Combination of plant phenolics and isoquinolinium alkaloids protects gingival fibroblast and improves post-extraction healing after lower third molar extraction

Karin Chytilova[1], Adela Galandakova[2], Katerina Valentova[2,3], Peter Tvrdy[1]

Aims. The effect of polyphenolic fraction of *Lonicera caerulea* (PFLC) and alkaloid fraction of Macleaya cordata (AFMC) mix on the production of inflammatory mediators in human gingival fibroblasts pretreated with lipopolysaccharide (LPS) was investigated. In addition, protective effects of mucoadhesive paste containing combination of PFLC and AFMC (0.05% and 0.01%, respectively; n=15, Group A) and placebo (n=15, Group B) were evaluated in patients after surgical extraction of lower third molars.

Methods. Gingival fibroblasts were pre-treated with LPS (10 μg/mL; 24 h) and PFLC/AFMC (25/0.25; 50/0.25; 100/0.25; 25/0.5; 50/0.5; 100/0.5 μg/mL) in serum-free medium was applied for 4 h. Then the interleukin-6 (IL-6), reactive oxygen species (ROS) generation, level of intracellular glutathione (GSH) and expression of cyclooxygenase-2 (COX-2) were evaluated.

The study was a 6-day, single-center, randomized, double-blind and placebo-controlled trial consisting of two parallel treatment arms. A modified Oral health impact profile questionnaire including both general oral condition and extraction related questions, was used to evaluate the oral condition and other changes before (day 0) and on the days 1, 3 and 6 after surgical extraction.

Results and Conclusion. The combination of PFLC with AFMC caused a reduction of ROS generation, reduced IL-6 production and suppressed the expression of COX-2. In group A the paste treatment contributed to improvement of oral health-related quality of life. Topical application of PFLC and AFMC into the extraction wound improved post-extraction site wound healing probably by antioxidant and anti-inflammatory mechanisms.

Key words: *Lonicera caerulea*, *Macleaya cordata*, anthocyanins, isoquinolinium alkaloids, gingival fibroblast, third molar surgery

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[1]Department of Oral and Maxillofacial Surgery, University Hospital, I. P. Pavlova 6, 779 00 Olomouc, Czech Republic
[2]Department of Medical Chemistry and Biochemistry, Faculty of Medicine and Dentistry, Palacky University Olomouc, Czech Republic
[3]Institute of Microbiology, Czech Academy of Sciences, Prague, Czech Republic
Corresponding author: Adela Galandakova, e-mail: adela.galandakova@upol.cz

INTRODUCTION

Gingivitis and periodontitis are the most widespread diseases affecting mankind. The main factor associated with these diseases is dental plaque harboring pathogenic bacteria. Extracellular substances, produced by bacteria in a mature plaque, include polysaccharides, toxins, antigens, enzymes and lactic acid and these play an important role in the development of inflammatory oral diseases[1]. A critical role is ascribed to a major constituent of bacterial walls, lipopolysaccharide (LPS) which stimulates the production of inflammatory mediators like reactive oxygen species (ROS), cytokines and prostaglandins. The latter are produced by cyclooxygenases, present both as constitutive (COX-1) and inducible (COX-2) isoforms[2]. Periodontal pathology is present in at least 25% patients in the lower third molar (M3) region. It is often associated with asymptomatic M3s in otherwise periodontally healthy patients and is difficult to eliminate effectively. The prevention of progression of periodontal disease is often given as justification for M3 removal. On the other hand, M3 removal can create or exacerbate periodontal problems in some cases[3].

It is well known that regular oral care decreases bacterial masses and reduces the frequency of oral diseases. Mechanical dental hygiene in combination with active ingredients such as fluorides and chemical plaque inhibitors, e.g. chlorhexidine, can reduce the bacterial count[4,5]. But not all available means are suitable for long term use. On the other hand, some herbal extracts can also reduce bleeding, improve the gingival index and are safe for long time period. In this regard, there is worldwide interest in plant polyphenols[6,7] and isoquinolinium alkaloids[8].

