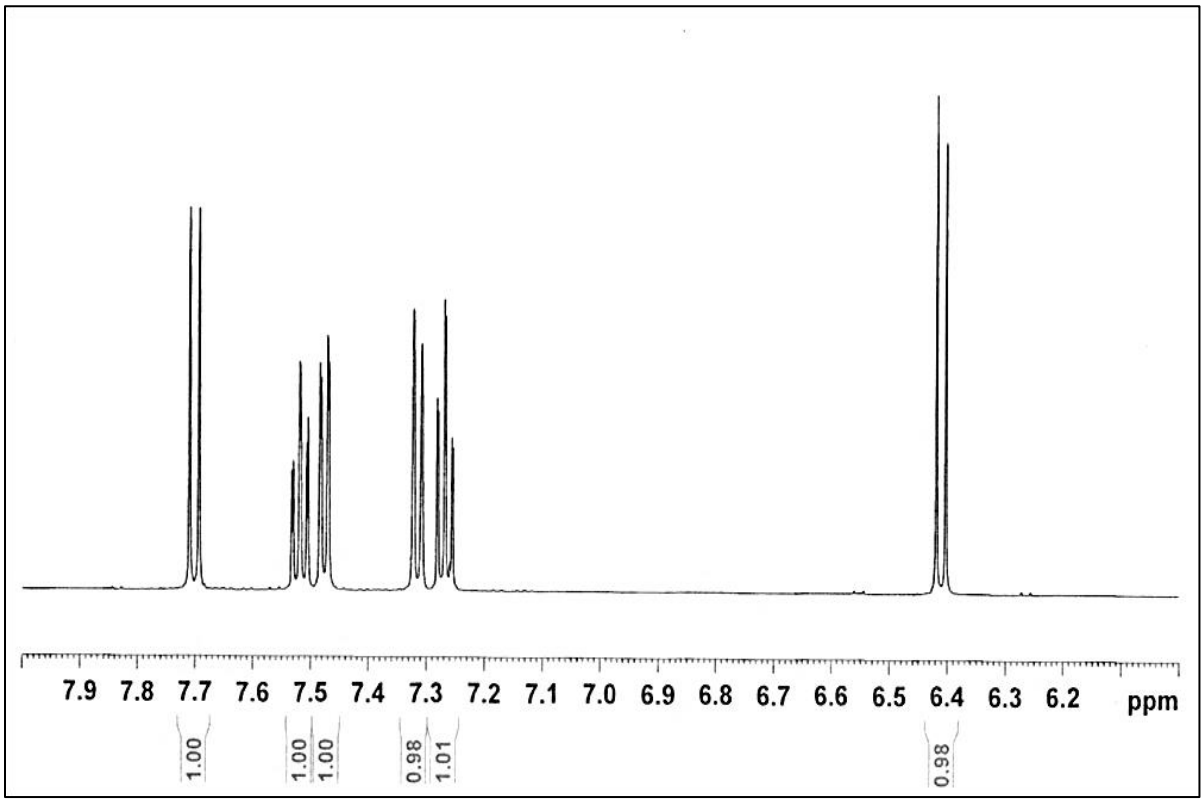

Fig.S3 the ^{1}H NMR spectrum of compound $_{8}$C at 600 MHz using $CDCl_3$solvent. Compound $_{8}$C isolated from *Monechma ciliatum* seed's aqueous extract.

