CHAPTER 4

DISCUSSION

Medicinal plants are used widely throughout the world, sometimes used as the alternative therapy for people who do not have access to trade mark drugs, especially those in developing countries such as Thailand. Traditional drugs are used from the older experience but blinded of true scientific knowledge. Today, medicinal plants are broadly studied in medication but studies in dental and oral diseases are rare.

Periodontal disease, the silent epidemic of oral disease, is an important global public health concern and probably the most common chronic infectious disease in humans. [17, 18, 64] It is a multifactorial complex disease involving multiple bacterial species and host cell interactions, the combined effect of which causes the destruction of soft tissue and bone. [15, 16, 18] In the USA, one of the richest and most developed country, more than 90% of the population are afflicted with the disease. Severe periodontal disease in the USA affects 14% of adults aged 45-54 and 23% of the population aged 65-74 years. [65] In a national survey in the United Kingdom (UK), 79% of dentate adults had bleeding gums, 88% had calculus and 69% had periodontal pockets, including 10% with deep pockets. [32] In a survey conducted in southern Thailand the prevalence of periodontal attachment loss $\geq$ 4 mm was found to be 92% in adults aged 30-39 years and 100% for those aged 50-59 years. [66] Periodontal diseases are also expensive to treat; in 1995-96 the total cost in the UK for a simple one visit periodontal treatment was £104 million, with two- and three-visit treatments costing another £30 million. [32] Moreover, many recent studies have reported the relationship between periodontal and systemic diseases. [67] This disease may be a predisposing factor in systemic conditions such as in the development of cardiovascular disease, specifically, myocardial infarctions, atherosclerotic diseases, infective endocarditis, type 2 diabetes/obesity, risk of preterm delivery, and low birth weight. [67- 69]

Part I of this study consists of the preparation of seven Thai medicinal plant extracts. All parts of the plants used in this study were extracted by absolute ethanol. Ethanol is well known as a solvent of many plant extracts, it is easy to buy and the cost is relatively low. These extracts were sticky and wet, so all of the wet parts were freeze dried except *Sc* bark and

Rn leaves. All of them were dissolved with DMSO. DMSO is usually used to improve the solubility of hydrophobic parts in aqueous solutions. [70] In this study, *Ao* leaves, *Sc* leaves and bark, *Pn* pericarp, and *Rn* leaves were better dissolved than *Ao* bark and *Tb* dried-fruit.

We are aware that the selected solvent types may affect our results. Ahmad, *et al.*, reported that the different solvent types, water, hexane and alcohol, were given the different antimicrobial properties of some Indian medicinal plants. [71]

Moreover, the starting concentrations of our plant extracts were wide ranging (such as *Ao* leaves, *Rn* leaves, *Sc* leaves and bark, and *Pn* pericarp; 50 mg/ml and *Ao* bark and *Tb* dried-fruit; 250 mg/ml). The starting concentration may affect the antibacterial property of the plant extracts. So, further studies could focus on solvent types and starting concentrations.

Part II consisted of the experiments to investigate the antimicrobial activity of seven Thai medicinal plant extracts against recognized periodontopathic bacteria *Aa*, *Pi*, and *Pg*. While little is known about the effect of these plant extracts on oral microorganism, this is the first report that *Ao* leaves and bark, *Sc* leaves and bark, *Pn* pericarp, and *Rn* leaves and *Tb* dried-fruit have the inhibitory effects and can kill *Pg* as well as *Pi* (not including *Sc* bark, *Pn* pericarp and *Rn* leaves). All of the extracts also have the inhibitory effects on *Aa*, however only *Ao* bark, *Tb* dried-fruit and *Sc* leaves have the potential to kill *Aa*.

The antibacterial activity of the plant extracts in our study is in agreement with previous studies. For example, Kudi, *et al*, reported that the leaves and bark of Nigerian *Ao*, by the hole-plate diffusion method, showed activity against gram-negative bacteria *E. coli* and *P. aeruginosa*. [2] Akinpelu, also found that *Ao* bark extract presented a wide spectrum of antibacterial activity being effective against 13 bacterial isolates such as *S. aureus, E. coli, Bacillus cereus, P. aeruginosa* and also against *Klebsiella pneumoniae* strain resistant to streptomycin. [55]

Shafi, *et al*, investigated the antibacterial activity of *Sc* leaves essential oils by disk diffusion method. [72] The study found that antibacterial activity was effective against six bacterial isolates, namely *S. aureus, E. coli, Bacillus sphaericus, Bacillus subtilis* (*B. subtilis*), *P. aeruginosa* and *Salmonella typhimurium* (*S. typhimurium*).

