ประสิทธิศักย์ของสารสกัดมาตรฐานบัวบก อีซีเอ 233 ในการรักษาแผลร้อนในชนิดไม่รุนแรง

นางสาว ชลันดากร เรืองประเสริฐกิจ

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Efficacy of standardized extract of *Centella asiatica* ECa 233 in the management of minor aphthous ulceration

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การวิจัยนี้เป็นการศึกษาประสิทธิศักย์ของยาป้ายปากซึ่งประกอบด้วยสารสกัดมาตรฐานบัวบก อีซีเอ 233 ในการรักษาแผลร้อนในชนิดไม่รุนแรง เปรียบเทียบกับยาป้ายปากไตรแอมซิโนโลนความเข้มข้น 0.10% โดยทดลองด้วยวิธีการสุ่มคัดเลือกและมียาหลอกเป็นกลุ่มควบคุม กลุ่มตัวอย่างเป็นกลุ่มประชากรทั่วไปที่สนใจเข้าร่วมโครงการจากการประชาสัมพันธ์และประกาศในที่สาธารณะ อาสาสมัครที่เข้าร่วมโครงการมีจำนวน 120 ราย ส่วนใหญ่อายุ 21 ปี เป็นเพศชาย 37 ราย และเพศหญิง 83 ราย ซึ่งเป็นแผลร้อนในมาไม่เกิน 48 ชั่วโมงก่อนเข้าร่วมโครงการ โดยจะได้รับการสุ่มคัดเลือกและแบ่งเป็น 3 กลุ่ม คือ กลุ่มที่ได้รับยาหลอก (กลุ่มควบคุม) กลุ่มที่ได้รับยาป้ายปากอีซีเอ 233 ความเข้มข้น 0.05% และกลุ่มที่ได้รับยาป้ายปากไตรแอมซิโนโลนความเข้มข้น 0.10% ซึ่งอาสาสมัครจะป้ายยา 3 ครั้งต่อวัน เป็นระยะเวลา 10 วัน และอาสาสมัครจะเป็นผู้บันทึกข้อมูลประจำวันคือขนาดแผล ระดับความปวด และแผลใหม่ที่เกิดขึ้นระหว่างเข้าร่วมโครงการลงในสมุดบันทึก โดยวันแรกที่เข้าร่วมโครงการผู้วิจัยจะถ่ายภาพลักษณะแผล จากนั้นนัดตรวจติดตามผลและการลงบันทึกข้อมูลเพื่อประเมินการหายของแผลในวันที่ 3 และ 10 ผลการศึกษาพบว่าค่าเฉลี่ยขนาดแผลในวันแรกที่เข้าร่วมโครงการเท่ากับ 8.14±0.75, 7.80±0.66 และ 8.74±0.76 และผลค่าเฉลี่ยระดับความปวดในวันแรกที่เข้าร่วมโครงการซึ่งประเมินด้วย VAS เท่ากับ 6.53±0.30, 6.63±0.26 และ 6.75±0.29 ในกลุ่มที่ได้รับยาหลอก กลุ่มที่ได้รับยาป้ายอีซีเอ 233 ความเข้มข้น 0.05% และกลุ่มที่ได้รับยาป้ายไตรแอมซิโนโลนความเข้มข้น 0.10% ตามลำดับ ขนาดแผลของกลุ่มควบคุมจะเพิ่มขึ้นสูงสุดในวันที่ 3 ก่อนที่จะค่อยๆลดลงและหายสนิท ซึ่งมีค่าเฉลี่ยในการหายของแผลเท่ากับ 8.65±0.25 วัน ในทางตรงกันข้ามกลุ่มที่ได้รับ 0.05% ECa 233 และ 0.10% ไตรแอมซิโนโลน พบว่าขนาดแผลลดลงตั้งแต่วันที่ 1 และลดลงอย่างต่อเนื่องจนมีความแตกต่างอย่างมีนัยสำคัญในวันที่ 3 เมื่อเทียบกับกลุ่มควบคุม โดยมีค่าเฉลี่ยในการหายของแผลเท่ากับ 6.63±0.23 และ 6.58±0.25 วัน ตามลำดับ ผลการลดระดับความปวดพบว่าทั้ง 0.05% ECa 233 และ 0.10% ไตรแอมซิโนโลน สามารถลดความปวดได้อย่างชัดเจนตั้งแต่วันที่ 1 และลดความปวดได้ดีกว่ากลุ่มควบคุมตั้งแต่วันที่ 3 อย่างมีนัยสำคัญ($p<0.05$) รวมทั้งไม่พบอาการไม่พึงประสงค์จากการใช้ยาป้ายอีซีเอ 233 ในระหว่างการเข้าร่วมโครงการ 11 วัน จากผลการศึกษาแสดงให้เห็นว่าอีซีเอ 233 ความเข้มข้น 0.05% ในรูปของยาป้ายปากน่าจะประสิทธิภาพเทียบเท่ากับไตรแอมซิโนโลน ในการลดขนาดและความปวดของแผลร้อนในชนิดไม่รุนแรง ซึ่งในอนาคตต้องศึกษาเกี่ยวกับความสามารถในการป้องกันการกลับเป็นโรคซ้ำของ ECa 233 เพื่อเป็นการสนับสนุนถึงความแรงของประสิทธิภาพและความปลอดภัยในการใช้ ECa 233 รักษาผู้ที่เป็นโรคแผลร้อนในชนิดไม่รุนแรงต่อไป

สาขาวิชา เภสัชวิทยา ลายมือชื่อนิสิต .....

ปีการศึกษา 2552 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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CHALANDAKORN RUENGPRASERTKIT : THESIS TITLE. (EFFICACY OF STANDARDIZED EXTRACT OF *CENTELLA ASIATICA* ECa 233 IN THE MANAGEMENT OF MINOR APHTHOUS ULCERATION) THESIS ADVISOR : ASSOC.PROF. MAYUREE H. TANTISIRA, Ph.D., THESIS CO-ADVISOR: ASSOC.PROF. NAULCHAVEE HONGPRASONG, D.D.S., M.D.S., THAI BOARD (PERIODONTOLOGY), ASSOC.PROF. BOONYONG TANTISIRA, Ph.D., 104 pp.

A randomized, single-blind, placebo controlled trial was conducted to evaluate the efficacy of an oral paste containing a standardized extract of *Centella asiatica* on minor aphthous ulcer (MiRAU) in comparison to 0.10% triamcinolone (TA) oral paste. One hundred and twenty individuals, median age of 21 years, 37 males and 83 females, presented at least one MiRAU with an onset of no longer than 48 hours at entry (day 0) were recruited by an announcement to the public. They were randomly divided into 3 groups of treatment receiving placebo oral paste (control), 0.05% ECa 233 oral paste and 0.10% TA oral paste. Subjects were instructed to apply the medication by themselves 3 times a day for 10 days. In addition they were asked to make a daily recording of ulcer size, degree of pain and occurrence of new ulcer in a given daily booklet. Photograph was taken on day 0 and at the follow-up on day 3 and day 10. Initial mean ulcer size was 8.14±0.75, 7.80±0.66 and 8.74±0.76 mm^2 and initial pain score assessed by a visual analog scale (VAS) was 6.53±0.30, 6.63±0.26 and 6.75±0.29 in control, 0.05% ECa 233 and 0.10% TA treated groups, respectively. Ulcer size in control group increased to its peak value on day 3 then gradually declined with mean time to complete healing of 8.65±0.25 days. In contrast a prompt decrease was noted on day1 and continued to decrease to reach a statistically significant difference from control on day 3 in both 0.05% ECa 233 or 0.10% TA treated group which accordingly exhibited complete healing in 6.63±0.23 and 6.58±0.25 days. Reduction of pain by 0.05% ECa 233 and 0.10% TA was clearly demonstrated on day 1 and pain score in both groups became significantly different from control group on day 3. In addition, no adverse effect was reported during the course of treatment of 11 days. Base on the results observed, 0.05% ECa 233 seems to be as effective as 0.10% TA in reducing the ulcer size as well as ulcer associated pain in MiRAU. Further study on the prevention of recurrence of MiRAU would strengthen the potential of ECa 233 as an effective and safe alternative treatment of MiRAU.

