

In Vitro and In Vivo Studies of a Bioadhesive Gel Containing Volatile Oil Extracted from Fruits of *Zanthoxylum limonella* Alston

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Abstract- Fruits of *Zanthoxylum limonella* Alston, one of the spices traditionally used for toothache relief, were hydro-distilled to obtain about 4.3% yield of volatile oil (ZL). 30% of ZL in a bioadhesive gel was prepared for in vitro permeation and in vivo tests. In vitro permeation of total phenolic contents of the ZL gel through porcine esophagus, as the model mucous membrane, using Franz diffusion cells indicates an average steady-state rate of 0.1 ± 0.02 mgGAE/cm²/h at 37 C (n = 5) without lag time. In vivo study in buccal pressure-wounds of rats indicates that licking behaviour was obvious and could be inhibited by gels containing xylocaine or ZL but not fluocinolone. Histological analysis of the inflamed cells in excised oral wounds showed that it took 1 day for fluocinolone gel to significantly reduce the number of inflamed cells and 2 days for ZL gel (p<0.05 both). Comparative wound assessments (edema and erythema) indicate mild anti-inflammatory activity of ZL gel. Thus, phenolic components of ZL could readily permeate through mucous membrane and ZL gel showed potential benefits for topical use in oral wound.

Index Terms- *Zanthoxylum limonella* Alston, total phenolic content, in vitro permeation, in vivo buccal wounds.

I. INTRODUCTION

Pressure-wounds on mucous membrane in the buccal cavity introduce discomforts and inflammation which manifests swelling, redness, heat, and pain. Complementary and alternative medicine employs natural products, particularly those derived from dietary or spice ingredients for topical applications, is well accepted [1]. *Zanthoxylum limonella* Alston., family Rutaceae, is locally known in Northern region of Thailand as one of the spices, namely Ma-Kwaen. Phytochemicals of *Zanthoxylum spp.* reveals richness of flavonoids and limonoids in volatile oils [2]. Fruits of *Z. limonella* are traditionally used in toothach relief [3] and reported to contain about 30% of limonene as the major component [4]. Limonene exhibits anti-inflammatory via cyclooxygenase and 5-lipoxygenase inhibition [5]. D-limonene is clinically investigated as anti-cancers and treatment of gastroesophagus reflux and gallstone dissolution [6].

This research aims to investigate in vitro permeation and in vivo effect of a bioadhesive gel containing volatile oil from *Z. limonella* so as to prove the traditional use using a modern formulation.

II. MATERIALS AND METHODS

a. Extraction and product preparation

Fruits of *Zanthoxylum limonella* Alston. were separated for hydro-distillation to obtain volatile oil (ZL). Locally harvested and dried cobs of purple waxy corns (*Zea mays L. ceritina* Kulesh.) from an open-pollinated variety (Kao Kum) were grinded and extracted in water at 80C for 30 min and freeze-dried (A).

PEG40 (Sigma-Aldrich, USA), 0.1% sodium polyacrylate (GMP, Bangkok, Thailand) and 0.1% carbomer934P (GMP, Bangkok, Thailand) were premixed to form clear gel in isotonic phosphate buffer saline at pH 7.4. ZL gel was obtained by mixing 30% of ZL with the premixed gel. Similarly, gels of fluocinolone (0.1%) or xylocaine (1%) were prepared by mixing the drug concentrates in the same premixed gel base. 30% ZL and 10% anthocyanin extract (A) were mixed to form ZLA gel.

b. In vitro skin permeation study

Franz diffusion cells (0.5 cm² diffusion area, 5 ml receptor volume) were mounted with porcine esophagus, used as the barrier membrane. 0.1% albumin solution would be used as the medium in the receptor site and incubation temperature mimic the physiological condition at 37°C. Receptor medium was withdrawn and immediately replaced with equal volume of fresh medium. The samples were collected at 0 (baseline) to 24 h. Agitation stir at 600 rpm was performed the whole time in the experiment. Samples from receptor medium was analyzed amount of total phenolic content using folin-ciocalteu method.

c. Pretreatment of Animals and Animal tissue

Adult Wistar rats (*Rattus norvegicus albinus*) about 350-400 g body weight were recruited and treated in accordance to ethical principles of animal experimentation of the National Research Council of Thailand after institutional committee review (AEKKU-NELAC 23/2557). Double-blinded implemented between investigators who conducted animals to obtain wound tissue samples and those who measured inflamed cells of the tissue samples. Rats were separated groups (n = 5-6 each), blank, fluocinolone, xylocaine, ZL and ZLA gels. All treatments were applied twice daily.