Other studies that found antibacterial activity of *Pn* were those by Meledez, *et al*, Prashanth, *et al*., and Holetz, *et al*., which all investigated the antibacterial activity of *Pn*

pericarp and found that the antibacterial activity was effective against both gram negative and positive bacteria. [59, 73, 74]

Ahmad, *et al*., showed that Indian *Tb* fruits had antibacterial activity against *B. subtilis*, *Proteus vulgaris*, *S. typhimurium*, *E.c*oli and *S. aureus*. [71]

Although all these plants seem to affect a wide range of bacterial species, there are considerable variations in concentrations among them. In this study, we found that *Ao* bark, *Tb* dried-fruit and *Sc* leaves were effective against three periodontopathic bacteria. The most effective being *Ao* bark and *Tb* dried-fruit which had MBC of *Pg* at 1.95 and 0.48 mg/ml, respectively. Akinpelu, found that *Ao* bark extract, at a concentration of 20 mg/ml contained antibacterial activity against 13 bacterial isolates. [55] Another study by Prashanth, *et al*., showed that *Pn* fruit rind had MIC of gram negative and positive such as *S. aureus, E. coli* at 1.5-50 mg/ml. [74] Holetz, *et al*., found that *Pn* fruits have MIC of *S. aureus* 62.5 µg/ml and more than 1,000 µg/ml of other standard strains in the study. [59] Machado, *et al*., reported that *Pn* pericarp had MIC against methicillin-resistant *S. aureus* at the value of 250 mg/ml. [6] Voravuthikunchai, et al., also reported that aqueous extract of *Pn* fruit shell had *E. coli* MIC and MBC values of 0.19 and 0.39 mg/ml, respectively. [75] These different results may be related to many factors, such as plant extract preparation, parts of plant used, plant ingredients, geographic region and age of plant collected.

Not only do the kinds and sources of plants need to be established but also their natural ingredients and the technical laboratory used. The crude extracts were composed of compound ingredients with stains, which made the interpretation of results by sight much more difficult. For example, the results of screening antibacterial activity by the plate diffusion method of *Sc* bark, *Pn* pericarp and *Tb* dried-fruit showed dark brown stain with the difficulty to investigate the clear zones. However, these may relate to other factors as well, i.e. the diffuse properties of tested materials, low viscosity of the gel, solubility of the solvent used, the natural dark brown stain, and non-homogenous tested materials.

Tb dried-fruit crude extracts contained stain making interpretation of the growth of organisms by the broth microdilution method difficult. However, these wells could be obtained by observing the growth on the blood agar plates.

We also tested the antibacterial activity against *Aa*, *Pg* and *Pi* of 25% and 50% DMSO for intra experimental control. The results showed that both concentrations of DMSO

were not effective for testing bacteria. We conclude that DMSO has no effect on the antibacterial activity of our studied plant extracts.

This *in vitro* study shows that all of the studied plant extracts were able to inhibit and kill *Pg* but were incomparable to metronidazole. Among the studied plants, *Ao* bark and leaves showed the best effect to inhibit *Pg* while *Tb* dried fruit was the most effective against *Pg*. It seems that *Aa* is difficult to kill. The plant extract concentrations against *Aa* were higher than other bacteria species in the study. It is quite clear that the development of effective concentrations of the plant extracts that aim to inhibit *Aa* may effect *Pg* and *Pi* too, while the effective concentrations of the plant extracts (except *Sc* bark and *Rn* leaves) that aim to kill *Pi* may also effect *Pg* and *Aa*.

The first goal of periodontal therapy is to reduce the microbial challenge by limiting or eliminating its related inflammatory response, thus allowing the periodontium to recover its health. [10-13] However, conventional periodontal treatment by scaling and root planing may sometimes fail to eliminate periodontopathic bacteria because of an insufficient access for proper instrumentation. [40, 41, 43] Therefore, antibiotics, both locally and systemically, have been introduced to reinforce the mechanical therapy and to support host defenses in overcoming the pathogenic role of subgingival microorganisms that persist after conventional therapy. [39-41, 45, 46] Nevertheless, systemic antibiotics provide positive outcomes but some adverse side effects. [39-41, 45, 46] For this reason the use of locally administered antibiotics, for instance, tetracycline, doxycycline, minocycline and metronidazole, which aim to maintain a high concentration of the drug within a well-confined area at the diseased sites, are now being investigated as an alternative mode of treatment. [46-48, 50]

Trade mark drugs are widely used in developed countries such as USA. In Thailand the main factor hindering access to these drugs is the high price. In addition antibiotic resistance has become a major worldwide health care problem over the past 20 years. [42] For these reasons the development of medicinal herbs with antimicrobial activity in the local treatment of periodontal disease is of interest to both developing countries, such as Thailand, and developed ones.