^{13}C NMR of compound **$_8$C**

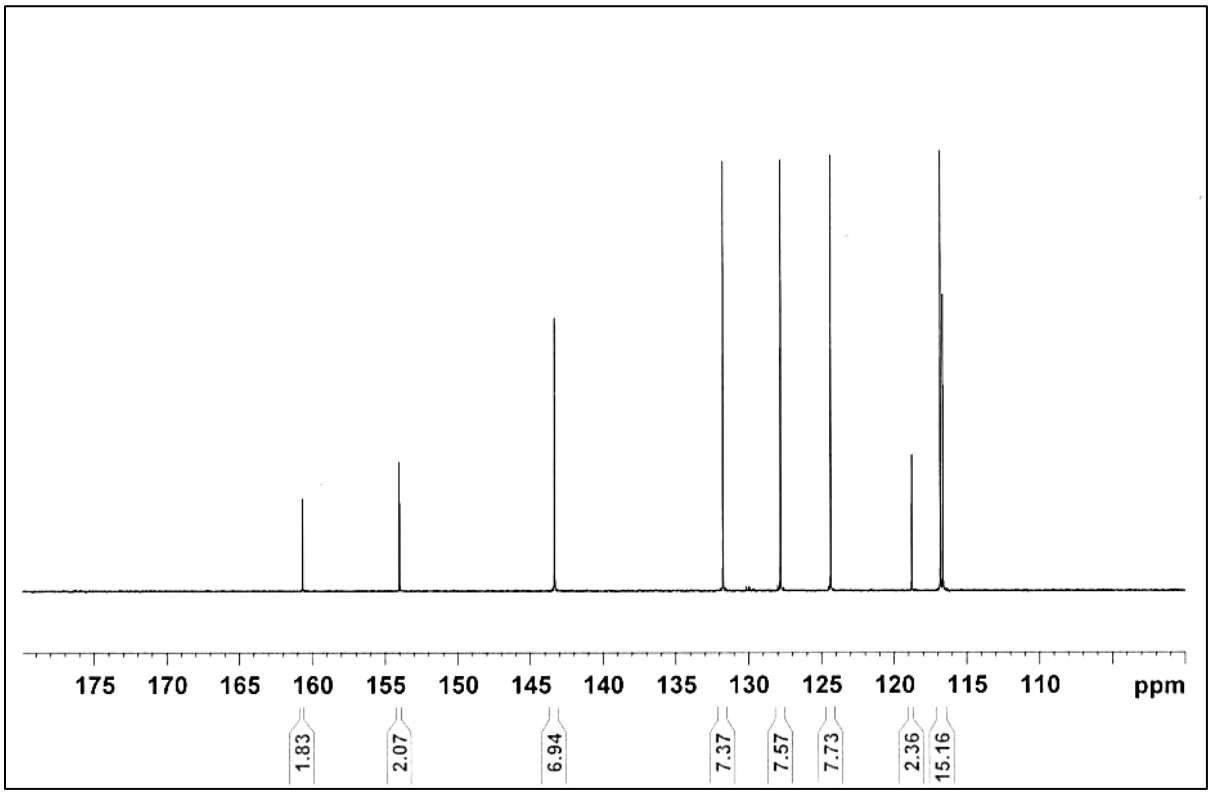

Fig.S4 the ^{13}C NMR spectrum of compound $_8$C at 150 MHz using $CDCl_3$solvent. Compound $_8$C isolated from *Monechma ciliatum* seed's aqueous extract.