Prior to the treatment, oral wound induction and tissue sampling were conducted as previously described [7]. In brief, anesthetized rats (by intraperitoneal injections of Nembutal) were subjected to 5-mm oral biopsy punching in the inner oral cavity. Wound size was monitored by observing through a periodontal probe (Hu-Friedy Mfg.co., LLC, U.S.A) with digital photographs

using a pen digital microscope (Andonstar professional Eletronic, China). At the predetermined time, the rat was sacrificed and the oral wound was excised. After excision, each piece of the wound tissue was subjected to hematoxylin and eosin staining (H&E, Sigma-Aldrich, U.S.A.) with cross-section cut (about 4 mm thick).

d. Measurement of inflamed cells

Each slide of tissue samples was randomly selected for 3 zones which were taken as digital photographs by using Zeiss Axiovert 25 Light Field microscope (AxioCam, Germany) at a magnification of 40. All of the photographs were systematically gridded for referencing frames of 7 cm × 7 cm of each box (each of box were sub-divided to be 1.75 cm × 1.75 cm per box) within an area of 49 cm² using the Photoshop program. Three photographs from the same tissue of the same rat were then randomly selected for 10 areas which were counted for numbers of inflamed cells, exhibiting as blue spots stained by H&E [8]. Two observers who determined the cells were interpersonal validated using wound and normal tissue samples by paired t-test and justified until non-significantly different measurements were obtained (*p* > 0.05) prior to start. Percentage of inflamed cell reduction in the tissue sample was compared with that treated with the blank gel on wound of the same day, using the following equation:

$$\% \text{ reduction} = \frac{B-T}{B} \times 100 \quad (\text{eq. 1})$$

where B = number of inflamed cells of wound tissue treated with the blank gel

T = number of inflamed cells of wound tissue treated with a treatment gel

e. Statistical analysis

Statistical significance was determined by one way analysis of variance (ANOVA) and post hoc test. The data was analyzed by using mean different with *p* values less than 0.05 was considered significance.

III. RESULTS

ZL from the fruits, about 4% yields, was clear light-yellow volatile oil with characteristic pungent smell. ZL gel was clear pale yellow bioadhesive gel. Burst effect was observed with

total phenolic contents permeated from the gel due to partition favor. Steady state permeation of total phenolic from ZL gel was saturated after about 12 h, possible due to limited solubility in the aqueous receptor. The steady-state permeation rate of total phenolic components of about 0.1 μg/cm²/h was obtained.