In Thailand, one medicinal herbal product is *Andrographis paniculata* gel (AP gel), a biodegradable gel administered to be subgingival area, and shown to have *in vitro*

antibacterial activity against *Pg*. [54] Atsawasuwan. *et al*., found that the use of AP gel seemed to produce the same adjunctive effects as obtained from the metronidazole gel. [76] The 25% metronidazole gel has been used as an adjunct in periodontal treatment for many years.

In our study, the use of crude plant extracts may contain a wide diversity of molecules with often unknown biological effects. For example, in the leaves of *Ao*, two main chemical groups (flavonoids and tannin) have been identified and *Pn* is rich in hydrolysable tannins too. [60, 77] Machado, *et al*., concluded that allagitannins are the principle components responsible for the antimicrobial action of *Pn*. [6]

So these five local Thai medicinal plants may be promising to delvelop as an adjunctive, locally administered treatment in periodontitis patients. Within this respect, the results of this study support to a certain degree the traditional medicinal use of these plants for their antibacterial activity against three periodontopathic bacteria species. Further investigation to isolate the active substances of these plant extracts will be of interest.

In order to develop these medicinal plants as an adjunctive periodontal treatment, not only their antimicrobial activity is of concern but also the safety use in humans. In Part III the cytotoxicity of these plant extracts on human gingival connective tissue fibroblasts (HGF) was done. HGF may represent soft connective tissue cells of the periodontium.

In this experiment, the toxicity of the studied plant extracts was investigated by MTT colorimetric assay in order to measure cell survival. The MTT assay has been used because of its precision, easiness, safety, low cost with large number of samples and rapidity to measure viable cells. [78, 79] The chemical structure of MTT is 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide. The tetrazolium ring in MTT is usually cleaved by dehydrogenase, an enzyme actively produced in mitochondria of viable cells. The yellow color of MTT was changed to a blue shade because of activity from viable mitocondria fibroblasts. [78, 79]

Darveau *et al*., reported that surfactants and antibiotics were resisted by dental plaque biofilms. [37] Quirynen *et al*., asserts that clinicians must be aware of the potential resistance of periodontopathic bacteria. [42] For these reasons, the effective concentration that should used in humans may need to be higher than that in *in vitro*. So the cytotoxicity experiments in this study were done at and over the MIC of each plant extract.

This *in vitro* study showed that the extracts from *Ao* leaves and bark and *Sc* leaves were non toxic to HGF. Moreover, *Ao* bark, *Sc* leaves and *Tb* dried-fruit extracts at some

tested concentration and tested hours promoted the proliferation of HGF. This was supported by another study regarding medicinal plants such as aloe vera and chitosan, which had efficacy on promoting cell proliferation. [80-82]

At 12 tested hours, some plant extracts such as *Tb* dried-fruit (5.95 mg/ml), *Sc* bark (12.5 mg/ml), and *Pn* pericarp (6.25 mg/ml) showed cytotoxicity on HGF. Interestingly, there were more HGF deaths at 12 tested hours before recovering later at 24 and 48 hours. These may due to HGF adapting themselves from immediate contact to new agents and media.

Konan, et al., showed preliminary results of acute toxicity tests of extracts from *Ao* leaves in mice. [77] The crude extracts of *Ao* leaves in this study did not produce toxic symptoms in rats in doses up to 2,000 mg/ml. This plant extract is usually tolerated by rats when doing biochemical analyses of renal and hepato-bililary functions, such as the level of urea, creatinine, tranaminases and alkaline phosphatase. In that study the authors used the Ames test in *S. typhimurium* stain TA97, TA100, TA102 and the bone marrow micronucleus test in mice for genotoxicity. The effect of *Ao* leaves extract was shown to induce frameshift, base pair substitution and damage to the chromosomes. However, this effect was less harmful than the clastogenic effect of ciclophosphamide used as a positive control. The main components of *Ao* leaves are flavonoids and tannins. [77] Havesteen, and Fernandes, *et al*., found that the compounds extracted from *Ao* leaves may react as a hepatoprotector since it was shown to be a polyphenolic-rich extract, particularly in flavonoids that are an antioxidant component. [83, 84] For instance, flavonoids reduced glutathione *tert*-butylhydroperoxide-induced and the lipid peroxidation that may strongly contribute to cellular damage. Moreover, Satyanarayana, *et al.*, reported that the main structure of *Ao* leaves, such as flavonoids and tannins, possess antioxidant properties and these components may inhibit nephrotoxicity. [85] The beneficial effects of tannins against nephrotoxicity were also reported by Yokozawa, *et al.* [86] In general, polyphenols are free radical scavengers, a feature that may explain their protective activity against nephrotoxicity. From previous studies, *Ao* leaves are unlikely to affect hepatic and renal function.