^{1}H NMR of compound **B4**

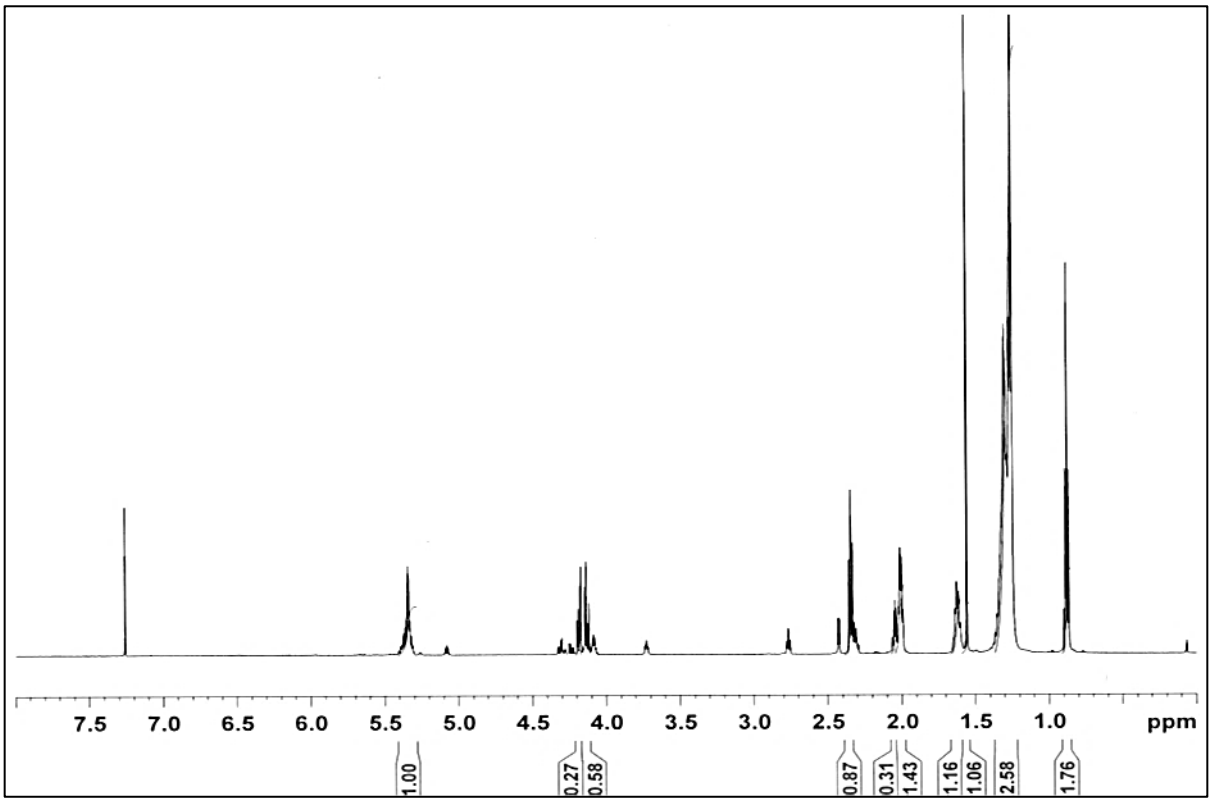

Fig.S5 the ^{1}H NMR spectrum of compound B4, which isolated from *Monechma ciliatum* seed's aqueous extract at 600 MHz ($CDCl_3$).

^{1}H NMR of compound **B5**

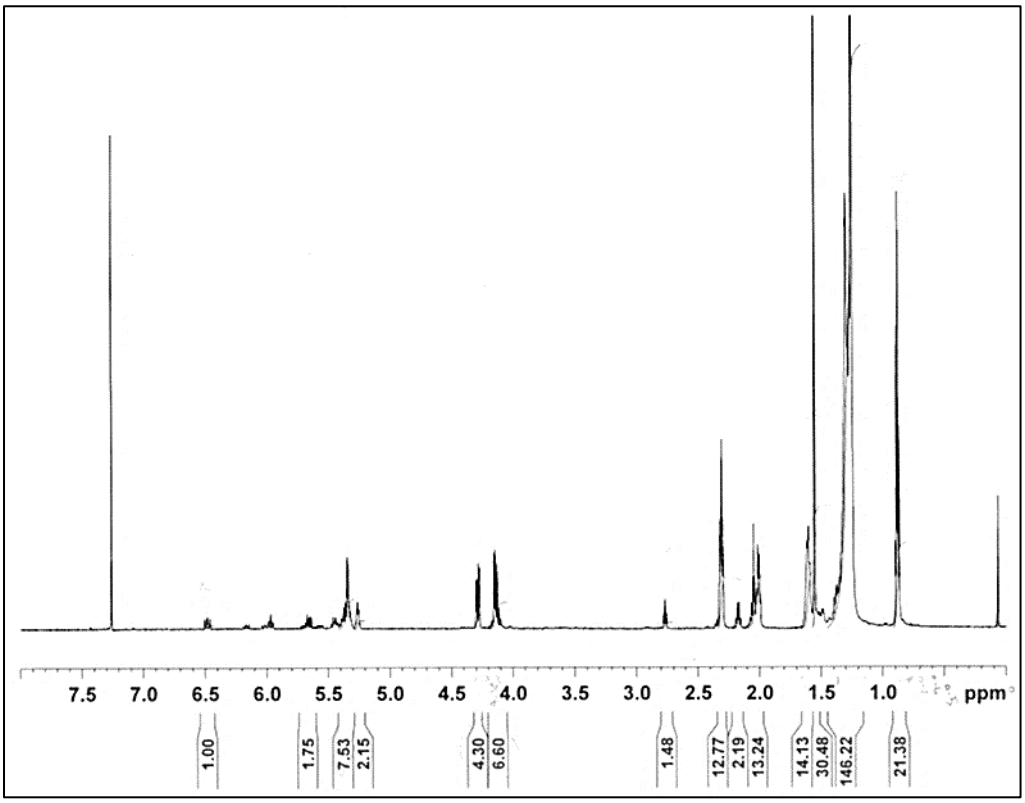

Fig.S6 the ^{1}H NMR spectrum of compound B5, which isolated from *Monechma ciliatum* seed's aqueous extract at 600 MHz ($CDCl_3$).