We have shown that the freeze-dried fruit of *Lonicera caerulea* (blue honeysuckle, Caprifoliaceae) and polyphenolic fraction of the fruits (PFLC) inhibited biofilm formation and adhesion of *Candida parapsilosis*, *Staphylococcus epidermis*, *Escherichia coli, Enterococcus faecalis*, and *Streptococcus mutans* at *in vitro* level[9]. PFLC reduced ROS production, intracellular glutathi-

one (GSH) depletion and suppressed expression of interleukin-6 (IL-6) and COX-2 in LPS-stimulated human gingival fibroblasts[10]. Similar results were obtained with the alkaloid fraction of *Macleaya cordata* (plume poppy, Papaveraceae) (ref.[11]). Sanguinarine and chelerythrine, main alkaloids of *M. cordata, Sanguinaria canadensis* and *Chelidonium majus*, have a long history of use in reducing gingival inflammation and plaque formation at *in vivo* level[8]. SAMITAL® - highly standardized botanical extracts of *M. cordata, Vaccinium myrtillus* and *Echinacea angustifolia* was shown to decrease the severity of chemo/radiotherapy-induced oral mucositis in patients with head and neck cancer[12].

The aim of this study was assess the effect of *L. caerulea* polyphenolics and *M. cordata* alkaloids combination at different concentrations on the level of GSH, production of ROS, expression of IL-6 and COX-2 in LPS-stimulated human gingival fibroblasts. Additionally, we developed a mucoadhesive paste containing the mix of both plant fractions. The pilot double-blind and placebo-controlled trial was designed to evaluate the short-term clinical effects of topical paste application in alveolus healing after lower third molar extraction.

MATERIALS AND METHODS

Plant extracts

L. caerulea L. berries were harvested in Lipník nad Bečvou (Central Moravia; Czech Republic; 2008). The fruits were frozen and stored at -20 °C. The fraction of *L. caerulea* phenolics (PFLC) was prepared and analyzed for phenolic acids and anthocyanins as described previously[9]. PFLC contained 0.74% (w/w) phenolic acids (chlorogenic, caffeic, ferulic, protocatechuic, gentisic, rosmarinic and vanillic) and 77% anthocyanins (60% cyanidin-3-*O*-glucoside). The alkaloid fraction of *M. cordata* (AFMC) was supplied by Phytobiotics Futterzusatzstoff, GmbH (Eltville, Germany). The content of sanguinarine was 44.9% and chelerythrine, 14.7%.

Materials

2,2´-dinitro-5,5´-dithiobenzoic acid was purchased from Serva (Germany). COX-2 rabbit polyclonal antibody, actin (1–19) goat polyclonal antibody, horseradish peroxidase conjugated goat anti-rabbit and rabbit anti-goat antibodies, Western Blotting Luminol Reagent were supplied by Santa Cruz Biotechnology (USA). Dihydrofluorescein acetate was from Fluka Chemie (Germany). Human interleukin-6 ELISA kit (Quantikine®) was obtained from R&D Systems (USA). Protease inhibitor cocktail tablets (Complete™) were purchased from Roche (Germany). Dulbecco's modified Eagle's medium, heat-inactivated foetal calf serum, stabilised penicillin–streptomycin solution, pamycon, colinomycin, trypsin-EDTA solution, neutral red, Triton X-100, Lipopolysaccharide (LPS; from *Escherichia coli* 055:B5), Immun-Blot™ PVDF (polyvinylidene difluoride) membrane, KODAK BioMax light film, phenylmethylsulfonyl fluoride and all other chemicals were purchased from Sigma–Aldrich (USA).

Paste

The pastes used in this study were prepared using current Good Manufacturing Practices at FAVEA Ltd. (Koprivnice, Czech Republic). The pastes composition used for the clinical trial consisted of (% w/w): Plant extracts paste: PFLC 0.05, AFMC 0.01, paraffin oil 46.8, Blanose 41.0, polyethylene A-C 5.0, peppermint aroma 3.0, Polidocanol 1.0, Nipabutyl 0.1; Placebo paste: Food dye E163 0.02, paraffin oil 46.9, Blanose 41.0, polyethylene A-C 5.0, peppermint aroma 3.0, Polidocanol 1.0, Nipabutyl 0.1. Purified water was used to attain 100%. The pastes were indistinguishable in appearance and had a pH of 5.9. The paste was packed in an aluminum tube with a codename. Identification code designation was at the end of the study.