The most toxic plant extract in our study was *Rn* leaves. This extract showed cytotoxic effect at 2 times MIC (25 mg/ml) at every tested time point.

Pn pericarp also showed cytotoxicity on HGF at 2 times MIC (6.25 mg/ml) at 12 hours. In agreement with our study, Squillaci and Di Maggio reported that severe acute gastric

inflammation and even death can occur due to consumption of decoction of the tree bark, and to a lesser extent pericarps of fruit. [87] Also the study of Vidal, *et al*., reported that whole fruit extracts have been known to cause congestion of internal organs and elevated creatinine *in vivo*. [88] Moreover, severe allergic reactions from eating pomegranate fruit and esophageal cancer from chronic consumption of roughly ground pomegranate seeds have been reported. [88, 89] Nevertheless, Fatope, *et al*., found that pomegranate seed oil was non-toxic to brine shrimp larvae. [89]

Further studies are also needed to confirm our findings, perhaps using different extraction methods.

It is widely accepted that the pathogenesis of periodontitis involes many inflammatory mediators i.e. IL-1, IL-6, PGE_2. [4, 15] The presence of PGE_2 has also been measured in high levels in the gingival crevicular fluid of periodontitis patients and has been associated with attachment and bone loss. [23-25] Part IV of these serial experiments selected PGE_2, the pronounced inflammatory cytokine in periodontitis, as the model to study the anti-inflammatory effect of these Thai medicinal plant extracts. *Ao* leaves and bark, *Tb* dried-fruit, *Sc* leaves and *Pn* pericarp were included in this experiment because of their effectiveness against *Pg* and absence of toxicity on HGF.

In vitro anti-inflammatory studies are very limited since inflammatory processes are related with many cytokines. However, this is the first preliminary study that shows the effect of plant extracts, such as *Ao* leaves, *Sc* leaves and *Pn* pericarp, to reduce the proinflammatory mediator, PGE_2.

Due to the limitation of our study we could not do triplicate the experiments. So the statistical analysis was done by using the non parametric Kruskal-wallis test. Comparison with controls showed that the efficacy of plant extracts on reduction of PGE_2 was small but the trend was noticed. We found that extracts of *Ao* leaves 3.125 mg/ml and *Sc* leaves 6.25 mg/ml reduced approximately 0.14 times when compared with the controls (PGE_2 production at 12 hours). The extracts continued their effects further to 24 hours, at 0.13 times compared to the controls. At 6 hours tested time, many plant extracts, such as *Tb* dried-fruit 59.5 mg/ml, *Pn* pericarp and *Sc* leaves 62.5 mg/ml, showed a trend to increase PGE_2 production but did not show a significant difference compared to the control. However, after 12 and 24 hours PGE_2 levels tended to decline closely to the control. These results may be due to the higher concentration of

studied plant extracts or the studied time periods (6, 12, and 24 hours) were inappropriate and so unable to indicate the true effect.

The results point to time-course related factors and the concentration of the crude extracts used in the study. Future experiments need to address the effective substances, doses, varying time interval and longer tested times.

Based on an internet search, to date there have been no reports about the efficacy of these plant extracts on *in vitro* PGE_2 reduction like in our study. Ibewuike *et al*., found the ability of *Ao* stem bark extract to produce 90% inhibitition of prostaglandin production from bovine seminal vesicles. [56] Other studies, O.A. Olajide, *et al*., evaluated the methanol extract of *Ao* stem bark for activity against LPS induced septic shock in mice. [57] The results showed that pre-treatment with *Ao* bark extracts (25-200 mg/kg) caused a dose-dependent and significant ($p<0.05$) reduction in the elevated levels of alanine and aspartate aminotransferasees in the sera of D-galactosamine-primed mice injected with LPS. The highest dose of the studied extract (200 mg/kg) produced a 100% protection against death from sepsis. Muruganandan, *et al*., 2001, [5] investigated the ethanolic extract of *Sc* bark for its anti-inflammatory activity *in vivo*. They found a potent anti-inflammatory action of this plant against different phases of inflammation without any side effect on gastric mucosa.