^{1}H NMR of compound **2**

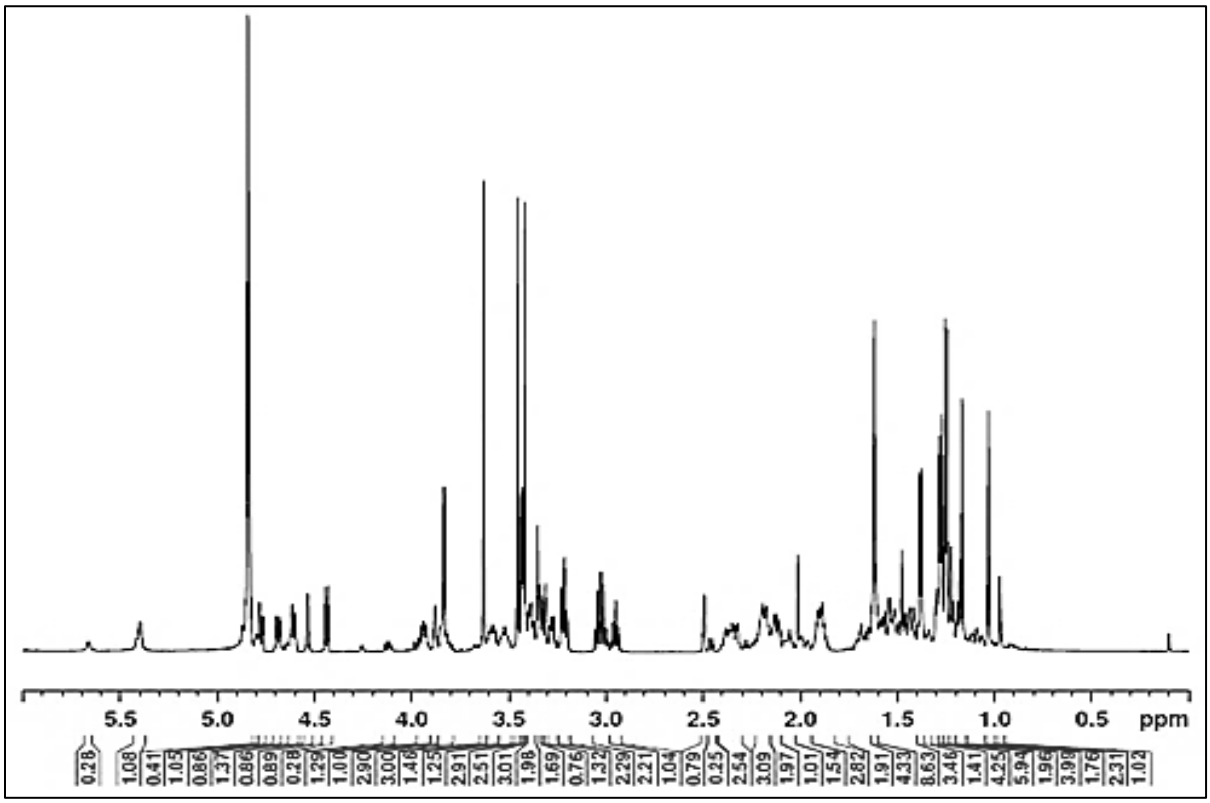

Fig.S7 ^{1}H NMR spectrum of compound **2** isolated from aqueous extract of *Solenostemma argel* leaves (600 MHz, CD_3OD).

