Research protocol: part 1

Project summary

Extraction of impacted molar teeth is a common procedure performed by oral surgeons and general dentists while postoperative pain is a significant adverse event after surgery. Inflammation and oxidative stress are both two important key players in the development, enhancement, and maintenance of pain, which they are almost invariably involved in pain after impacted mandibular third molar surgery. Thus, a non-steroidal anti-inflammatory drug (NSAID) such as ibuprofen is prescribed to the patients after the dental surgery. Curly kale (*Brassica oleracea* L.) is an edible vegetable containing different acylated and nonacylated flavonoids (e.g. quercetin, kaempferol, and cyanidin and derivatives of p-coumaric, ferulic, sinapic, and caffeic acid), their glycosides, and complex hydroxycinnamic acids. Meanwhile, anthocyanin (ACN), a group of flavonoid polyphenols, is commonly found in many plants, which possesses potent anti-inflammatory and antioxidant effects and mitigates various pathology of disease states. Nonetheless, the empirical evidences of ACN for alleviating pain in patients after impacted mandibular third molar surgery are few, despite its potentials. This study aims to assess and compared the analgesic and anti-inflammatory properties of ACN-rich kale extract and ibuprofen when administered to the patients after mandibular third molar surgery. The study was conducted on patients who required the surgical removal of impacted mandibular third molars under local anaesthesia. The study subjects (n = 20, 8 male/12 female, aged 18-25 years) were randomized for giving the kale extract (500 mg ACN equivalent) or ibuprofen (400 mg) capsules for 7 days or vice versa after removal of each impacted tooth. All patients were assessed for pain using VAS scoring on the 1st–7th postoperative days and determined levels of α-amylase activity, MMP and TGF-β2 concentrations in the saliva collected before and on the 1st, 4th and 7th postoperative days. We expect that patients who took the kale extract (study group) had significantly less pain and inflammation on postoperative days, which showed clinically and statistically significant results when compared with the orally-administered ibuprofen (control group) after mandibular third molar surgery.

General information

- Protocol title: Comparing Anti-Inflammatory Effect between Ibuprofen and Water Extract of Curly Kale Leaves in Patients Following Wisdom Tooth Extraction: A Randomized Controlled Study (Protocol number: 65/2565, Date: 12 December 2022)
- Name and address of the sponsor/funder.
    1. Residency Training Program in Oral and Maxillofacial Surgery, Faculty of Dentistry, Chiang Mai University
    2. Royal Project Foundation Fund, Chiang Mai, Thailand
- Name and title of the investigator(s) who is (are) responsible for conducting the research, and the address and telephone number(s) of the research site(s), including responsibilities of each.

Investigator	Address	Responsibility
1. Vuttinun Chatupos, D.D.S. Assistant Professor	Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand	Supervisor, Surgeon
2. Sansanee Neelawatanasook, D.D.S.	Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand	Investigator, Surgeon, Data collector
3. Tidanut Sangutai, D.D.S.	Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand	Investigator, Surgeon

4. Atit Khanutwong, D.D.S.	Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand	Investigator, Surgeon
5. Somdet Srichairatanakool, Ph.D. Professor	Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand	Analysis of α-amylase, MMP9 and TGF-β2

- Name(s) and address(es) of the clinical laboratory.

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Rationale & background information