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LIST OF ABBREVIATIONS

BD	Behcet's disease
bFGF	basic fibroblast growth factor
CAT	catalase
CD	Crohn's disease
CIA	collagen-induced arthritis
cm	centimeter
CMV	cytomegalovirus
COX-2	cyclooxygenase-2
CPK	creatine phosphokinase
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
g	gram
GPx	glutathione peroxidase
GSH	glutathione
GSHPx	glutathione peroxidase
GST	glutathione S-transferase
HIV	human immunodeficiency virus
hsp	heat shock protein
HSV	hepes simplex virus
HU	herpitiform ulceration
IC_{50}	half maximal inhibitory concentration
i.g.	intragastric
i.p.	intraperitoneal injection
Ig	Immunoglobulin
IKK	inhibitor of kappaB kinase
IL	Interleukin
IκB-α	Inhibitor kappa B-alpha
kDa	kilodalton

kg	kilogram
LDH	lactate dehydrogenase
LPO	myocardial lipid peroxides
LPS	lipopolysaccharide
m	meter
MaRAU	major recurrent aphthous ulceration
mcg	microgram
MDA	malonaldehyde
mg	milligram
MiRAU	minor recurrent aphthous ulceration
ml	milliliter
mm	millimeter
mm^2	millimeter squared
MPO	myeloperoxidase
NF-κB	nuclear factor kappa B
ng	nanogram
NO	nitric oxide
PCR	polymerase chain reaction
pg	pictogram
PGE2	prostaglandin E2
RAS	recurrent aphthous stomatitis
RAU	recurrent aphthous ulceration
rG-CSF	recombinant granulocyte-colony stimulating factor
S.E.	standard error
SOD	superoxide dismutase
TA	triamcinolone acetonide
TNF-α	tumor necrosis factor α
TGF β	transforming growth factor-β
TβRI	TGF β Receptor I
VAS	visual analog scale

VEGF	vascular endothelial growth factor
VZV	varicella-zoster virus
w/w	weight by weight
μM	micromolar
μg	microgram

CHAPTER I

INTRODUCTION

Background and Rationale

Recurrent aphthous ulceration (RAU), typically manifested as an erosion of epidermal layer of non-keratinized oral mucosa such as the labial and buccal mucosa, is a common oral mucosal disease with the frequency of 5-20% in general population worldwide, the cause of this disease is poorly understood (Khandwala et al., 1997; Natah et al., 2004; Scully, 2006). It clinically manifests as small round to ovoid lesion, covered by a yellow-white pseudomembranous film, and surrounded by an erythematous (red) inflammatory halo. RAU is classified into 3 specific classes: minor (MiRAU), major (MaRAU), and herpetiform (HU) according to the size, number and duration of the ulcers. Among them, MiRAU is the most common form, consisting of 70-87% of the population with RAU. MiRAU is clinically manifested as 1-5 small round to ovoid lesions with less than 1 cm in diameter (Woo and Sonis, 1996; Shashy and Ridley, 2000). The specific cause of MiRAU remains unknown but probably involved multifactorial process and several initiating factors such as trauma, stress, infective agents, nutritional and immunologic factors. The disease was associated with significant pain that interfere with eating, speaking and swallowing. The treatments are primarily aimed at pain relief, speeding healing and reducing recurrence. A variety of topical and systemic therapies including antibiotics, enzymatic preparations, non-steroidal anti-inflammatory drugs, analgesic drug and corticosteroids have been used to relieve pain, reduce the duration of the ulcers and to prevent recurrence (Saxen et al., 1997; Ylikontiola et al., 1997; Chandrasekhar et al., 1999; Murray et al., 2005). Varying degree of success has been reported and in some cases significant side effects were noted (Herlofson and Barkvoll, 1994; Kerr et al., 2003; Delavarian et al., 2007).

Centella asiatica (Umbelliferae), also known as gotu kola is commonly found in many parts of Asia, China, India, Sri Lanka, Indonesia and Madagascar. *Centella asiatica* is a pantropical plant in Thailand that is normally consumed as a tea or juice

(Punturee et al., 2005). It is a medicinal plant containing mainly asiaticoside, asiatic acid, madecassoside and madecassic acid. Traditionally, *Centella asiatica* has been used for the treatment of eczema, psoriasis, and leprosy (Sampson et al., 2001). It is extensively evaluated for a wide spectrum of pharmacological activity including improving learning and memory (Gadahad et al., 2008), antimicrobial, antifungal (Jagtap et al., 2009), antioxidant, anti-inflammatory (Jayashree et al., 2003; Yun et al., 2008) and wound and ulcer healing (Maquart et al., 1999; Cho et al., 2003; Cheng et al., 2004; Lu et al., 2004; Kimura et al., 2008; Yun et al., 2008). Furthermore, lesion size in minor aphthous ulceration was significantly reduced by an oral gel containing a crude extract of *Centella asiatica* (Wangrangsimakul, 1999). In order to overcome a fluctuation of biologically active constituents normally exists in the crude extract of medicinal plants, activity-guided isolation was used to establish a standardized extract of *Centella asiatica* ECa 233 which is a white to off-white titrated extract of *Centella asiatica* containing triterpenoids at least 80% and the ratio of madecassoside and asiaticoside is always kept at 1.5 ± 0.5. Recently wound healing effects on burn wound and the safety profile of ECa 233 have been demonstrated in animals (Tantisira, 2009; Wannarat et al., 2009).

Objective

The present studies aimed to evaluate clinical efficacy of a standardized extract of *Centella asiatica* ECa 233 oral paste in comparison to 0.1% triamcinolone oral paste and placebo on minor recurrent aphthous ulceration (MiRAU), using a randomized, single-blind, placebo-controlled design.

Hypothesis

The standardized extract of *Centella asiatica* ECa 233 oral paste has efficacy in the management of minor recurrent aphthous ulceration (MiRAU).

CHAPTER II

LITERATURE REVIEWS

Recurrent aphthous ulceration (RAU)

An inflammatory condition of unknown etiology characterized by painful, recurrent (single or multiple) ulcerations of the oral mucosa (Natah et al., 2004). Recurrent aphthous ulceration take three clinical forms (Table 1): minor aphthous ulcers (MiRAU), major aphthous ulcers (MaRAU) and herpetiform ulcers (HU) (Woo and Sonis, 1996; Greenberg and Pinto, 2003).

1. Minor recurrent aphthous ulceration (MiRAU)

MiRAU also called Mikulicz's, aphthae or mild aphthous ulcers is the most common variety that affects about 80% of patients with RAU. Ulcers are small less than 1 cm in diameter and number less than 10, usually between 1 and 3, oval or round shallow ulcers usually with a grey-white pseudomembrane enveloped by a thin erythematous halo. It usually occurs on non-keratinized mobile surfaces particularly the buccal and labial mucosa and floor of the mouth but is uncommon on the gingival, palate or dorsum of the tongue. MiRAU is the most common form of childhood RAU. These lesions recur at varying frequencies (from every few years to almost constantly). The ulcers heal within 10-14 days without scarring.

2. Major recurrent aphthous ulceration (MaRAU)

MaRAU, described by Sutton in 1911 as periadenitis mucosa necrotica recurrens, that may affect about 10% of patients with RAU. Ulcers are larger (usually greater than 3 cm) and deeper. Because of the greater depth, they are more painful, slower to heal. These lesions are similar in appearance to MiRAU, but they are larger than 10 mm in diameter, single or multiple. These ulcers have a predilection for the lips,

soft palate and fauces (but can affect any site). They persist for up to 6 weeks and often heal with scarring. MaRAU usually has its onset after puberty. It is chronic and persists for up to 20 years or more.