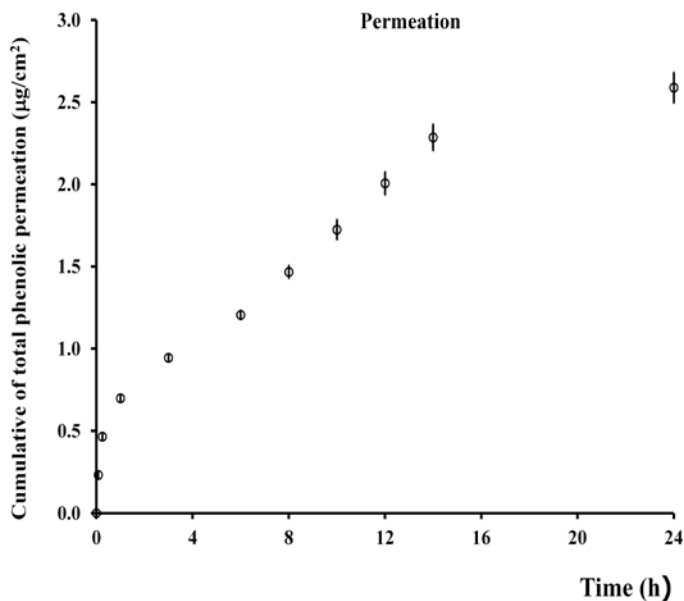


Fig. 1: Cumulative amount of total phenolic permeation study of Z.limonella gel

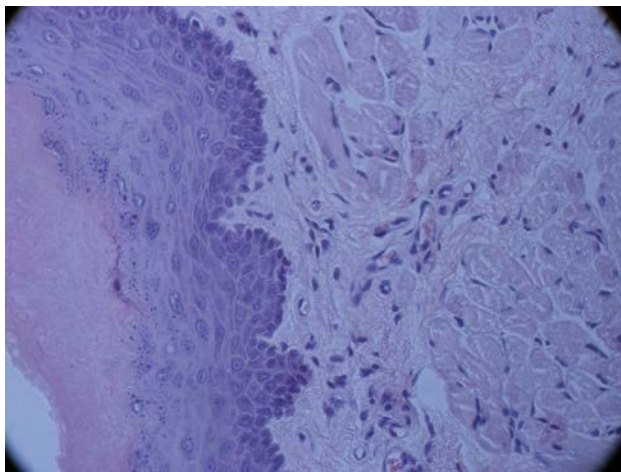
Behaviour and wound assessments in the buccal cavities of rats were shown in Table 1. Licking, but not grooming, was obvious in all rats with pressure buccal wounds, particularly on day 1. Xylocaine and ZL inhibited licking in all rats, but not fluocinolone due to the taste of the drug. Edema and erythema were overall suppressed by ZLA > fluocinolone > xylocaine or ZL. Therefore, ZL exhibited similar activity as xylocaine and addition of anthocyanins enhanced the activity in the same manner as fluocinolone.

TABLE I: Numbers of rats classified by by grading in the behaviour and wound assessments.

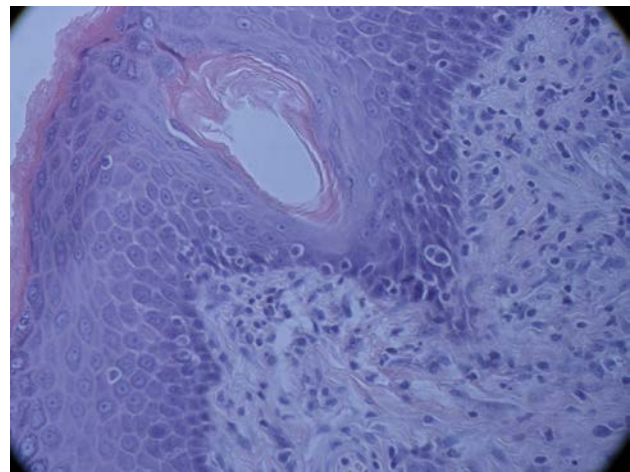
| Gel | Count/ Grade | Behavior assessment (number of rat(s)) | | | | | | | | | | | | Buccal wound assessment (number of rat(s)) | | | | | | | | | | | |
|-------------------------------------|-----------------|--|----|----|----|----|----|-----------------------|----|----|----|----|----|--|----|----|----|----|-----------------------|----|----|----|----|----|----|
| | | Licking ¹ | | | | | | Grooming ¹ | | | | | | Edema ² | | | | | Erythema ² | | | | | | |
| | | b | d0 | d1 | d2 | d3 | d4 | b | d0 | d1 | d2 | d3 | d4 | d0 | d1 | d2 | d3 | d4 | d5 | d0 | d1 | d2 | d3 | d4 | d5 |
| Blank (n = 5) | 0 | 5 | 0 | 5 | 3 | 3 | 2 | 2 | 2 | 0 | 3 | 0 | 1 | 0 | 0 | 0 | 2 | 3 | 3 | 0 | 0 | 1 | 3 | 4 | 2 |
| | 1-2 | 0 | 1 | 0 | 2 | 2 | 3 | 3 | 3 | 5 | 2 | 3 | 3 | 3 | 4 | 5 | 3 | 2 | 2 | 4 | 4 | 4 | 2 | 1 | 3 |
| | >2 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 2 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 |
| Fluocinolone (n = 6) | 0 | 6 | 0 | 5 | 5 | 5 | 6 | 1 | 2 | 5 | 2 | 1 | 2 | 0 | 2 | 1 | 2 | 2 | 2 | 0 | 2 | 3 | 2 | 3 | 2 |
| | 1-2 | 0 | 2 | 1 | 1 | 1 | 0 | 4 | 4 | 1 | 2 | 4 | 3 | 6 | 4 | 5 | 4 | 4 | 4 | 6 | 4 | 3 | 4 | 3 | 4 |
| | >2 | 0 | 4 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 2 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Xylocaine (n = 6) | 0 | 6 | 0 | 6 | 6 | 6 | 6 | 2 | 5 | 6 | 6 | 6 | 6 | 0 | 2 | 2 | 3 | 4 | 5 | 0 | 2 | 3 | 2 | 3 | 4 |
| | 1-2 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 1 | 0 | 0 | 0 | 0 | 6 | 4 | 4 | 3 | 2 | 1 | 6 | 4 | 3 | 4 | 3 | 2 |
| | >2 | 0 | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Z.limonella (n = 6) | 0 | 6 | 0 | 6 | 6 | 6 | 6 | 2 | 4 | 4 | 5 | 5 | 6 | 0 | 2 | 2 | 2 | 3 | 5 | 0 | 3 | 3 | 3 | 3 | 4 |
| | 1-2 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 2 | 2 | 1 | 1 | 0 | 6 | 4 | 4 | 4 | 3 | 1 | 6 | 3 | 3 | 3 | 3 | 2 |
| | >2 | 0 | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Z.limonella + Anthocyanins (n=6) | 0 | 6 | 0 | 6 | 6 | 6 | 6 | 2 | 5 | 6 | 6 | 6 | 6 | 0 | 4 | 4 | 5 | 5 | 6 | 0 | 2 | 2 | 2 | 2 | 4 |
| | 1-2 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 1 | 0 | 0 | 0 | 0 | 6 | 2 | 2 | 1 | 1 | 0 | 6 | 4 | 4 | 4 | 4 | 2 |
| | >2 | 0 | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