Curly kale (*Brassica oleracea* L.) belongs to the Brassicaceae family, similar to Chinese kale, cabbage, broccoli, and cauliflower. There are several varieties, distinguished by differences in plant characteristics such as long green leaves, green or purple curly leaves, smooth or serrated leaf edges, and purple or green stems depending on the variety. In Thailand, there are five popular varieties of kale: curly green kale, lacinato kale (also known as dinosaur kale or Siberian kale), purple curly kale, scarlet kale, and red Russian kale. Kale is easy to cultivate and manage, similar to other vegetables in the Brassica family. It takes about 45 days to harvest, and the plants can live for 1-2 years depending on care. It is also known as the "queen of greens" and recognized as a superfood due to its high nutritional value, including vitamins, protein, fiber, and minerals, compared to other vegetables in equivalent quantities. Additionally, kale contains up to three times more calcium than cow's milk and up to 14 times more calcium than spinach, which contributes to bone density. Moreover, it boosts levels of vitamin K in the body, as well as vitamins A, C, which improve vision and promote healthy skin due to improved blood circulation, reducing wrinkles [1,2]. Dietary supplementation with kale for 12 weeks has been found to reduce triglyceride and low-density lipoprotein-cholesterol (LDL-C) levels and prevent inflammation in mice fed a high-fat diet, although it did not control fat accumulation or insulin resistance [3]. Anthocyanins, the pigments responsible for red, purple, and blue colors in plants and fruits, serve as antioxidants and anti-inflammatory agents, contributing to the prevention of various chronic diseases such as cardiovascular disease, diabetes, fatty liver, and cancer. To prevent hypercholesterolemia, the adhesion of monocytes on vascular endothelial cells is reduced [4,5]. This condition is associated with diabetes mellitus by lowering hyperglycemia and glycated hemoglobin (hemoglobin A1c), as well as reducing insulin resistance and increasing proinsulin hormone production [3,6]. Additionally, it prevents fatty liver disease in rats fed a high-fat diet by lowering total cholesterol levels in the blood and increasing the expression of antioxidant enzyme genes, including catalase, NAD(P)H:quinone reductase, glutathione reductase, and superoxide dismutase [7]. It also prevents the occurrence of colon cancer and hepatocellular carcinoma by inhibiting the proliferation, movement, invasion, and apoptosis of hepatocellular carcinoma cells [8,9]. Moreover, it inhibits oxidation and destroys free radicals, preventing leukemia and hereditary cancer [10,11]. Anthocyanin is the largest group of phytochemicals found in vegetables and fruits, and it should be considered and utilized efficiently by extraction methods such as single or mixed solvent extraction (e.g., acidic solvent) alone or in combination with friendly organic substances to obtain high amounts of anthocyanin phytochemicals.

Anthocyanins (ACN) are of great interest to researchers due to their antioxidant properties and ability to reduce inflammation, thereby protecting against chronic diseases. Kale is cultivated for consumption as food and beverages, and processed kale leaf products can be used as dietary supplements to enhance consumer health and well-being, increasing the shelf life and stability of harvested kale. Kale contains anthocyanins found abundantly in fresh leaves and kale extract, which can reduce levels of free radicals and oxidative stress effectively. It has the potential to inhibit the growth of bacteria that cause acne and skin cell damage, providing benefits for facial and overall skin health [2]. Studies using scanning

electron microscopy have shown that extracts of *Hibiscus sabdariffa*, *Brassica oleracea* (var. capitata f. Rubra), and *Beta vulgaris*, rich in phenolic compounds such as anthocyanins and flavonoids, have anti-growth properties against bacterial strains such as *Staphylococcus aureus* and *Klebsiella pneumonia* [12]. In terms of skin health, sweat and sebaceous glands release organic compounds such as 2-nonenal, acetic acid, 2-hexanone, 6-methyl-5-hepten-2-one, benzaldehyde, allyl methyl sulfide, γ-octanolactone, γ-decanolactone, isovaleraldehyde, hexanal, and 2-pentanone, contributing to the occurrence of body odor and skin conditions. Consuming New Zealand blackcurrant extract powder containing 6 grams (equivalent to 138.6 milligrams of anthocyanins) daily for 7 days reduced the levels of these volatile organic compounds compared to a placebo group [13]. The results of this research provide valuable guidelines for increasing the value of kale in terms of health, economics, and the cosmetics industry, thereby promoting sustainable development.

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin, indomethacin, ibuprofen, and diclofenac are used to alleviate pain, reduce fever, and relieve inflammation. In dentistry, they are commonly used to manage postoperative pain, especially after procedures like wisdom tooth extraction. They work by inhibiting the action of cyclooxygenase enzymes (COXs), which accelerate the conversion of arachidonic acid into prostaglandins. However, the use of these drugs can lead to side effects such as gastrointestinal irritation, gastritis, ulcers, decreased platelet aggregation, and gastrointestinal bleeding. Many researches aimed at finding herbs and plant extracts with similar properties is valuable for reducing these side effects while enhancing the efficacy of NSAIDs. This can help replace the use of NSAIDs in Thai traditional medicine, alternative medicine, and integrative medicine, ultimately reducing the cost of purchasing these anti-inflammatory drugs. Previous studies have shown that various plants have analgesic and anti-inflammatory properties, including *Sapindus trifoliatus*, *Sapindus mukorossi*, *Sapindus Saponaria* [14,16], and *Dimocarpus longan* Lour [17]. These properties are attributed to compounds such as polyphenolics, flavonoids, anthocyanins, terpenes, and alkaloids found in these plants.