3. Herpitiform ulceration (HU)

The third and least common variety of RAU. The name is derived from the supposed resemblance to the intraoral lesions of primary herpes simplex virus (HSV) infection, but HSV cannot be isolated from HU lesions or from any other forms of RAU. In general, RAU is confined to the freely movable or lining oral mucosal surfaces, while recurrences of the HSV are usually found on oral mucosa bound to bone such as gingival and hard palatal mucosa (Table 2). HU arises in about 1-10% of patients with RAU, being characterized by multiple recurrent crops of widespread, small, painful ulcers , that may be distributed throughout the oral cavity, usually in the posterior part of the mouth and typically heal within 14 days without scarring. There maybe up to 100 ulcers at a given time, each measuring 2-3 mm in diameter, although they tend to fuse to become large and irregular. It has been suggested that HU might have a female predisposition and also a later age of onset than other RAU types or represent a spectrum of oral disorders manifesting as recurring ulcers.

Most patients have only one to three ulcers, and some have recurrences only two to four times each year. Other may have almost continuous disease activity with new ulcers developing as older ulcers heal, or may have ulcers associated with systemic diseases.

Table 1 Characteristics of the different types of RAU (Porter et al., 2000).

	Minor	Major	Herpetiform
Sex ratio	M = F	M = F	F > M(?)
Age of onset (years)	5–19	10–19	20–29
Number of ulcers	1–5	1–10	10–100
Size of ulcers (mm^2)	< 10	> 10	1–2*
Duration (days)	4–14	> 30	< 30
Rate of recurrence (months)	1–4	< Monthly	< Monthly
Sites	Lips, cheeks, tongue, floor of mouth	Lips, cheeks, tongue, palate, pharynx	Lips, cheeks, tongue, pharynx, palate, gingiva, floor of mouth
Permanent scarring	Uncommon	Common	Uncommon

* Can be larger if there is a fusion of ulcers.

Table 2 Recurrent aphthous ulcers vs. Herpes simplex virus (Shashy and Ridley, 2000).

Descriptive	RAU	HSV
Location	Buccal/labial	Gingival
Size	> 3 mm	< 3 mm
Vesicles	No	Yes
Number	1 to 3	> 10
Cluster	No	Yes
Pain	Yes	Yes
Viral isolate	Negative	Positive

Diagnosis of RAU

The diagnosis of RAU is based on history and primarily clinical findings. When the clinical diagnosis is in question, the clinician can differentiate with laboratory. Supplemental diagnostic laboratory tests such as cytology, culture, polymerase chain reaction (PCR), serology and biopsy. Cytology may also detect viral features suggestive of HSV ulcers, which would eliminate RAU from consideration. A culture could identify specific bacterial or viral infection. Biopsy can be used to rule out deep fungal infection (Zunt, 2001).

These supplement tests, while not definitive for RAU. There is no specific diagnostic test, but there is a need to exclude other possible causes of RAU as following (Table 3):

Table 3 Some systemic diseases resembling ulceration like recurrent aphthous ulceration (Scully and Porter, 2008).

Disease	Comment
Behcet's disease	Aphthous-like ulceration is a cardinal feature but may be more severe, and is more likely to comprise major or herpetiform ulcers, or both. Patients also have recurrent genital ulceration, cutaneous disease (usually papulopustular lesions or erythema nodosum), ocular disease (typically posterior uveitis) and a range of gastrointestinal, neurological, renal, joint and haematological abnormalities.
MAGIC syndrome	A possible variant of Behcet's disease, comprising major aphthae and generalised inflamed cartilage.

Table 3 Some systemic diseases resembling ulceration like recurrent aphthous ulceration (Scully and Porter, 2008) (continued).

Disease	Comment
Sweet syndrome (acute neutrophilic dermatosis)	Patients have ulceration similar to RAU, but with sudden onset of fever, leucocytosis and well-demarcated cutaneous, plum-coloured skin papules or plaques. There is an associated malignancy (such as acute myeloid leukaemia) in half of patients.
PFAPA syndrome	Comprises periodic fever, aphthae, pharyngitis, and cervical adenitis. Although rare, it tends to occur in young children. Although throat cultures may be negative about two thirds of children improve after tonsillectomy. An immunologically mediated pathogenesis has been suggested by the good response to prednisolone, cimetidine (by suppression of T lymphocyte function on tonsillectomy).
Cyclic neutropenia	Cyclic reduction in circulating levels of neutrophils about every 21 days. Patients develop recurrent oral ulceration, fever, cutaneous abscesses, upper respiratory tract infections and lymphadenopathy. Other oral complications include severe gingivitis and aggressive periodontitis which can be treated with recombinant granulocyte colony stimulating factor (rG-CSF).
HIV disease	Aphthous – like ulceration may be seen independent of the necrotising ulceration of HIV infection.

Crohn's disease (CD)

Crohn's disease (CD), aphthous lesions are regarded as possible precursors of typical intestinal involvement. CD is an inflammatory bowel disease in which the wall of one or more segments of the gastrointestinal tract becomes thickened, inflamed and swollen (Hizawa et al., 1994). The criterion for inclusion was confirmed aphthous lesions within the gastrointestinal tract with histologically verified epithelioid granuloma. The degrees of aphthous lesions in the small intestine and the colon were graded by small bowel radiography, barium enema examination and colonoscopy. Typical small intestinal CD occurred in four of seven patients with marked aphthous lesions of the small intestine, whereas colonic CD occurred in two of eight patients with such aphthous lesions of the colon (Matsumoto et al., 2000).

Behcet's disease (BD)

Behcet's disease (BD) is a multi-system inflammatory disorder and can present with oral ulcers similar to RAU, is a vasculitis of unknown cause that dominated clinically presents with ocular, oral and genital manifestations. The ocular signs are uveitis and iritis, which can lead to blindness. Typically, the patient will have a hypopyon (leukocytes in the anterior chamber of the eye) (Table 4) (Rogers, 1997; Scully and Porter, 2008). The cause of BD remains unknown, although an autoimmune reaction triggered by an infectious agent in a genetically predisposed individual has been suggested (Al-Otaibi et al., 2005).

Table 4 Principal clinical features of Behcet's disease (Scully and Porter, 2008).

System	Features
Gastrointestinal	RAU
	Ileo-caecal ulceration
Urogenital	Scrotal or penile ulcers, or both
	Vulval or vaginal ulcers, or both
	Perianal ulcers
	Epididymo-orchitis
Dermatological	Papules
	Pustules
	Erythema nodosum
	Ulcers
	Cutaneous pathergy response
Ocular	Anterior or posterior uveitis
	Retinal vasculitis
Musculoskeletal	Arthralgias
	Arthritis
	Fatigue
Neural	Headaches
	Dural sinus thrombosis
	Parenchymal inflammatory lesions
	Meningo-encephalitis
Vascular system	Superficial thrombosis
	Deep venous thrombosis
	Arterial occlusion or aneurysms, or both

Pemphigus

Pemphigus occurs in older patients (generally those older than 60 years), with onset usually in the fifth decade, and presents as tense bullae on the inner thighs and flexor surfaces of the forearms and axillae. It begins as vesicles and bullae that rupture and ulcerate. Immunofluorescence will show IgG autoantibodies on the surface of the suprabasal cell layers. Oral manifestations of pemphigoid follow the cutaneous lesions and immunofluorescence will show deposition of IgG and C3 in a linear pattern at the basement membrane. This leaves raw, red areas of skin which can be very sore and painful. The raw areas of skin heal without scarring, but the affected skin may become more pigmented (darker) (Bell and Rogers, 1982).

Periodic Fever, Aphthous Stomatitis, Pharyngitis and Cervical Adenitis Syndrome (PFAPA Syndrome)

The PFAPA syndrome, of periodic fever characterized by abrupt onset of fever, malaise, aphthous stomatitis, tonsillitis, pharyngitis and cervical adenopathy has been described only in pediatric patients, which includes systemic onset juvenile idiopathic arthritis, cyclic neutropenia, and the expanding group of hereditary fevers. It usually begins before the age of 5 years and in most cases resolves spontaneously before age 10. It differs from those autoinflammatory fevers in that it is a non-hereditary syndrome (Matoussi et al., 2008; Padeh et al., 2008).