TABLE II: Numbers of inflamed cells of buccal tissue samples in each treatment group (n = 3)

| Xylocaine | | Fluocinolone | | ZL gel | | ZLA gel | | | |
|-----------|-----------|--------------|------------|-----------|-----------|---------|------|------|------|
| NT | T | NT | T | NT | T | NT | T | | |
| 28.6±5.8 | 40.06±6.4 | 33.4±5.8 | 17.7±13.6* | 32.8±2.5 | 18.0±19.7 | 22.8 | 8.5 | 19.8 | 2.5 |
| nd | nd | 23.7±10.4 | 21.5±8.3 | 43.0±4.2 | 27.4±6.9 | nd | nd | nd | nd |
| 25.5±10.9 | 30.8±7.4 | 32.7±1.1 | 32.8±2.5 | 33.06±2.8 | 29.9±2.8 | 21.7 | 15.4 | 22.4 | 14.0 |
| 21.4±11.5 | 27.9±8.3 | nd | nd | nd | nd | nd | nd | nd | nd |
| nd | nd | 25.7±8.3 | 28.6±2.7 | 31.50±5.0 | 23.3±8.4 | 19.3 | 12.8 | 18.3 | 2.2 |



(A) blank gel



(B) ZL gel

Fig 2 : Histological photographs of cross-sectioned buccal wounds of rats treated with blank and ZL gel, stained with hematoxylin and eosin (H&E) (magnitude 40×)

Fig. 2 demonstrates histology of cross-sections in the areas of buccal wounds treated for 4 days with ZL gel compared with the same blank gel. The stained inflamed cells were larger and densely packed with clearly defined nucleus. For confirmation, the numbers of inflamed cells were counted and compared in TABLE II which indicates significant lower numbers of inflamed cells after fluocinolone treatment for 1 day or ZL for 2 days ($p < 0.05$, both) Thus, ZL showed potential anti-

inflammatory effect, although slower action than fluocinolone. This effect was obtained by topical application using the formulated gel.

IV. CONCLUSION

In conclusion, phenolic components of ZL could readily permeate through mucous membrane and help reduced

inflammation such as edema and erythema. Thus, ZL gel showed potential benefits for topical use in oral wound.

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