In nature, ACN are found ubiquitously in fruits and vegetables like cherries, grapes, colored rice, cabbage, broccoli, and kale, have antioxidant and anti-inflammatory properties. They inhibit inflammation and the growth of cancer cells in both laboratory experiments and the human body [18,19]. Additionally, consuming ACN-rich cherry juice has been shown to reduce pain and inflammation in muscle tissue [20]. Extracts from *Coriandrum sativum* leaves, rich in ACN, have been found to be more effective in alleviating pain in Swiss mice than aspirin [21]. Purple corn extract, containing high levels of ACN, prepared in capsule form, reduced pain effectively in a orofacial anti-allodynia test compared to aspirin in mice [22]. Furthermore, the peel of *Myrciaria floribunda*, widely cultivated in Brazil, has anti-inflammatory properties and pain relief by acting on opiate receptors, similar to the pain-relieving effects of morphine [23]. No toxicity in leafy greens, but leafy green smoothies have been produced for general consumption as organic food [24-27], promoting health benefits. Kale extract has been found to be non-toxic to human liver cells (HepG2) and cow liver cells. Rats given high doses of ACN for 28 days showed no signs of toxicity [29]. In a randomized, controlled, double-blind clinical trial, anthocyanin extracts equivalent to 125 milligrams of anthocyanins from corn extracts for 12 months did not prevent radiation-induced skin toxicity in breast cancer patients. However, nutritional supplementation with anthocyanins helped patients tolerate and safely undergo radiation therapy [30].

Study goals and objectives

Study goals: Developing a product: Kale extract capsules and evaluating their efficacy in relieving pain and inflammation in patients after wisdom tooth extraction.

Objectives: To compare the effectiveness between the kale extract capsule product and ibuprofen in reducing pain and inflammation in patients after lower third molar extraction

Study design

Subjects: All 20 volunteers were patients who came to surgical removal procedures at the Oral and Maxillofacial Surgery Clinic, Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand. Each participant will undergo extraction of one lower third molar on each side, randomized to either the control group or the experimental group. There will be a one-month interval between extractions on each side. The entire research procedure will be conducted by the same oral surgeon

For inclusion criteria:

- Age between 18-25 years.
- Patients with good health status (American Society of Anesthesiologists: ASA class I and II)
- Presence of impacted lower third molars on both left and right sides with similar mesioangulation, requiring surgical extraction, and free from pathologic lesion
- have no chronic diseases, and no history of allergy to ibuprofen

For exclusion criteria:

- Patients who were pregnant, heavy smokers, allergic to paracetamol, ibuprofen, or any anesthetic medications (e.g., mepivacaine and epinephrine-related drugs).
- Patients who had pain related to impacted lower third molars or facial/jaw regions on the day of surgery or admit pain.
- Patients who had taken medication or dietary supplements that could impact the wound healing process.
- Patients who experienced psychological or communication disorders

For discontinuation criteria:

- Patients could give up or withdraw from the study at any time.
- Unable to revisit for next follow-up

Methodology

Sample Size Calculation

Sample size was calculated according to the data obtained from the study conducted by Rauten et al. [31] in combination with data obtained from the study conducted by Yamano et al. [32] using the STATA® version 16.0 software (StataCorp, LLC, College Station, TX, USA). Initially, the total sample size was comprised of 16 individuals, who were then divided into two groups (n = 8 each). However, the researchers decided to increase the sample size to a total of twenty individuals, which were then divided into two groups (n = 10 each) by including additional participants.

Study Intervention

By using the Block Randomization method, participants were divided into two groups. Each participant will undergo extraction of one lower third molar on each side. One side will be the control group while the other side will be the experimental group, with a one-month interval between sides. The entire research procedure will be conducted by a single oral surgeon. Group 1 patients were instructed to take an ibuprofen tablet (400-mg size, Heidi-400, Siam Bheasach Company Limited, Bangkok, Thailand), taking 1 tablet four times a day after meals for 4 days after tooth extraction and Group 2 patients were instructed to take a 500 mg capsules of kale extract, taking 1 capsule four times a day after meals for 4 days after tooth extraction. Their medical histories were recorded and a physical examination was conducted prior to the tooth extraction procedure. The step-by-step procedure for tooth extraction, along with the potential risks associated with the treatment, was explained to the subjects. First, a local anesthetic solution containing 4% articaine hydrochloride with epinephrine (dilution 1:100,000) (Septanest® SP, Septodont, France) was injected by using the inferior alveolar nerve block and long buccal nerve block technique. The local anesthetic was administered and 10 minutes were allowed to pass for the action to take place. The tooth was then extracted using forceps. The wound was sutured with silk size 3-0. After the surgery, patients were instructed to bite down on a gauze pad for one hour and advised on post-operative care. Each group