Cell culture

Human gingival fibroblasts, obtained from medically healthy donors who were clinically free of periodontal disease, were isolated and cultivated as described previously[10]. Samples of gingiva were obtained from patients undergoing surgical removal of M3s at the Department of Oral and Maxillofacial Surgery (University Hospital Olomouc). The tissue acquisition protocol adhered to the requirements of the local Ethics Committee. All patients signed written informed consent.

For all experiments, the gingival fibroblasts were seeded on plates at a density of $1 \cdot 10^5$ cells/cm^2. Cells were used between the 3rd and 10th passages for experiments. For treatment of cells, the stock solutions of PFLC/AFMC mix (final concentration 25/0.25; 50/0.25; 100/0.25; 25/0.5; 50/0.5; 100/0.5 μg/mL, 2000 × concentrated) were prepared in dimethylsulfoxide (DMSO). The final concentration of DMSO in serum-free medium was thus 0.5 % v/v. LPS stock solution (10 mg/mL) was prepared in sterile water.

LPS treatment

For the evaluation of PFLC or AFMC effects on LPS-treatment, gingival fibroblasts were pre-treated with LPS (final concentration 10 μg/mL; 24 h) in serum-free medium. The cells were then washed with PBS and PFLC/AFMC (25/0.25; 50/0.25; 100/0.25; 25/0.5; 50/0.5; 100/0.5 μg/mL) in serum-free medium was applied for 4 h. Then the media were collected and immediately frozen (-80 °C) for the measurement of IL-6. Cells were washed with PBS, harvested and ROS generation, level of intracellular GSH and expression of COX-2 were evaluated.

Cell viability assay

Viability was assayed by neutral red (NR) uptake by lysosomes[13].

Determination of ROS production

ROS production was monitored using the dihydrofluorescein acetate assay[14].

Determination of intracellular GSH

GSH concentration was measured as described previously[10].

Determination of IL-6

Interleukin-6 level was determined using specific immunoassays (Quantikine®). The assay was performed according to the manufacturer's protocols.

Expression of COX-2

The amount of COX-2 was investigated using Western immunoblot analysis as described previously[10]. The used antibodies are specified in part "Materials".

Design of study and patients

The study was conducted according to the guidelines laid down in the 2008 Declaration of Helsinki and all procedures involving human subjects were approved by the Ethics Committee of the University Hospital and Faculty of Medicine and Dentistry, Palacký University in Olomouc (reference 96/05). The study was a 6-day, single-center, randomized, double-blind and placebo-controlled trial consisting of two parallel treatment arms. It was conducted between September 2009 and August 2010 at the Department of Oral and Maxillofacial Surgery of University Hospital, Czech Republic.

Participants meeting all of the inclusion criteria and none of the exclusion criteria (Table 1) and consenting to study participation were randomly divided into two equal groups (n=15): group A (PFLC + AFMC) and group B (placebo).

A total of 30 subjects (10 females and 20 males) aged between 18 – 34 years and indicated for surgical extraction of an impacted (36%) or retained (64%) lower third molar, were selected. Written informed consent was obtained from all patients. Participants were healthy individuals with no systemic diseases, not taking any medication and none had undergone such therapy in the previous six months. No subject had a history of known sensitivity or oral mucosal tissue reaction to pastes. At the baseline visit, the patient´s demographic data were registered, including age, sex, risk factors like smoking and pre-existing morbidities (Table 2).

The healing effect of both pastes was evaluated in patients after surgical extraction of M3. At baseline, the patients had to have the community periodontal index

Table 1. Eligibility criteria.

Inclusion criteria	Exclusion criteria
• females or males with age between 18–35 years • community periodontal index of treatment needs (CPITN) ≤1 an plaque index (PL I)=0 • indication for surgical extraction of an impacted or retained lower third molar under local anesthesia • the same surgeon for all patient • healthy individuals • follow-up (first, second, third and sixth day after surgical extraction)	• age under 18 and over 35 years • bad oral hygiene (CPITN≤1, PL I >1) • surgical extraction of lower third molar under general anesthesia • not the same surgeon, who extracted lower third molar • antibiotic treatment during the study • patients with a history of medical or surgical events that could affect the study outcome or place the subject at risk, including cardiovascular disease, gastrointestinal problems, metabolic, renal, hepatic, neurological, sexually transmitted diseases or active musculoskeletal disorders • use of any medicaments, narcotics, heavy episodic drinking of alcohol • simultaneous participation in another clinical trial and participation in a clinical research trial within 30 days prior to randomization

Table 2. Baseline parameters.