^{13}C NMR of compound **2**

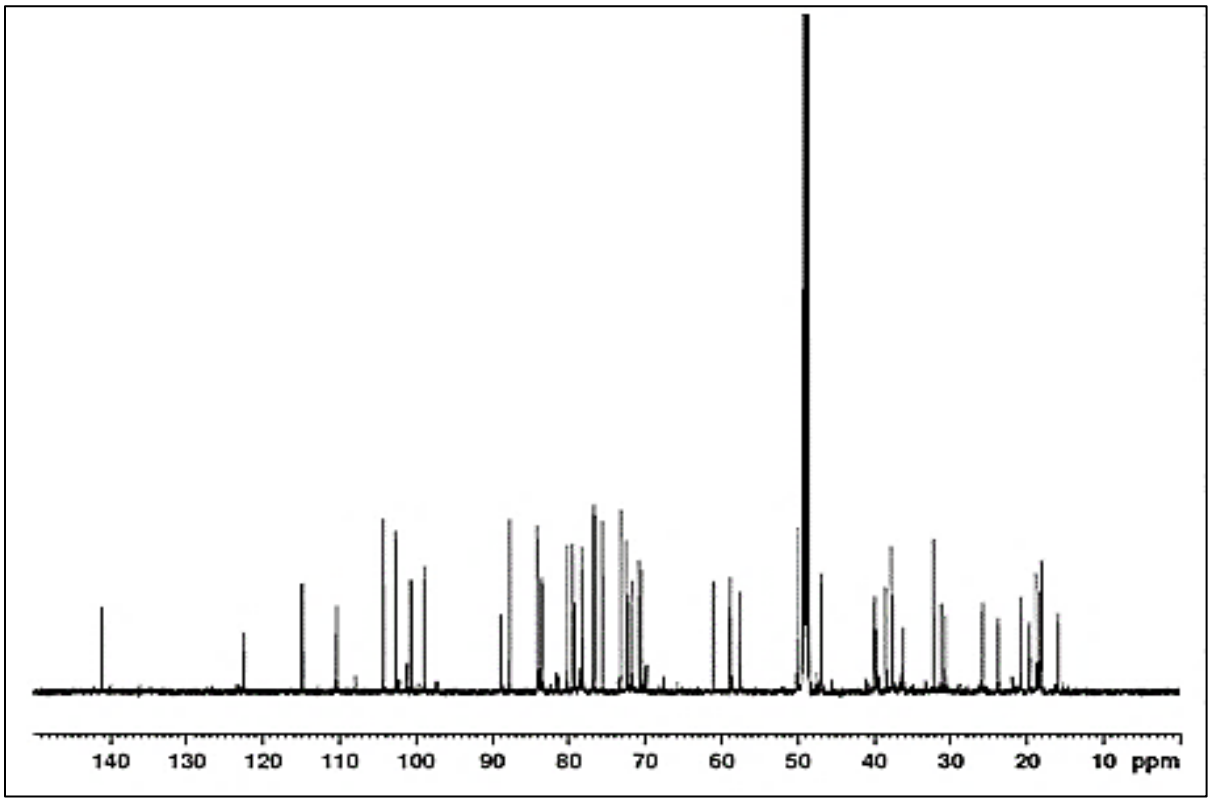

Fig.S7 ^{13}C NMR spectrum of compound **2** isolated from aqueous extract of *Solenostemma argel* leaves (150 MHz, CD_3OD).

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Dedication

I dedicate this thesis to:

My families and friends

For their generous love and support.

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Sara,,,

Summary

Oral illnesses are one of the critical chronic diseases. Of them, periodontal disease (periodontitis) is a major oral health problem, caused by bacterial inflammation of the tissues supporting the teeth. Numerous diverse bacteria exist simultaneously in the oral cavities of patients with periodontal disease. Mainly, *Porphyromonas gingivalis*, a gram-negative, asaccharolytic, obligate anaerobic rod bacterium, appears to be the prime etiological agent associated with the progression of the inflammatory events underlying periodontal disease. *P. gingivalis* produces several virulence factors in the outer cell membrane. The primary stage of bacterial pathogenesis begins with the adhesion of bacterial cells to host tissues followed by colonization, which provides nutrients and allows bacteria to survive, grow, and produce further virulence factors. These virulence agents include numerous molecules associated with adherence and colonization, such as fimbriae, lipopolysaccharides, proteases like gingipains, and hemagglutinins. The most important virulence factors that *P. gingivalis* produces are the hemagglutinins and gingipains, which play a vital role in the infection process through facilitating adhere of *P. gingivalis* to the surface of host cells following by acquisition of heme through erythrocyte binding and causes lysis and aggregation of erythrocytes via several proteolytic enzymes to release the heme moiety from the hemoglobin molecule as a nutrient.

The development of anti-adhesive agents against *P. gingivalis* hemagglutinins and gingipains could be a promising cytoprotective strategy to prevent the harmful effects of long-term bacterial infection. Over the past few years, several plants derived natural products have been described for oral disease treatment and as adjuvant remedies that aim to reduce inflammation through interaction with the adhesion of *P. gingivalis* to host cells. In this study, we aimed to screen a group of Sudanese medicinal plants that are used traditionally in the Sudanese folkloric medicine

against *P. gingivalis* TDC60 proliferation and virulence factors. Consequently, thirty-eight organ parts from twenty-five plant species were collected from Khartoum state, Sudan (The voucher specimens were prepared and identified at the faculty of agriculture and faculty of forest, university of Khartoum, Khartoum, Sudan) to investigate the potential inhibitory activities of their aqueous extracts. Thereafter, the research aimed to isolate, purify and identify the biologically active compounds of the most active plants extract on *P. gingivalis* proliferation and specific virulence factor/s.