Coeliac disease

Celiac disease is a disease of the small intestine characterized by villous atrophy that impairs with nutrient absorption and improves upon withdrawal from wheat gliadin barley, rye and oat prolamines of the diet (Mihailidi et al., 2003). It is caused by gluten sensitivity of the small intestines. In epidemiologic studies, the prevalence of celiac disease rates of 1:120 to 1:300 has been reported in Western Europe. Diagnosis of celiac disease may be somewhat hard to achieve in some of the patients due to the

wide spectrum of signs and symptoms. Other systems may be affected by the disease such as dermatitis herpetiformis in the skin. Some studies that Celiac disease prevalence in patients with RAS is higher than in the normal population studies, in the literature investigating the relationship between RAU and Celiac disease. Therefore RAU may be the presenting sign of the disease. Aydemir and his colleague (2004) have repeated that 2 (4.8%) out of 41 subjects having RAS were diagnosed as Celiac disease.

Epidemiology of RAU

RAU has been estimated that 20% of the general population will suffer from RAU at some time in their lives. Population studies have found RAU in about 2% of Swedish adults examined, though a history compatible with RAU is far more common (Axell and Liedholm, 1990). RAU prevalence varies from 5 to 66% of the population depending on the group studied. RAU seems to be infrequent in Bedouin Arabs but is especially common in North America and is more common in western countries. There may be a female predominance in some adult communities, and 40% of selected groups of children can have a history of RAU. A high prevalence and severity of disease has been found in students with a high socio-economic background (Natah et al., 2004; Eris et al., 2007).

Factors predisposing to RAU

Age and sex

The prevalence of RAU detected during oral examination (average time point prevalence) was found to be about 1% in children of developed countries, but 40% of selected groups of children can have a history of RAU, with ulceration beginning before 5 years of age and the frequency of affected patients rising with age (Porter et al., 1998). In the adult population, the first ulceration appears before the age of 30 in 60-

85% of patients. A slight predominance was found for females, and there may also be a female predisposition in affected children. A decreased prevalence has been noted in males, though not females, over the age of 50 in the Scottish population whereas population studies found a decrease in prevalence with age in both sexes in the Swedish population (Natah et al., 2004).

Family

In some individuals, RAU may have a familial basis, perhaps more than 40% of RAU patients having a vague family history of oral ulceration. Patients with a positive family history of RAU may develop oral ulcers at an earlier age and have more severe symptoms than affected individuals with no family history of oral ulceration (Porter et al., 1998). There is an increased likelihood of a child developing RAU to 90% when both parents have ulcers and there is a high correlation of RAU in identical twins, but only 20% when neither patient has RAU. Nevertheless, there is a clear variability in host susceptibility with a polygenic inheritance but penetrance on other factors (Scully et al., 2003).

Food hypersensitivity

Some studies have noted an increased prevalence of atopy among RAU patients, but others have failed to find any significant correlation. Some patients exhibit allergies to a variety of foods, food dyes and food preservatives such as gluten, cow's milk, cheese, chocolate, nuts, azo dyes and flavoring agents, but did not find any significant association of RAU with 3 specific food items (tomatoes, strawberries and walnuts) strict elimination diets resulted in improvement and resolution of otherwise persistent ulcers in 25 to 75% of patients (Woo and Sonis, 2001; Besu et al., 2009). Some patients correlate the onset of their ulcers to exposure to certain food, but controlled studies have failed to disclose a causal role despite the fact that certain foods triggering positive skin-prick reactions will elicit pain when they are topically applied to

aphthous ulcers. In one report, leukocytes from patients with RAU released histamine in response to environmental and food antigens. Dietary manipulation significantly improves RAU in only rare instances (Porter et al., 2000). Furthermore, a double-blind study using gluten-free and gluten-supplemented diets found that both groups showed a significant symptomatic improvement, indicating a marked placebo response (Scully et al., 2003).

Hormonal changes

There are a few patients whose RAU remits during pregnancy and oral contraceptives (Scully et al., 2003). The studies had reported that a minor subset of women with RAU have cyclical oral ulceration related to the onset of menstruation or the luteal phase of menstrual period. Almost no men developed RAU after the age of 50, whereas 10% of women having their first episode between 50-59 years had been reported. However, the association between RAU and menopause has not been established (McCartan and Sullivan, 2006).

Drugs

Exposure to certain drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) (diclofenac, phenylacetic acid and proprionic acid) rarely given rise to oral ulcers similar of RAU. Potassium channel blocker such as nicorandil (used in cardiac disease) and some other drugs may produce aphthae-like ulcers, but the onset typically is in older people and related temporally to the drug used which differentiates them from true aphthae (Scully et al., 2003). However, such types of ulcers usually occur as an adverse side effect and disappear with discontinued usage of the drug.

Zinc Deficiency

The improvement of RAU with zinc sulphate supplementation were described in an open trial and in a case report of aphthous ulcers with zinc deficiency and immunodeficiency, but such improvement could not be confirmed in later studies. The level of serum of zinc of 40 cases of RAU was found to be on a lower level within normal range, and serum copper was also normal. Oral zinc treatment using 50-200 mg of zinc sulphate appeared to alleviate the symptoms in a great majority of patients. So far no information exists on the association of RAU and other trace element (Bor et al., 1990).

Hematinic deficiencies

Though some studies deny an etiologic relationship between RAU and deficiencies of folic acid or iron, deficiencies of vitamin B1, B2, B6 or B12 have been demonstrated to be twice as a common in RAU patients than in controls (Piskin et al., 2002; Koybasi et al., 2006). Hematinic deficiencies have been found in about 20% of patients with RAU (Thongprasom et al., 2002; Burgan et al., 2006). Low serum ferritin levels were found in 8-12% of patients with RAU, compared with 3-5% in control and the level did not differ in different subtypes of RAU (Piskin et al., 2002). A cohort of Scottish patients found that 28.2% of patients with RAU had deficiencies of vitamins B1, B2 and B6. Fifty eight patients with RAU receiving vitamin B12 replacement therapy were found to improve symptoms of RAU (Garcia et al., 2009; Volkov et al., 2009).

Environmental factors

Stress

Earlier studies have documented an association between RAU and a variety of psychological factors such as anxiety, repressed hostility, depression, psychological life stress, as well as job related and other stressors (Pedersen, 1989). Patients have often subjectively associated the appearance of RAU with increased stress. Psychological

illness has been proposed to initiate some episodes of RAU and there are sparse data to suggest that some patients may benefit from antidepressant therapy (Porter et al., 1998; Gallo et al., 2009). Environmental or emotional stress has been reported to precede the first RAU outbreak in 60% of patients and in approximately 21% of recurrent cases. A more recent study, in which the relaxation or imagery treatment program was used demonstrate a significant decrease in the frequency of ulcer recurrence for all treated subjects (Albanidou-Farmaki et al., 2007).

Smoking and Tobacco use

There actually appears to be an inverse relationship in the development of RAU and use of any form of tobacco. In one epidemiologic study, all groups using tobacco of one form or others had a lower incidence of RAU than nonsmokers (Woo and Sonis, 1996). Paradoxically, the majority of patients with RAU are nonsmokers, for instance, in a more recent study only 9% of RAU patients were found to be active smokers compared with 25% among the control subjects. Tobacco may increase keratinization of the mucosa, which in turn may render the mucosa less susceptible to ulceration. Subjects who quit smoking are less likely to develop RAU if they use nicotine replacement therapy as compared with those who do not use nicotine replacement therapy. The nicotine is probably the most likely protective factor. Its effects may result from influences on nerve function, although these agents may also exert direct anti-inflammatory effects. However, the mechanism by which cigarette smoking protects against RAU is still unknown (Tuzun et al., 2000).

Local trauma

Patients with RAU are predisposed to develop ulcers at sites of trauma. Whether local trauma such as anesthetic injection, dental treatment or toothbrushing would or would not trigger aphthous ulceration in these patients is still unknown (Kvam et al., 1987).