Patients	Group A (n=15, PFLC+AFMC)	Group B (n=15, placebo)
Gender - Woman	10	8
Gender - Men	5	7
Age (years)	23.1 (19–28)	22.4 (18–34)
Diagnosis: Impacted lower third molar	5	7
Diagnosis: Retained lower third molar	10	8
OHIP - before extraction	7.0 ± 1.8	6.3 ± 1.6
CPITN before extraction	1	0.5
PL I before extraction	0	0

of treatment needs (CPITN) ≤ 1 and plaque index (Pl I) = 0. The surgical extraction was performed under local anaesthesia (articaine + epinephrine) and the extraction wound was washed with physiological solution. A total of 1 ml bioadhesive paste was directly applied into the wound by the surgeon (the same for all patients) on the 1st, 2nd and 3rd day. Pasta in an aluminum tube was randomly selected by the surgeon from the 30 coded tubes. None of the participants knew the contents of the tube. A modified Oral health impact profile (OHIP) questionnaire including both A (general oral condition) and B (extraction related questions) (Appendix Table 2) was used to evaluate the oral condition and other changes before (day 0) and on the days 1, 3 and 6 after extraction. The recovery for each OHIP item was defined as the number corresponding to a total of 5 points on a Likert-type scale[15]. The scale included the following responses: never (coded 0), hardly ever (coded 1), occasionally (coded 2), fairly often (coded 3), and very often (coded 4). The OHIP scale ranged from 0 to 64 with higher scores indicating poorer oral health-related quality of life (QoL). Pain intensity after each paste application was evaluated using a visual analogue scale (0 = no pain, 4 = unbearable pain). Moreover, need for analgesic and antibiotic treatment after extraction was recorded.

Statistical analysis

In vitro, each experiment was repeated at least three times and the data are reported as the means ± SD. The Student t-test was used for statistical analysis. The significance of the differences between visits in the clinical trial was analysed by a one-way analysis of variance (ANOVA). Normality of data was assessed using Shapiro-Wilk test. Student t-test with Bonferroni correction, Bartlett´s and Tukey´s tests of significance were used for normally distributed data. The Friedman test and Wilcoxon paired test with Bonferroni correction and McNemar´s tests were used for qualitative or non-normally distributed data (OHIP questionnaires). Fisher's exact test with Bonferroni correction was used to compare the groups on separate questions of the OHIP questionnaire. Data were analyzed using SPSS version 15 (SPSS Inc., Chicago, USA). The level of confidence required for significance was selected at $P<0.05$.

Patient consent

Written informed consent was obtained from all patients.

Statement of Clinical Relevance

The extraction of the impacted mandibular third molar is often attended by complications (inflammation, pain, swelling). We want to find some topical mucoadhesive gel with anti-inflammation, antioxidant properties and patients could it apply alone daily to the wound.

RESULTS

No visible alterations to the morphology of gingival fibroblasts treated with different concentrations of PFLC + AFMC (25/0.25; 50/0.25; 100/0.25; 25/0.5; 50/0.5; 100/0.5 μg/mL) was found using an inversion microscope. No cytotoxic effects were detected by NR viability assay for the tested concentrations range after 24 h treatments (data not shown).

No mixture of PFLC with AFMC had any influence on the GSH level (data not shown). Treatment of cells with LPS (10 μg/mL, 24 h) resulted in the reduction of GSH level (70.8% of control). After the PFLC + AFMC application GSH increase was found. A statistically significant effect was found only for the mix PFLC + AFMC 100/0.5 μg/mL (Fig. 1(i)).

Dihydrofluorescein acetate assay was used for ROS determination. Treatment of cells with PFLC + AFMC at all concentration had no effect on the basal level of intracellular ROS production (data not shown), while stimulation of the cells with LPS (10 μg/mL, 24 h) caused a high production of ROS. Treatment of cells with PFLC + AFMC (25/0.25, 50/0.25, 100/0.25 μg/mL) led to dose-dependent reduction of ROS (Fig. 1(ii)).