receive medication as above. Both groups also receive amoxicillin 500 mg (SANOMOX - G 500, SEVEN STARS PHARMA, Thailand), taking 1 tablet after meals, four times a day for 5 days, while Paracetamol 500 mg (500-mg size, Tylenol-500, OLIC (Thailand), Company Limited, Phra Nakhon Si Ayutthaya, Thailand) is taken every 4 hours as needed for supplementary pain relief for 4 days. If the patient has a VAS score greater than or equal to 5, they will receive ibuprofen 400 mg (400-mg size, Heidi-400, Siam Bheasach Company Limited, Bangkok, Thailand).

Evaluation of post-operative outcomes

Patients will be evaluated and data recorded, including: visual analog scale (VAS) pain scores from day 1 to day 7 post-surgery and the time of first analgesic intake and total analgesic consumption.

Saliva Collection

Before collection of saliva, patients were asked to abstain from eating and drinking for one hour. Saliva samples were collected from each patient at three different time points (before tooth extraction, 3 days after tooth extraction, and 7 days after tooth extraction) using a plastic micropipette tip, then transferred into microtubes (1.5 mL capacity) and kept frozen in a freezer at -20ºC for up to 3 months for analyses of α-amylase activity, MMP9 and TGF-β2 concentrations.

Assay of salivary α-amylase activity

Salivary α-amylase activity was determined using a Megazyme reagent kit (NEOGEN Corporation, Wicklow, Ireland) according to the manufacturer's instruction, which is based on the Ceralpha procedure established by McCleary et al. [33]. In principle, an oligosaccharide substrate defined as non-reducing-end blocked *p*-nitrophenyl maltoheptaoside (BPNPG7) is hydrolyzed by endo-acting a-amylase in the presence of excessive α-glucosidase to liberate glucose and free chromogenic *p*-nitrophenol (PNP) giving a yellow-colored product (e_{mM} = 18.1, l_{max} at 400 nm). Before use, a vial of Amylase HR reagent containing 125 unit of α-glucosidase and 54.5 mg of BPNPG7 was reconstituted in 10.0 mL of distilled water. In assay, 0.2 mL aliquots of Amylase HR reagent solution and saliva samples were pre-incubated separately at 40 °C for 5 minutes. Then, 0.2 mL of the pre-equilibrated saliva was added directly to the tubes containing Amylase HR reagent solution (0.2 mL) and the mixture was incubated at 40 °C for exactly 10 minutes (from time of addition). At the end of the 10-minute incubation period, 3.0 mL of stopping reagent (1% tri-sodium phosphate buffer, pH 11) was added exactly and the tube contents were stirred vigorously. Finally, absorbance (A) values of the solutions and the reaction blank were read at 400 nm against distilled water. A standard curve was constructed by using different PNP concentrations. For quality control, the reagent kit shows absolute specificity for a-amylase, standard deviation value of 0.0189, standard errors of mean <5% and a coefficient of variation of 4.05%. Ceralpha unit (CU) of a-amylase activity was calculated by using the formula of a-Amylase activity (CU/mL) = DA_{400} x 4.7 x dilution. Accordingly, international unit (IU) was derived by multiplying CU by 4.6.

Measurement of MMP-9 and TGF-β2 Concentrations

Salivary MMP-9 and TGF-β2 concentrations were determined using sandwich-type enzyme-linked immunosorbent assay (ELISA) kits (ABBEXA Company Limited, Bar Hill, Cambridge, UK) according to the manufacturer's instructions. In terms of quality control, the human MMP-9 ELISA kit (Catalogue numbers: abx050165) indicated sensitivity of 0.1 ng/mL, coefficient of variation < 10% for intra-assay and inter-assay, while the human TGF-β2 ELISA kit (Catalogue numbers: abx153267) indicated sensitivity of < 5.7 pg/mL and a coefficient of variation of < 10% for intra-assay and < 12% for inter-assay.

Safety considerations

Risks from wisdom tooth extraction:

1. Bleeding from the surgical wound may occur within the first 24-48 hours.

Prevention: Instruct patients not to rinse their mouth. If bleeding occurs during surgery, consider using a hemostatic agent.
Treatment: Instruct patients to bite on gauze for 60 minutes. If bleeding persists, consider reopening the wound to apply a specific hemostatic agent.