Infectious factors

Bacterial agents

An association between RAU and oral streptococci has long been suggested as important in the pathogenesis of RAU, either as direct pathogens or as an antigenic stimulus culminating in the genesis of antibodies that may conceivably cross-react with keratinocyte antigenic determinants. In a serological test, *Helicobacter pylori* dose not appear to be of etiologic significance in the development of RAU. *Helicobacter pylori* has been detected in lesional tissue of ill-defined oral ulcers, and PCR in up to 72% of examined RAU ulcers, but the frequency of serum IgG antibodies to *Helicobacter pylori* is not increased in RAU (Iamaroon et al., 2003; Fritscher et al., 2004). The initial L-form isolated from RAU patients was typed that as *Streptococcus sanguis*, but later analysis disclosed that this organism was actually a strain of *Streptococcus mitis*, or rather *Streptococcus oralis* a bacterium frequently present in the dental plaque of patients with Behcet's disease (Gordon et al., 1967; Elsheikh and Mahfouz, 2005). Nevertheless, the evidence is still incomplete and reasons for the limited oral involvement of RAU in comparison with the multisystemic nature of Behcet's disease remain unclear (Freysdottir et al., 1999; Natah et al., 2000).

Viral agents

An association of RAU with adenoviruses has been suggested and the possible association of RAU with herpesviruses 1-6 has recently been reviewed (Porter et al., 1998). Other studies failed to detect HSV antigen in the biopsies, and HSV cannot be cultured from the RAU lesions (Embil et al., 1975). Using PCR identified RNA complementary to HSV-1 in circulating mononuclear cells in four out of eight patients with RAU (Studd et al., 1991). Antivirals such as acyclovir, highly effective against HSV, appear to have only equivocal clinical effect on RAU (Pedersen, 1992). Further studies have detected VZV-like DNA, CMV-DNA, Epstein-Barr virus (EBV-DNA) in some oral

ulcer biopsy specimens from some RAU patients (Pedersen and Hornsleth, 1993). Furthermore, VZV DNA can be detected in lesional tissue by PCR (Porter et al., 1998). Antibodies to CMV may be significantly clevated in some RAU patients, and CMV DNA has been detected in ill-defined oral ulcerations in non-HIV-infected persons (Rogers, 1977). An association between EBV and the early ulcerative lesions of RAU and Behcet's disease has been proposed, but the data are based upon a very small group of patients (Porter et al., 2000).

Overall, the evidence for involvement of viruses such as VZV, CMV, HSV and EBV in RAU is conflicting. It is possible that RAU is a non-specific response with multiple etiologies and represents the final common pathway of mucosal inflammations. The dormant herpesviruses might be reactivated by the immuno-dysregulation known to be associated with RAU.

Immunopathogenesis

The pathogenesis of RAU involves a predominantly cell-mediated immune response in which tumor necrosis factor α (TNF-α), plays a major role. TNF-α induces inflammation by its effect on endothelial cell adhesion and neutrophil chemotaxis (Healy et al., 1997). Patients with RAU may have increased levels of peripheral blood $CD8^+$ T-lymphocytes or decreased $CD4^+$ T-lymphocytes, in the affected and non-affected oral mucosa of RAU patients (Albanidou-Farmaki et al., 2007). A mononuclear (lymphocytic) cell infiltrate in to the epithelium in the preulcerative stage followed by a localized popular smelling due to keratinocyte vacuolation surrounded by a reactive erythematous halo representing vasculitis (Scully et al., 2003). Other cytokines such as natural killer (NK) and interleukin (IL)-2, IL-10 cells play a role in RAU (Lin et al., 2005). Serum immunoglobulin levels are generally normal, though increase in serum IgA, IgD, IgG and IgE have all been reported in different groups of RAU patients (Olszewska et al., 2006). Normal or reduced immunoglobulin levels have been observed in other groups of RAU patients (Olszewska et al., 2006; Guimaraes et al., 2007).

Clinical Features

RAU clinically manifests as small round to ovoid lesions, with a crateriform base. For 24-48 hours preceding the development of a minor aphthous ulcer subjects may experience a burning sensation or pricking in the mucosa, erythema of the surrounding mucosa may be observed. Within a day or so an ulcer develops with a breakdown of the epithelium, oval or round ulcer with covered by grey-white pseudomembrane centre and erythematous halo develops, and continues to increase in size with accompanying pain (Shashy and Ridley, 2000).

Signs and Symptoms of RAU

Besides the pain and discomfort experienced when the ulcers are present, especially pain is noted to increase with consumption of spicy, acidic liquids and hot foods, as well as from irritation caused by oral muscular movements or rough foods. Few other signs and symptoms accompany these lesions. Occasionally, a patient may have submandibular lymphadenopathy with soreness on node palpation, but temperature elevation and other constitutional signs and symptoms usually are lacking (Kutcher et al., 2001).

Management of RAU

Since the etiology of RAU remains unknown, there is no specific therapy reliably effective for RAU. Patients with RAU, which is possibly secondary to systemic disease, require referral to an appropriate specialist for detailed evaluation and suitable therapy (Porter et al., 1998). Some patients have mild outbreaks, whereas others have severe and longer episodes. Thus, therapy should be tailored to each patient individually (Scully et al., 2003). There is a multitude of therapies for RAU, the goals of therapy are (Shashy and Ridley, 2000):

1. Control the pain of the ulcer and decrease other symptoms.

2. Promote ulcer healing, reduce ulcer number and size.

3. Prevent recurrence or increase disease-free periods.

The best treatment is the one which will control ulcers for the longest period with minimal unwanted effects.

Topical agents

A variety of topical medications and rinses have been used with variable success. Topical agents are preferred as they lack serious side effects (Scully and Porter, 2008).

Corticosteroids

Topical corticosteroids are considered the mainstay of therapy, are non invasive, relatively benign in regards to adverse effects and have proven to be a beneficial treatment of RAU in numerous studies. The use of corticosteroids in the treatment of RAU is based on the assumption that RAU is an acute inflammatory lesion (Yoke et al., 2006). The major concern of possible adrenal suppression with long-term or repeated use or both, has rarely been addressed. The only major problem with the long term use of these agents is the occurrence of oral candidiasis, which may necessitate the need for antifungal lozenges or mouth rinses (Scully and Porter, 2008).

Triamcinolone acetonide (TA), one of synthetic glucocorticoid of long-acting, has been used for the treatment of inflammatory disease. TA is beneficial in the management of RAU because of their anti-inflammatory effect and anti-immunologic properties of suppressing T cell function (Yoke et al., 2006). Triamcinolone 0.1% (Kenalog in Oral base) can be applied to ulcers two to four times a day (Scully et al., 2003). This preparation also provides a protective local coating for the ulcer. Triamcinolone and fluocinonide gel in oral base have been used to decrease the duration of ulcers and increase the time between episodes (Shashy and Ridley, 2000).

Other anti-inflammatory agents

Amlexanox®

Amlexanox® or aphtheal® or aphthasol® (5% amlexanox® paste), is a topical anti-inflammatory and anti-allergic drug, which can inhibit the formation and release of histamine and leukotrienes from mast cell, neutrophils and mononuclear cells (Liu et al., 2006). There have been a number of studies of the efficacy and safety of 5% amlexanox paste in the management of RAU indicating some beneficial effects in healing aphthous ulcers, but its exact mode of action is unknown. It has been available in the USA (approved by the FDA) and Canada and may become available in the UK (Murray et al., 2005). Several studies have demonstrated that 5% amlexanox oral paste applied to ulcers two to four times daily may considerably reduce their pain, size, erythema and speed up healing. In addition of erythema and exudation of patients with RAU were also reduced by Amlexanox (Liu et al., 2006). Adverse effects to amlexanox paste are rare and comprise a transient mild stinging at the site of application (Khandwala et al., 1997).

Non steroidal anti-inflammatory drug (NSAIDs)

Diclofenac

Topical diclofenac in hyaluronan has been showed to provide remarkable relief from pain of RAU. Having been transported to the site of inflammation, diclofenac produces a potent anti-inflammatory action by inhibiting cycloxygenase, thereby blocking the synthesis of protaglandins (PGs) that produce sensitization of primary afferent neurons (Saxen et al., 1997).

Benzydamine

Benzydamine hydrochloride mouthwash or lignocaine gel is non steroidal anti-inflammatory drug (NSAIDs) that has been assessed formally but did not aid healing (Scully and Porter, 2008).

5-aminosalicylic acid

Topical 5-aminosalicylic acid or mesalamine® or mesalazine® applied three times daily, significantly reduced the duration and number of ulcers. The mechanism of action is thought to be a reduction of protacyclin synthesis, inhibition of oxygen metabolite production by polymorphonuclear cell, or inhibition of the release of leukotrienes from mucosa (Porter et al., 2000).