After 24 h, LPS (10 μg/mL) induced significant production of IL-6 which was almost undetectable in untreated cells. Treatment with PFLC + AFMC (25/0.25; 50/0.25; 100/0.25; 25/0.5; 50/0.5; 100/0.5 μg/mL) statistically significantly ($P<0.05$) reduced the IL-6 level (Fig. 1(iii)).

The effect of PFLC + AFMC (25/0.25; 50/0.25; 100/0.25; 25/0.5; 50/0.5; 100/0.5 μg/mL) was confirmed at the protein level by Western immunoblotting. COX-2 expression was drastically increased by LPS stimulation (Fig. 1(iv), line 2) and reduced by PFLC + AFMC application. The effect of PFLC + AFMC was more potent for the dose with lower concentration of AFMC (0.25 μg/mL). The maximal inhibition of COX-2 was found in the cells treated with PFLC + AFMC at the concentration of 25/0.25 and 100/0.25 μg/mL (Fig. 1(iv), line 3 and line 5, respectively).

Patient recruitment is displayed in Fig. 2(i). Of the 125 patients who underwent surgical extraction of impacted or retained third lower molar performed the same surgeon, 95 patients did not meet the inclusion criteria or met some of the exclusion criteria. The remaining 30 (18 – 34 years) eligible patients were enrolled and were randomized to two groups: Group A (n=15, PFLC + AFMC) and Group B (n=15, placebo). The groups were similar with regard to baseline characteristics (Table 2). The extraction duration was not significantly different between the groups ($P=0.640$, data not shown). The application of pastes is depicted in Fig. 2(ii).

In relation to the QoL, the preoperative (day 0) OHIP score in groups A and B was 7.5 and 6.6, respectively. On day 1, the values increased to 21.7 and 26.1 and successively decreased to 13.9 and 17.5 on day 3 and to 10.1 and 10.6 on day 6 after extraction. Non-significant improvement of the post-extraction QoL in both experimental