The thesis consists of five chapters. Chapter one is a general introduction, research objectives, and thesis outlines. In chapter two, details of non-specific inhibitor isolated from *Origanum vulgare* leaves restrains *P. gingivalis* TDC60 growth and virulence factors. Chapter three focuses on isolates from *Monechma ciliatum* seeds' extract that hampered *P. gingivalis* TDC60 hemagglutinins, while chapter four demonstrated the influence of long-chain fatty acids using sub-MICs model against *P. gingivalis* TDC60 pathogenicity. Finally, chapter five shows argeloside I, from *Solenostemma argel* leaves, a candidate hostile to the pathogenicity of *P. gingivalis* TDC60. Primarily, the crude aqueous extracts from 38 plant organs of 25 plant species were screened to inhibit hemolysis, hemagglutination, gingipains, and bacterial growth, as illustrated in chapter two. Although several plants inactivated virulence factors (hemolysis, hemagglutination, and gingipains) independent of cell growth suppression, only three plants, namely *O. vulgare* (leaves), *Glycyrrhiza glabra* (areal parts) and *Salvia offinalis* (areal parts) hindered cell growth and virulence agents of *P. gingivalis*. Among them, the aqueous extract of *O. vulgare* provided low MIC values for heme aggregation, erythrocyte lysis, and gingipain. The inhibitory compound from *O. vulgare* leaves was isolated and characterized as a hydrolysable tannin-like compound that non-specifically suppressed *P. gingivalis* proliferation and virulence factors. Hydrolysable tannin-like

compound was characterized against tannic acid using HPLC, Prussian blue staining, and MALDI-TOF MS. The isolated compound inhibited the cell growth with a MIC of 0.05 $mg.mL^{-1}$ and gingipains (IC50 = 0.05 $mg.mL^{-1}$) as well as heme aggregation and erythrocyte lysis with a MIC of 0.0063 $mg.mL^{-1}$ for both virulence's.

The third chapter works were focused on the potent hemagglutinin inhibition found from *M. cilliatum* aqueous extract. Hemagglutinins are vital molecules, allow *P. gingivalis* to uptake iron and heme by attaching, aggregating, and lysing erythrocytes. To isolate and identify the inhibitory compounds, the water extracted from dry powdered seeds of *M. ciliatum* was partitioned using ethyl acetate followed by reversed-phase chromatography, thin-layer chromatography, ESI-MS, and NMR analysis resulting in the isolation of four compounds identified as oleic acid, coumarin, 1,2-dioleoylglycerol, and 1,3-dioleoylglycerol with MICs of 15–100 $\mu g.mL^{-1}$ against hemagglutination. This evidence of inhibitory activity will encourage the application of *M. ciliatum* effectively either as a functional food or therapeutic agent. Subsequently, in chapter four, we extended our study to compare the influence of long-chain fatty acids on hemagglutinins in correlation to *P. gingivais* growth and gingipains. Ten fatty acids with different saturation degrees and carbon chain length were selected for the first screening against *P. gingivalis* growth and hemagglutinins activity. Of them, unsaturated fatty acids showed lower MIC values compared to that of saturated fatty acids. Five unsaturated fatty acids were selected for further analysis using sub-minimum inhibitory concentration (sub-MIC) model against *P. gingivalis* growth, hemagglutination activity, and gingipains. The results showed that the sub-MICs dosages of unsaturated fatty acids significantly affected *P. gingivalis* growth and hemagglutinins and gingipain secretion. The N-terminal analysis of partially purified protein revealed that six protein bands corresponding to gingipain R1 hemagglutinin, lysine gingipain, and hemagglutinin genes

decreased inversely with the sub-MICs concentrations of unsaturated fatty acids. This section's results might support and expand the uses of the fatty acids as safe additives to impede undesired microbes to prevent periodontal diseases in the early stages.