Antimicrobials

Chlorhexidine

Chlorhexidine gluconate aqueous mouthrinse may be of some benefit in the management of RAU. Chlorhexidine can reduce the duration of RAU and increase the number of ulcer-free days, but cannot prevent the recurrence of ulcers. Chlorhexidine is generally used as a 0.2% w/w mouthrinse or 1% gel (Addy and Moran, 1997).

Triclosan

Triclosan mouthrinse reduces the number of ulcers, relieves pain and shortens the ulcerative phase by antimicrobial, anti-inflammatory and analgesic effects (Scully and Porter, 2008).

Cromolyn

Cromolyn has been used in patients with RAU. It is used as drops or lozenges in the dose of 20 mg four times daily to increase time between recurrences and decrease ulcer days. Cromolyn is a mast cell stabilizer and inhibits the release of histamine and leukotrienes from mast cells. It exact mechanism of action in RAU is unknown (Shashy and Ridley, 2000).

Antibiotics

Penicillin G potassium

Penicillin G potassium troches or cankercillin® is a narrow spectrum antibiotic with known bacteriocidal effects against oral bacteria, and has been demonstrated to reduce ulcer size , the time of healing and pain relief of RAU, by mechanisms which remain unclear. Penicillin G potassium had adverse effects such as sensation of pain or burning immediately following the application of the troche (Kerr et al., 2003).

Tetracyclines

Several studies of tetracyclines in the management of RAU have been conducted. Topical tetracyclines and doxymycine relieve the pain of RAU. It has been shown that tetracycline inhibited prostaglandin production, inhibited collagenase activities, suppressed leukocyte activities as well as the oxidative activation of their latent forms (Ylikontiola et al., 1997). Chlortetracycline or aureomycin® mouthrinse were recommended for 1 min four times daily. They were found to reduce the number of ulcers and relief pain of RAU. However, they should be avoided in children and pregnant or lactating mothers (Sahba and Mohammadalipour, 2005 Henricsson and Axell, 1985).

Systemic agents

Corticosteroids

Azathioprine or prednisolone or both can improve healing of severe RAU, but long-term use should be avoided as adverse effects will usually outweigh clinical benefit (Macphee et al., 1968; Femiano et al., 2003).

Immunomodulatory agents

Oxypentifilline

Oxypentifilline, also known as Trental® or pentoxifylline is anti-TNF-α agent, is an orally active methylxanthine derivative. It has immunosuppressive action such as interference in neutrophil adherence, inhibition of T- and B-lymphocyte activation and NK cell activity (Thornhill et al., 2007). Oxypentifilline prescribed at a dosage of 400 mg three times daily, significantly reduced the number of RAU after 1 month of treatment, but about 10% of patients developed gastrointestinal symptoms (Chandrasekhar et al., 1999).

Colchicine

Colchicine may be of some clinical benefit in the management for RAU. Colchicine at a dose 1.5 mg daily produced a significant reduction in pain score and frequency of ulcers. Possibly by virtue of its action on the microtubular function of polymorphonuclear leukocytes and interface in adhesion molecule expression. The adverse effects such as diarrhea and its can infertility in young males have been repeated (Porter et al., 1998).

Levamisole

Levamisole is believed to be a valuable immunopotentiator and encouraging results of its use in the treatment of RAU have been described. Levamisole at the dose 150 mg once daily improves symptom of RAU. It might reduce the duration of ulcers, but the adverse effects such as nausea, dysguesia and hyperosmia could discourage its use (Drinnan and Fischman, 1978).

Thalidomide

Thalidomide is the most reliably effective agent available for the management of RAU. Clinical benefit of thalidomide at the dose of 50-100 mg daily in patients of RAU and HIV-related oral ulceration is confirmed. However, the adverse effects such as teratogenicity and polyneuropathy seriously limit its application to short-term use, and in women who will not get pregnant (Revuz et al., 1990; Aweeka et al., 2001).

Nutrition supplements

Individuals with RAU possibly related to nutritional deficiencies may occasionally benefit from nutritional replacement which can be of value in patients with RAU of unknown cause as well. Vitamin B12 or zinc sulphate or both are benefit in the management patients with RAU (Volkov et al., 2005). Vitamin B12 at the dose of 1000 mcg daily has been found to significantly reduce the duration, number and pain of ulcers (Volkov et al., 2009). Zinc sulphate at the dose of 50-200 mg once or twice daily, has been found to reduce duration, size and pain of ulcers (Bor et al., 1990).

Herbals

Few medicinal herbs are listed as anti-aphthous agents, medicinal plants preparations with anti-inflammatory and antibacterial have been used for reduction of pain and shortening of healing time of RAU. *Satureja khuzistanica* and its essential oil

preparations applied 5 drops four times daily, showed reduction of the healing time and relief pain of ulcers (Amanlou et al., 2007). *Zataria multiflora*, *Anthemis nobelis* and *Myrthus communis* reported to possess anti-allergic, anti-inflammatory and mild anti-bacterial activities, are approved for mucous membrane inflammations. They were applied 10 drops five times daily, to reduce duration of ulcers and relief pain of RAU (Jafari et al., 2003).

***Centella asiatica* (L.)**

Centella asiatica (L.) Urban, commonly known as asiatic centella, is a slender, creeping plant, root at the nodes (Figure 1). The herb is also a traditional vegetable in southeast Asia, China, India, Sri Lanka and Indonesia, Madagascar, South Africa, and is being introduced as a cultivated vegetable in developed countries (Zheng and Qin, 2007). It is a weekly aromatic smelling herb of the family Umbelliferae. It is used in the Ayurvedic system of medicine to treat various diseases. Fresh extracts of this plant have been used by the people of Java and the Malay Peninsula for many years, as both topical and internal agents, for healing of wounds and antioxidant properties (Hamid et al., 2002; Zainol et al., 2008). This plant is continued to be used within the framework of folk medicine (Brinkhaus et al., 2000).

Figure 1 *Centella asiatica* (L.) Urban (Umbelliferae).

1. Name and synonyms

Centella asiatica (L.) Urban, a perennial herbaceous creeper belongs to the family Apiceae (Umbelliferae) (Table 5), which has many common names including gotu kola or Indian Pennywort or Asiatic Pennywort (English). The synonym such as Hydrocotyle Asiatique (French), Idrocotyle (Italian), Tsubo-Kusa (Japannese), Brahma-manduki and Brahmi-Buti (Hindi), Asiatischer Wassernable and Indischer Wassernable (German), Tung Chian and Luei Gong Gen (Chinese) (Brinkhaus et al., 2000), Pegaga (Malaysia), 'Vallarai (India), Daun Kaki Kuda (Indonesia) (Somchit et al., 2004) and Bua-Bok (Thailand) (Punturee et al., 2005).

Table 5 Systematic classification (Taxonomy) of *Centella asiatica* (Brinkhaus et al., 2000).

Classification	Name
Kingdom	Eukaryota
Subkingdom	Embryophyta
Division	Spermatophyta
Subdivision	Angiospermae
Class	Dicotyledoneae
Subclass	Rosidae
Superorder	Aralianae
Order	Araliaes (Umbelliflorae)
Family	Apiaceae or Umbelliferae
Subfamily	Hydrocotyle
Genus	Centella
Species	asiatica

2. Botany

Centella asiatica is a tender umbelliferous plant with numerous creeping stems, are slender, creeping stolons, green to reddish green in color, interconnecting one plant to another. It is flourishing abundantly in moist areas and distributing widely in tropical and subtropical countries up to an altitude of 1800 m and tolerates dense shade. The plant is clonally propagated by long internodes and nodes, on which are borne reniform-cordate leaves are 2 to 6 cm long and 1.5 to 5 cm wide, with a crenate margin and 5 to 9 ribs, with palmate nerves, soft and hairless or with only a few hair. The petioles are 3 to 30 cm long. The pedicles are 1.2 to 4 cm long. The sepals of the epicalyx are oval to circular, with a membranous border and are about 2.5 to 3 mm long and 1.5 to 2.5 mm wide.