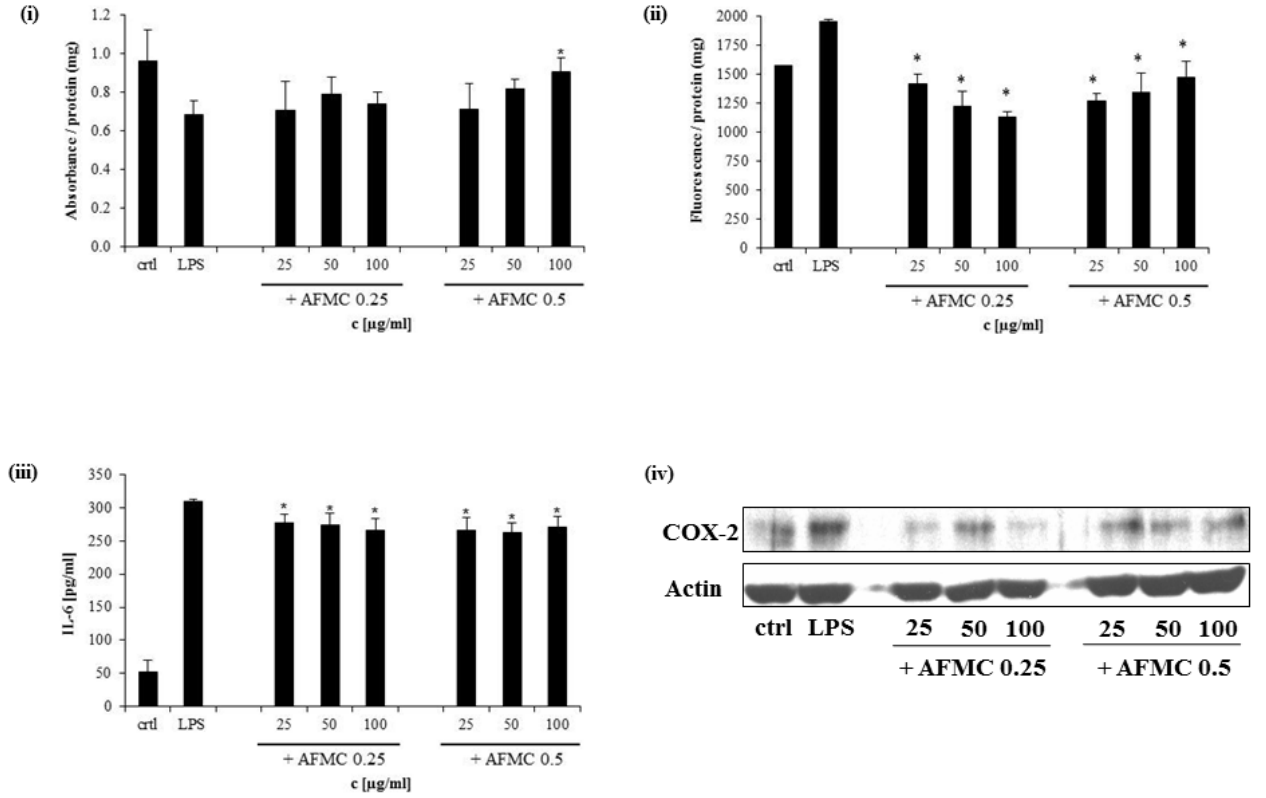

Fig. 1. Effects of PFLC/AFMC on LPS-stimulated (i) GSH depletion, (ii) ROS generation, (iii) IL-6 level and (iv) COX-2 expression. Fibroblasts were pre-treated with LPS (10 µg/mL, 24 h) and then incubated with PFLC/AFMC for 4 h. Control cells were incubated with DMSO (0.5 %, v/v, negative control). Data are expressed as mean ± SD (n=3) **P*<0.05 statistically different from LPS-treated cells (positive control).

groups was observed (Fig. 3(i)). When the OHIP items were evaluated separately, significant (*P*=0.034) improvement was noted only in item A3 (Difficulties in sleeping) on day 1 after extraction for group A (60.0%) compared to group B (100%). Overall, the extraction had the highest impact on items A1 (discomfort eating foods), A2 (problems with mastication), B1 (problems with mouth opening), B2 (facial swelling) and B8 (pain intensity), which most of the patients reported occasionally (2) to fairly often (3) on day 1 after extraction. On the other hand, nausea (item B5) was reported only very occasionally (rating < 1) regardless of treatment.

The first and second day after surgery, analgesics were the most used in group B (placebo). Patients used NSAIDs, namely *ibuprofenum*. The consumption of analgesics decreased in both groups on the third day after surgery and there were less drug users in group A (Fig. 3(ii)).

Pain intensity was monitored after each paste application every 2 h up to the evening. Mean pain intensity increased for all groups in the evening. The lowest mean pain intensity was reported on day 3. The higher mean pain intensity was found in group B (placebo) for all applications. The lowest mean pain intensity was found in group A for all applications (data not shown).

The topical application of pastes did not elicit any mucosal reaction. No adverse events were recorded. The pastes were well tolerated and did not show any unpleasant effect.

DISCUSSION

Inflammatory periodontal diseases are related to dental plaque formation and are influenced by microbial composition of plaque microbiota and the host`s immune system. The dry socket (alveolar osteitis) that may occur as inflammatory complications after M3 extraction is in many aspects similar. These conditions are accompanied by inflammatory response of the periodontal tissues mainly inhabited by gingival fibroblasts[16]. For this reason, there are ongoing efforts to formulate preparations which inhibit dental plaque formation and suppress inflammation. Research in this field is focused mainly on anti-microbial and anti-inflammatory properties of chemotherapeutic agents such as chlorhexidine and cetylpyridinium chloride and oral antibiotics such as tetracycline and doxycycline. However, side effects can occur when these chemical drugs are prescribed for an extended period, e.g. chlorhexidine is related to hypersensitivity and generalized allergic reactions[17]. Hence, the search for alternative agents continues and natural secondary metabolites isolated from plants used as traditional medicines are considered good alternatives. Among natural compounds, plant polyphenols and isoquinoline alkaloids are the most popular in oral care products. *In vitro* and *in vivo* studies proved that polyphenolics show in addition to their anti-septic, antimicrobial and anti-inflammatory properties, anti-oxidant activity against spontaneous oxidation[6,18].