The study in chapter five was focused on the isolation, purification, and identification of the most inhibitory compound in the aqueous extract of *S. argel* leaves that reveal the most potent inhibition activity against *P. gingivalis* proliferation among all test plants. We isolated and identified the inhibitory compounds by extracting *S. argel* leaves with distilled water, followed by several purification steps, including liquid-liquid separation, column chromatography, ^{1}H- and ^{13}C-NMR, and HR-ESI-MS resulting in a known pregnane glycoside, named as argeloside I. Argeloside I hindered cell growth of *P. gingivalis* TDC60 at 15 $\mu g.mL^{-1}$ and inhibited the hemagglutinins with a MIC value of 60 $\mu g.mL^{-1}$, as well as Arg and Lys specific gingipains with IC50 of 400 and 500 $\mu g.mL^{-1}$, respectively. The results show a new function for the pregnane glycosides, and argeloside I might be a preventing agent candidate for reducing the risk of *P. gingivalis* TDC60 and adhesion factors.

In conclusion, the current dissertation demonstrated the inhibitory activities of selected plants used traditionally in Sudanese folkloric medicine against *P. gingivalis* pathogenicity. Several compounds were introduced to the scientific community as candidates to hostile *P. gingivalis* and/or its virulence factors, including oleic acid, coumarin, 1,2-dioleoylglycerol, and 1,3-dioleoylglycerol, and argeloside I.

Summary in Japanese

要旨

歯周病は慢性的な口腔疾患であり、歯を支える歯肉組織の細菌性炎症によって引き起こされる。歯周病原菌は様々であるが、中でも *Porphyromonas gingivallis* は主要な病原因子を分泌し、歯周病の重症化を引き起こすと考えられている。*P. gingivalis* はグラム陰性・偏性嫌気性細菌で糖を資化出来ないため、歯周奥深くに接着してアミノ酸やヘムを主な栄養素として摂取する。接着しながら栄養素を獲得するために、線毛やリポ多糖を生産して細胞に定着し、プロテアーゼと赤血球凝集素を生産して歯肉組織や赤血球細胞を破壊し溶血を引き起こしてアミノ酸やヘムを獲得する。プロテアーゼの中でも、ジンジパインという強力なシステインペプチダーゼは、赤血球凝集素と共に多く生産され、赤血球分解や歯肉組織破壊に働く。そのため、ジンジパインと赤血球凝集素が病原性因子の中でも最も重要とされ、それらの阻害剤開発は、口腔医療の分野において急務である。

これまでの研究において、植物からの抽出物は口腔疾患の治療において炎症を軽減させるための補助療法に利用できる可能性が示されてきた。筆者の母国であるスーダンにも、伝統的に利用されている薬用植物は存在する。そこで本研究では、*P. gingivalis*TDC60 株を使用し、スーダンで使用されている薬用植物から、歯周病原菌の増殖と病原性因子を阻害する物質をスクリーニングすることを目的とした。スーダンのハルツーム大学の森林学部や農学部から 25 種類の植物種から葉や種子の加工物（乾燥等、計 38 種）を取り寄せ、それらの水抽出物を調

製するとともに、*P. gingivalis*TDC60 株の増殖や病原性因子の阻害活性を評価した。さらには、抽出物中に存在する阻害物質の単離・精製、及び同定を行った。

本論文は 5 つの章で構成され、第 1 章は本研究の背景、目的、及び概要を示す。第 2 章では、香辛料等としても利用される *Origanum vulgare* の葉から抽出された *P. gingivalis*TDC60 株の増殖や分泌される病原性因子の非特異的阻害物質について、第 3 章では、*Monechma ciliatum* の種子に由来する赤血球凝集素の阻害物質について、第 4 章では *P. gingivalis*TDC60 株の病原性に対するサブ MIC モデルを使用した、長鎖脂肪酸の阻害効果について、第 5 章では、ハーブ *Solenostemma argel* の葉の水抽出物から単離されたアルゲロシド I とその *P. gingivalis*TDC60 株の増殖や病原性に対する効果について述べる。

本研究は、38 サンプルの植物の器官（25 種の植物）の水粗抽出物のスクリーニングから始まる。これらの抽出物について、*P. gingivalis* TDC60 株が生産する溶血素、赤血球凝集素、ジンジパインの阻害効果、そして菌の増殖抑制効果を評価した。その結果、*O. vlugare* の葉、*Glycyrrhiza glabra* の表皮部、*Salvia offinalis* の表皮部で、*P. gingivalis* TDC60 の増殖と病原因子を共に抑制した。中でも *O. vlugare* の葉の水抽出物は病原因子に対する MIC 値が最も低く、その阻害物質を単離し、HPLC、プルシアンブルー染色、MALDI-TOF MS 解析を行った結果、加水分解性タンニン様化合物であることが明らかとなった。本化合物の *P. gingivalis* TDC60 の増殖に対する MIC は 0.05 $mg \cdot mL^{-1}$、ジンジパインに対する IC_{50} は 0.05 $mg \cdot mL^{-1}$、赤血球凝集素と溶血素に対する MIC は 0.0063 $mg \cdot mL^{-1}$ であった。