The petals are white, to purple or pink, born in small, rounded bunches (umbels) near the surface of the soil. Each flower is partly enclosed in two green bracts. The hermaphrodite flowers are minute in size (less than 3 mm), with 5-6 corolla lobes per flower. Each flower bears five stamens and two styles. The fruit is oval to globose and has a diameter of 2 to 5 mm. The mericarps are clearly flattened at the sides and usually have 7 to 9 ribs and are raised rugose (Brinkhaus et al., 2000; Zheng and Qin, 2007).

3. Chemical constituents

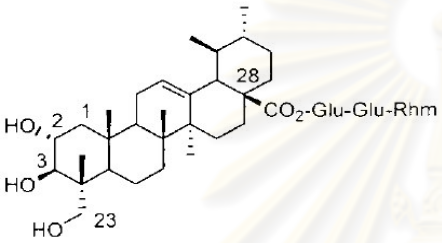
Various chemical constituents are reported in *Centella asiatica* such as asiaticoside, madecassoside, asiatic acid, madecassic acid, rhamnose, glucose, terpenoid, sitosterol, stearic acid, stigmasterol, fatty oil consist of glycerides of palmitic acid, linoleic acid, linolenic acid and ascorbic acid. It also contains calcium, iron and phosphate. These derive from the metabolism of acetate and phenylpropane, and belong to the terpenes and flavonoids (Brinkhaus et al., 2000; Jagtap et al., 2009). These compounds can be classified on the basis of their chemical structures as following (Newall et al., 1996).

1. Terpenoids : include
    - 1.1 α-pinene
    - 1.2 β-pinene
    - 1.3 Bornyl acetate
    - 1.4 Myrcene
2. Triterpenes: include
    - 2.1 madecassoside
    - 2.2 madecassic acid
    - 2.3 asiaticoside
    - 2.4 asiatic acid
    - 2.5 Centellic acid
    - 2.6 Centoic acid
3. Flavonoid glycosides: include
    - 3.1 Kaempferol-3-glycoside
    - 3.2 Quercetin-3-glycoside
4. Free amino acids: include
    - 4.1 Serine
    - 4.2 Aspartate
    - 4.3 Alanine
    - 4.4 Aminobutyrate
    - 4.5 Lysine
    - 4.6 Glutamate
    - 4.6 Threonine
5. Phytosterols
    - 5.1 β-sitosterol
    - 5.2 Stigmasterol
    - 5.3 Campesterol
6. Sugars
    - 6.1 Rhanose
    - 6.2 Arabinose

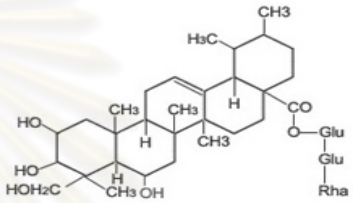
6.3 Fructose

6.4 Sucose

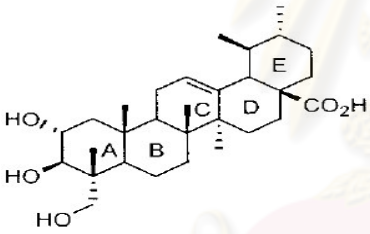
The substance of interested as therapeutics was the saponin-containing triterpene, acids and their sugar esters. The most important being: asiaticoside, madecassoside, asiatic acid and madecassic acid (Brinkhaus et al., 2000). Their structures are shown in Figure 2.



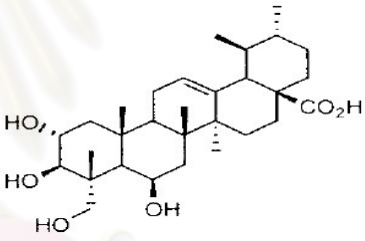
asiaticoside (1)



madecassoside (2)



asiatic acid (3)



madecassic acid (4)

Figure 2 Structure of asiaticoside (1), madecassoside (2), asiatic acid (3) and madecassic acid (4) (Jew et al., 2000).

4. Traditional applications

In common with most traditional phytotherapeutic agents, *Centella asiatica* is claimed to possess a wide range of pharmacological effects, being used for human wound healing, antifungal, antibacterial, anti-inflammations, antioxidant and other diseases.

- Skin disorders: as an universal use in wound healing and various skin disorders such as psoriasis, keloid, leprosy, ezema, burn, insect bites and lupus.

- Gastrointestinal disease: it is used to treat gastric ulcer, diarrhea, gastritis, colicky abdominal pain and dysentery.

- Neurological and psychiatric disease: it is used for mental disorder such as anxiety, psychiatric problems, epilepsy, improve memory and learning.

- Nephrological and urogenital disease: it is used as diuretic.

- Other disease: In addition, it were also claimed to be effectively used as anti-herpes simplex virus, anticancer, asthma, venous hypertension and microangiopathy.

- Microangiopathy: *Centella asiatca* have been shown to be efficacious in venous insufficiency (Zheng and Qin, 2007; Zainol et al., 2008).

5. Pharmacological activities

5.1 Central nervous system (CNS)

In animal model, aqueous extract of *Centella asiatica* at the dose of 200 and 300 mg/kg given orally for 14 days showed an improvement in learning and memory. Two doses of aqueous extract increased the number of avoidances in shuttle box and prolonged the step through latency in step through apparatus in a dose dependent manner, increase in the step down latency in step down apparatus and transfer latency (TL) in elevated plus maze. *Centella asiatica* showed decrease in the brain levels of malondialdehyde (MDA) and increased in levels of glutathione (GSH) (Veerendra Kumar and Gupta, 2002).

The extract of *Centella asiatica* at the dose of 300 mg/kg given orally for 60 days was effective in reducing brain regional lipid peroxidation (LPO) and increased superoxide dismutase (SOD) activity, by acting as a potent antioxidant exerted significant neuroprotective effect and proved efficacious in protecting rat brain against age related oxidative damage (Subathra et al., 2005).

The effect of *Centella* extract at the dose of 6 ml/kg given orally in rats for 6 weeks showed a significant increase in the dendritic length (intersections) and dendritic branching points along the length of both apical and basal dendrites. *Centella asiatica* extract has growth-stimulating neuronal dendritic in the hippocampal CA3 neurons (Gadahad et al., 2008).

A standardized aqueous extract of *Centella Aasiatica* at the dose of 5 mg/kg orally in prepubertal male mice pre-treat for 10 days were resistant against the 3-Nitropropionic acid (3-NPA)-induced oxidative stress and mitochondrial dysfunctions in brain. It increased level of glutathione (GSH), superoxide dismutase (SOD) and thiols in striatum and reduced level of malondialdehyde (MDA), hydroperoxide, succinic dehydrogenase and maleate dehydrogenase in mitochondria of brain regions (Shinomol and Muralidhara, 2008).

In a clinical, placebo controlled, forty one healthy volunteers of middle age participants received the *Centella asiatica* capsules at various doses ranging 3 g to 4 g (according to body weight) once daily for 2 months. It was showed that the *Centella asiatica* enhanced many of the cognitive test measured at different time between males and females (Dev et al., 2009). Potentially *Centella asiatica* is able to attenuate the age-related decline in cognitive function in healthy middle age and elderly (Wattanathorn et al., 2008).

5.2 Anti-infective effects

The extract of *Centella asiatica* contained both anti-HSV-1 and -2 activities, as determined by plaque inhibition assay. An inhibition of the production of infectious HSV-2 virions from infected Vero cells., the inhibitory effects of *Centella* extract were also substantiated by flow cytometric analysis of virus-specific antigens in the infected cells. The result showed that *Centella extract* demonstrated inhibitory effect on plague formation and also reduced the yield of HSV-1 and HSV-2 (Wirotesangthong and Rattanakiat, 2006).

The ethanol extract of *Centella asiatica* has been tested for antimicrobial activity by agar diffusion method compared with standard antibiotics ciprofloxacin (10 µg/ml). Zone of inhibition produced by ethanol extract in dose of 62.5, 125, 250, 500 and 1000 µg/ml against some selected strains. As data obtained the growth of inhibition of four bacterial strains varied largely included *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Propionibacterium vulgaris* and three fungal strains included *Aspergillus niger*, *Aspergillus flavus*, *Candida albicans* (Jagtap et al., 2009).