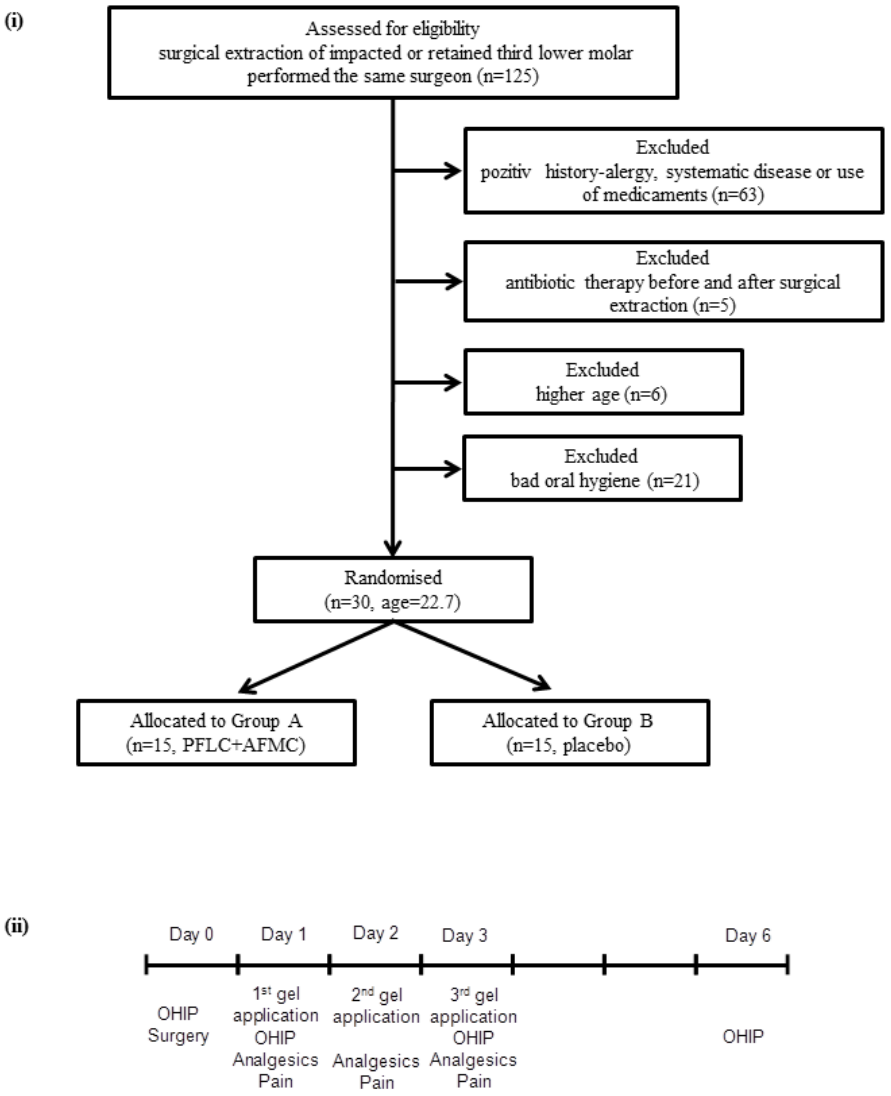

Fig. 2. (i) Flow chart of the clinical trial and (ii) design of the clinical trial.

In cells, LPS is one of the best-characterized stimuli that induce the transcription of genes encoding pro-inflammatory proteins including their products and ROS. The phenolic fraction of *L. caerulea* fruits inhibited the expression of inducible NO synthase, COX-2, nitric oxide and prostaglandin E2 production in LPS-stimulated RAW264.7 cells[19]. These authors compared the anti-inflammatory effect of PFLC major components, cyanidin 3-glucoside, cyanidin 3-rutinoside and chlorogenic acid with that of PFLC. Their anti-inflammatory effect was weaker than the effect of the complex phenolic fraction.

Isoquinoline alkaloids sanguinarine and chelerythrine are isolated from different species of the genera *Macleaya, Sanguinaria, Chelidonium* and *Fumaria* (Papaveraceae). These alkaloids or plant extracts containing both alkaloids have shown anti-microbial, anti-inflammatory, anti-oxidant and analgesic properties at *in vitro* and *in vivo* levels[20]. We reported that acute gingivitis can be effectively treated with topical application of a paste containing the alkaloid fraction of *C. majus* roots[21] or a toothpaste containing a mix of AFMC and phenolics of *Prunela vulgaris*[22].