第 3 章では、*M. cilliatum* の水抽出物から単離された強力な赤血球凝集素の阻害物質に焦点を当てた。赤血球凝集素は歯周病の疾病や重症化に主要な働きをする物質であり、本物質により *P. gingivalis* が赤血球を凝集し、溶解してヘムを獲得する。阻害物質の単離では、種子の乾燥粉末の水粗抽出物から溶媒抽出、逆相クロマトグラフィー、薄層クロマトグラフィーを行 4 つの化合物を単離し、その後 ESI-MS 及び NMR によりそれらの同定を行った。その結果、4 つの化合物はオレイン酸、クマリン、1,2-ジオレイルグリセロール、1,3-ジオレイルグリセロールと同定され、それぞれの赤血球凝集素に対する MIC は 15～100 μg·mL^{-1} であった。

続いて、第 4 章では、*P. gingivalis* TDC60 株の生育、ジンジパイン活性、赤血球凝集素活性の阻害に対する長鎖脂肪酸構造との関連について調べた。オレイン酸の構造を軸として、飽和度や炭素鎖長が異なる 10 種類の脂肪酸を選抜し、阻害の強さとの比較を行った結果、不飽和脂肪酸は飽和脂肪酸に比べて MIC 値は低い結果となった。さらに 5 つの不飽和脂肪酸を選抜し、*P. gingivalis* TDC60 株の増殖と病原性因子に対するサブ MIC を分析した結果、不飽和脂肪酸の処理は、*P. gingivalis* TDC60 株の増殖に加え、ジンジパインや赤血球凝集素の分泌にも大きな影響を与えた。

第 5 章では、*P. gingivalis* TDC60 株の増殖に対して最も強力な阻害活性を示したハーブ *S. argel* の葉の水抽出物に焦点を当て、阻害物質の単離と同定を行った。*S. argel* の葉の水抽出物から溶媒抽出、順相クロマトグラフィーを行って阻害物質を単離し、^{1}H-NMR、^{13}C-NMR、HR-ESI-MS を行って阻害物質を同定した。同定された物質アルゲロシド I は、MIC 値が 15 μg·mL^{-1} で *P. gingivalis* TDC60 株の増殖を抑制し、MIC 値が 60 μg·mL^{-1} で赤血球凝集素を阻害

し、IC_{50} 値が 400～500 $\mu g \cdot mL^{-1}$ でジンジパインを阻害した。本結果はプレグナン配糖体及びアルゲロシドⅠの新奇機能ある。

本博士論文研究により、スーダンの民間療法で伝統的に利用されている薬用植物がもつ、*P. gingivalis* に対する病原性の阻害機能を実証した。これらの植物にはオレイン酸、クマリン、オレイルグリセロール類、アルゲロシドⅠ等の化合物が存在し、それらが *P. gingivalis* の病原性や増殖に強く作用するためであることが考えられ、歯周病抑制に係る補助療法への利用可能性をサイエンスに紹介するものである。

LIST OF PUBLICATIONS

Publication related to chapter two:

Eltigani, S. A., Eltayeb, M. M., Bito, T., Ishihara, A., and Arima, J. (2019). Non-specific Inhibitor from *Origanum vulgare* Leaves Restrains *Porphyromonas gingivalis* Growth and Virulence Factors. International Journal of Agriculture and Biology, 23 (6):1068-1074.

Publication related to chapter three and four:

Eltigani, S. A., Eltayeb, M. M., Ishihara, A., and Arima, J. (2019). Isolates from *Monechma ciliatum* seeds' extract hampered *Porphyromonas gingivalis* hemagglutinins. Journal of Food Biochemistry, 43 (11): e13029.

Publication related to chapter five:

Sara A. Eltigani1, Mohamed M. Eltayeb, Tomohiro Bito, Tsuyoshi Ichiyanagi, Atsushi Ishihara, Jiro Arima. (2020). Argeloside I inhibits the pathogenicity 1 of *Porphyromonas gingivalis* TDC60. Journal of Bioscience and Bioengineering, in press.