2. Inflammation of the bone socket (Dry socket) or infected wounds may lead to the formation of pus.

   Prevention: Provide wound care instructions.

   Treatment: Place a surgical pack for dry socket and prescribe antibiotics with wound irrigation for infected wounds with pus.

3. Numbness or tingling sensation around the lips or tongue due to the proximity of nerves to the wisdom teeth or injection sites.

   Prevention: Assess nerve positions from radiographs.
   Treatment: Follow-up appointments with vitamin supplementation.

4. Broken roots may need to be left within the surgical wound, in case the roots are curved or close to nerves, and are less than 2 millimeters in size.

   Prevention: Perform surgery with caution.
   Treatment: Follow-up appointments for monitoring.

5. Adjacent teeth may experience of pain or moveable

   Prevention: Perform surgery with caution.
   Treatment: Follow-up appointments for monitoring.

6. Fracture of jaw bones

   Prevention: If there is a risk, advise patients to consume soft diet for the 1 month.
   Treatment: open surgery for repair fractured bones or tooth splinting.

Risks from anesthesia injection:

1. Allergic reactions to anesthesia or its components, such as magnesium sulfate, may include symptoms like difficulty breathing, facial swelling, tongue swelling, or throat swelling.

   Prevention: Review medication allergies.

   Treatment: Basic life support (BLS).

2. Overdose of anesthesia or vasoconstrictor drugs from injections may result in symptoms such as restlessness, tremors, excessive sweating, pale skin, difficulty breathing, increased blood pressure, or rapid heartbeat.

   Prevention: Aspirate before administering injections.
   Treatment: Basic life support (BLS).

3. Injection into the mucosal layer may cause small bruises or bleeding spots at the injection site.

Prevention: Administer injections slowly and avoid piercing blood vessels.
Treatment: Self-resolution.

4. Injecting medication into the bone carries risks such as needle breakage and sudden bone inflammation (<1%).

   Prevention: Avoid excessive needle pressure.
   Treatment: Surgery in case of needle breakage and prescribe antibiotics for bone inflammation.

Follow-up

Follow up will be done on 1st day and 4th day for saliva collection and post operation complication. Follow up on seven day after surgery for saliva collection, suture removal, of patient had complication as above (safety consideration) continue appointment for observe or treatment along each guideline.

Data management and statistical analysis:

Results/data were analyzed and expressed as mean ± interquartile range (IQR) using the STATA® version 16.0. Median α-amylase activity, MMP-9 and TGF-β2 concentrations in the saliva samples collected at different time points among patients who received paracetamol and ibuprofen were determined and compared using Mann-Whitney U test at a 95% confidence level. Proportions of LTHI values between patients who had received paracetamol and ibuprofen were determined and compared using Fisher's exact test at a 95% confidence level. The relationship between LTHI values, MMP-9 concentrations, and TGF-β1 concentrations were determined and compared using Spearman's correlation coefficient at a 95% confidence level. Accordingly, at least $P < 0.05$ was considered significantly different. Consolidated Standards of Reporting Trails (CONSORT) flowchart is available, which shows the progress of all participants through the trail, as shown in Fig.1.