The present pilot trial translated the results from our recent studies on human gingival fibroblasts[10,11]. These cells are a good model for further study of the inflammatory responses *in vivo*. The PFLC or AFMC significantly protected human gingival fibroblasts against LPS-induced pro-inflammatory mediators. Here we found that application of the mixture AFMC with PFLC to LPS-stimulated gingival fibroblasts resulted in significantly decreased IL-6 level, expression of COX-2 and ROS generation (Fig. 1). These results are in agreement with those of the aforementioned. However, for all concentrations of the combination of AFMC with PFLC, their antioxidant effect was stronger than that of PFLC or AFMC itself.

A major goal of the pilot double-blind clinical trial was to assess the effect of a mucoadhesive paste containing a mix of plant extracts AFMC and PFLC on wound healing. The results demonstrated non-significant improvement on the QoL, assessed using OHIP questionnaire in group A (Fig. 3(i)). There was also lower consumption of analgesics and lower pain intensity after extraction (Fig. 3(ii)).

CONCLUSION

The combination of the phenolic fraction of *L. caerulea* fruit and the *M. cordata* alkaloids showed beneficial effects on the antioxidant imbalance and inflammation stimulated by LPS in human gingival fibroblasts, mainly via suppression of ROS, IL-6 and COX-2 level. Topical treatment of the wound after extraction of third molar with the paste containing 0.05% PFLC and 0.01% AFMC can provide relief from pain and reduce the analgesic requirements of the patient. The paste had no negative effects on post-extraction site wound healing. Further more extensive *in vivo* study exploring other effects of the tested paste is required to elucidate its potential value in oral care.

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Author contribution: KCH, PT: contributed to the design of clinical study, obtained and analyzed the data, interpreted the results; AG: contributed to the design of *in vitro* study, obtained and analyzed the data, interpreted the results; KCH, PT, AG, KV: All authors contributed to the preparation of the manuscript and approval of the final version.

Conflict of interest statement: The authors state that there are no conflicts of interest regarding the publication of this article.

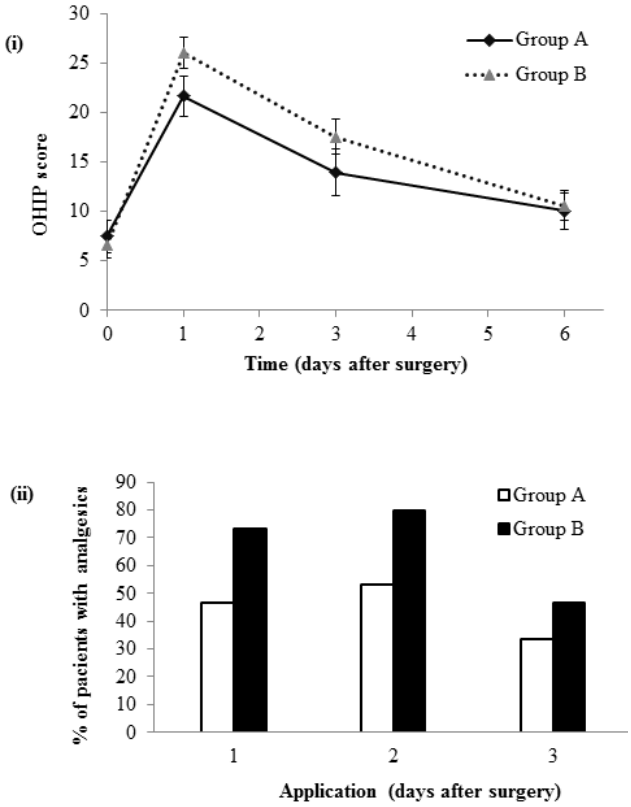

Fig. 3. Impact of paste topical application on (i) patient's quality of life (OHIP score) before (day 0) and after surgical lower M3 extraction (expressed as mean ± SE; n=15) and (ii) use of analgesics after surgical lower M3 extraction in the Groups A and B.

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Supplemental Material:

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