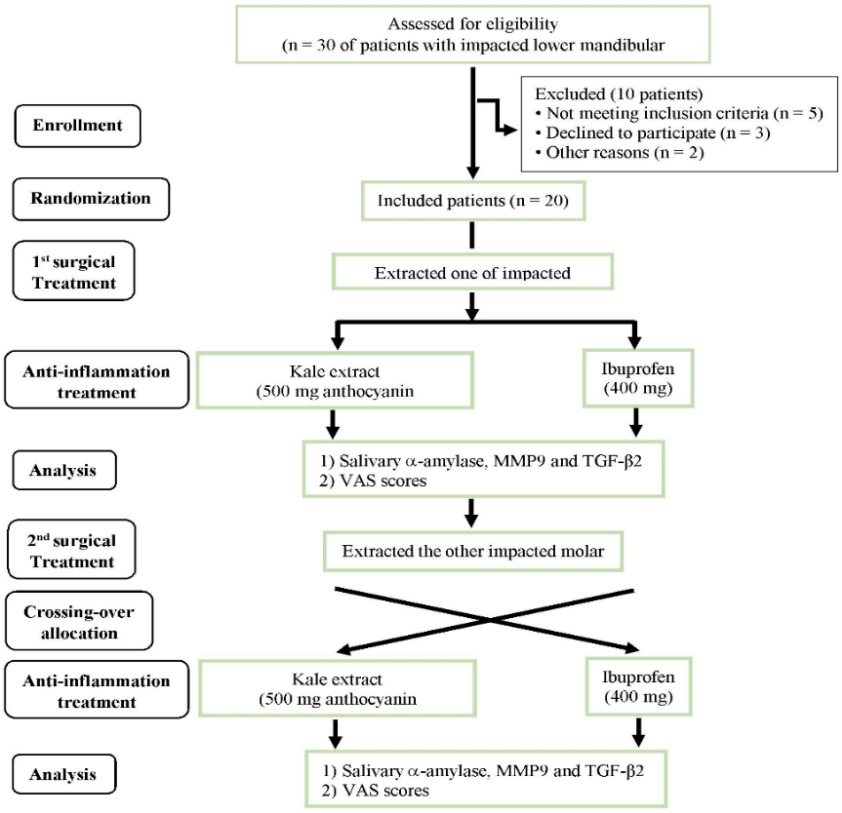

Figure 1. CONSORT flow diagram of the study design. Abbreviations: LTHI = Landry, Turnbull, Howley Index, MMP-9 = matrix metalloproteinase 9, TGF-β1 = transforming growth factor-beta 1, VAS = visual analogue scale.

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Quality assurance

The protocol should describe the quality control and quality assurance system for the conduct of the study, including GCP, follow up by clinical monitors, DSMB, data management etc.

Expected outcomes of the study

Curly kale leaves which are abundant with flavonoids (e.g. anthocyanins) could exert strong anti-inflammatory and wound healing effects on tooth extraction wound

Dissemination of results and publication policy

The protocol should specify not only dissemination of results in the scientific media, but also to the community and/ or the participants, and consider dissemination to the policy makers where relevant. Publication policy should be clearly discussed- for example who will take the lead in publication and who will be acknowledged in publications, etc.

Duration of the project: 12 months (from 01/01/2023 to 30/06/2023)

Activities	**Year 2023 (month)**											
	1	2	3	4	5	6	7	8	9	10	11	12
1. Wrote a study protocol												
2. Submitted a proposal for human experimentation												
3. Revised the proposal												
4. Approved the Human Ethical Clearance												
5. Recruited patients with impacted mandibular third molar												
6. Surgically removed and crossing-over intervened the participants with ibuprofen or kale extract												
7. Assessed VAS values and collected saliva												
8. Assayed the alpha-amylase activity, MMP9, TGF-β2												

Problems anticipated

This section should discuss the difficulties that the investigators anticipate in successfully completing their projects within the time frame stipulated and the funding requested. It should also offer possible solutions to deal with these difficulties.

Project management

This section should describe the role and responsibility of each member of the team

No.	Investigator	Role and responsibility
1	Vuttinun Chatupos, D.D.S. Assistant Professor	Supervisor, Surgeon
2	Sansanee Neelawatanasook, D.D.S.	Surgeon, Data collector
3	Tidanut Sangutai, D.D.S.	Surgeon
4	Atit Khanutwong, D.D.S.	Surgeon
5	Somdet Srichairatanakool, Ph.D. Professor	Analysis of α-amylase, MMP9 and TGF-β1

Ethics

Ethical approval for this project was granted by the Human Experimentation Committee of the Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand (Certificate Number: 65/2565, Date: December 16th, 2022). All patients were fully informed about the particulars of the study and have willingly provided their signatures in the consent forms. This study followed the guidelines of the Helsinki Declaration 2008: Ethical Principles for Medical Research Involving Human Subjects.

Informed consent forms

Reference document number 2

Research protocol: part 2

Budget: Total 51,200 Thai baht

No.	Items	Cost
1	Gauze sheet, cotton, antiseptic	3,000
2	Disposable dental care equipment	7,000
3	Alpha-amylase assay reagent (€305.00)	11,580
4	Sandwich ELISA kit for MMP9 (USD 526.93)	10,860
5	Sandwich ELISA kit for TGF-beta1 (USD 526.93)	18,760
	Total	51,200

Other support for the project

1. Residency Training Program in Oral and Maxillofacial Surgery, Faculty of Dentistry, Chiang Mai University supported disposable small equipment and consumable reagents
2. Royal project Foundation Fund paid for consumable alpha-amylase assay reagent and ELISA kits for MMP9 and TGF-β1.

Collaboration with other scientists or research institutions: No

Links to other projects: No

Curriculum Vitae of investigators: We have now provided the CV of all the investigators.

Other research activities of the investigators: All research projects have been finished.

Financing and insurance: No