The antibacterial and antifungal activity of the *Centella* extracts against sixteen microbial strains was determined by the disc diffusion method, Kanamycin (30 µg/disc) was used as the standard. The n-hexane, carbontetrachloride, chloroform and aqueous soluble partitionates of the methanoic soluble fractions of methanol extract of *C. asiatica* showed average zone of inhibition ranged from 7-15 mm, 8-12 mm, 8-16 mm and 8-13 mm, respectively, at a concentration of 400 µg/disc. The chloroform extract showed the highest activity against the growth of *Staphylococcus aureus* and *Vibrio parahemolyticus* having the zone of inhibition of 16 mm. The extract showed good activity against the growth of *Salmonella typhi*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Shigella boydii* for antifungal activity of *Centella* extract, the average zone of inhibition was found to be 12-15 mm against the growth of *Aspergillus niger* (14 mm) (Ullah et al., 2009).

5.3 Anticancer

The anticancer effect of asiatic acid on HepG2 human hepatoblastoma cells. Asiatic acid decreased viability and induced apoptosis of HepG2 human hepatoma cells in a dose-dependent manner starting from the concentration of 30 µM. Asiatic acid also increased intracellular Ca^{2+} level, which in turn enhanced p53 expression in HepG2 cells (Lee et al., 2002).

The anticancer effect of water extract of *Centella asiatica* has been noted on formation of azoxymethane (AOM)-induced aberrant crypt foci (ACF) and intestinal

tumorigenesis in male F344 rats. It has been founded that extract at a dose of 100 mg/kg significantly reduced the multiplicity of neoplasms in the small intestine. In addition, the extract was found to significantly decrease the number of larger ACF in the large intestine in the early stage. In the post-initiation stage, the extract significantly decreased the total number of ACF and the number of larger ACF (Bunpo et al., 2004).

The anticancer effect of asiatic acid on human melanoma SK-MEL-2 cells (skin cancer). Asiatic acid decreased cell viability in a time- and dose-dependent manner with an IC_{50} of approximately 40 μM. In addition, asiatic acid-induced activation of caspase-3 activity in a dose-dependent manner. Asiatic acid significantly blocked the induction of Bax and activation of caspase-3 (Park et al., 2005).

5.4 Antioxidant

The methanol extract of *Centella asiatica* at the dose of 50 mg/kg/day given orally in lymphoma-bearing mice for 14 days, significantly increased the anti-oxidant enzymes, like superoxide dismutase (SOD), catalase, glutathione peroxidase (GSHPx), and anti-oxidants like glutathione (GSH) and ascorbic acid (Jayashree et al., 2003).

The antioxidant effect of aqueous extract of *Centella asiatica* at the dose of 200 mg/kg given orally into rats for 3 weeks to study its effect on myocardial marker enzymes and antioxidant enzymes in adriamycin induced cardiomyopathy. *Centella* extract significant prevented these alterations and restored the antioxidant enzymes included superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST) and glutathione peroxidase (GPx). *Centella* extract reduced level of myocardial lipid peroxides (LPO), creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) (Gnanapragasam et al., 2004).

The antioxidant effect of given orally 0.3% *Centella asiatica* extracts and 5.0% *Centella asiatica* powder in Spraque Dawley rats for 25 weeks. Results showed that administration of H_2O_2 (0.1%)-induced oxidative stress in drinking water of the rats, *Centella asiatica* extract and powder reduced malonaldehyde (MDA) levels and Increased superoxide dismutase (SOD) levels and catalase activity (Hussin et al., 2007).

5.5 Anti-inflammatory activity

The effect of water extract of *Centella asiatica* at the dose of 10, 30, 100 and 300 mg/kg intraperitoneally (i.p.) given to rodent models, was studied using acetic acid-induced writhing and hot-plate method in ICR Balb/c mice and prostaglandin E_2 (PGE_2)-induced paw edema in rats. The water extracts of *Centella asiatica* revealed significant anti-inflammatory activity, showing a significant dose-dependent reduction in the number of writhing with approximately 13%, 45%, 64% and 85% of inhibition respectively. Furthermore significant and dose-dependent prolongation of the response latency in the hot-plate test was elicited by the extract (Somchit et al., 2004).

The anti-inflammatory effect of asiatic acid at the concentrations of 30, 60 and 120 μM on LPS-induced nitric oxide (NO) and prostaglandin E_2 (PGE_2) production were investigated in RAW 264.7 macrophage cells. The results showed that asiatic acid inhibited protein and mRNA expression levels of inducible iNOS and cyclooxygenase-2 (COX-2) enzymes in a concentration-dependent manner. In addition, asiatic acid dose-dependently reduced the production of IL-6, IL-1β and TNF-α. Asiatic acid inhibited the NF-κB activation induced by LPS and this was associated with the abrogation of IκB-α degradation and with subsequent decreases in nuclear p65 and p50 protein levels. Moreover, the phosphorylations of IKK, p38, ERK1/2, and JNK in LPS-stimulated RAW 264.7 cells were suppressed by asiatic acid in a dose-dependent manner (Yun et al., 2008).

The anti- rheumatoid arthritic effect of madecassoside at the dose of 10, 20 and 40 mg/kg given orally for 21 days on collagen II (CII)-induced arthritis (CIA) in mice were examined. Madecassoside reduced clinical scores, and elevated the body weights in a dose-dependent manner. Madecassoside reduced the serum level of anti-CII IgG and suppress CII-stimulated proliferation of lymphocytes from popliteal lymph nodes in CIA mice. Histopathological examination indicated that madecassoside alleviated infiltration of inflammatory cells and synovial hyperplasia as well as protected joint destruction. In vitro, madecassoside was ineffective in the activation of macrophages caused by lipopolysaccharide (Liu et al., 2008).

The anti-inflammatory activity of madecassoside at the dose of 3, 10 and 30 mg/kg once daily were investigated by intragastric (i.g.) from 21 to 42 days after immunization on collagen-induced arthritis (CIA) in DBA/1J mice, evaluated by hind paw swelling, polyarthritis index, and histological examination. In vitro proliferation of spleen cells was examined using 3-[4,5-dimethylthylthiazol-2-yl]-2,5diphenyltetrazoliumbromide (MTT) assay. The results showed that madecassoside dose-dependently suppressed the clinical arthritis score and joints tissues pathological damage, reduced the proliferation of spleen cells, plasma levels of TNF-α and IL-6, synovial tissues prostaglandin E_2 (PGE_2) production and cyclooxygenase-2 (COX-2) protein expression (Li et al., 2009).

5.6 Wound healing

The effect of titrated extract from *Centella asiatica* (TECA), a reconstituted mixture of 3 triterpenes extracted from the plant, asiatic acid, madecassic acid and asiaticoside at the dose of 40 mg/injection in the wound chamber model were demonstrated. Chambers were collected at days 7, 14, 21 or 28 for biochemical analysis or histological examination. It was found that TECA increased dry weight, DNA, total protein, collagen and uronic acid contents. Peptidic hydroproline was also increased, showing an increased remodeling of the collagen matrix in the wound. In addition, the 3 components were also able to stimulate glycosaminoglycan synthesis (Maquart, 1999).

The activity of asiaticoside has been studied in normal and delayed-type wound healing. In guinea pig, topical applications of 0.2% solution of asiaticoside twice daily for 7 days to punch wounds produced 56% increase in hydroxyproline, 57% increase in tensile strength, increased collagen content and improving epithelization. In streptozotocin induced diabetic rats, where healing is delayed, topical application of 0.4% solution of asiaticoside over punch wounds increased hydroxyproline content, tensile strength, collagen content and epithelisation thereby facilitating the healing. Asiaticoside was effective by the oral route at the dose of 1 mg/kg for 7 days in the

guinea pig punch wound model. It promoted angiogenesis in the chick chorioallantoic membrane model at 40 µg/disk concentration (Shukla et al., 1999).

The effects of *Centella asiatica* extract orally administrated at the dose of 0.05 g/kg, 0.25 g/kg and 0.50 g/kg could prevent ethanol induced gastric lesions in rats. *Centella* extract significantly inhibited gastric lesions formation (58% to 82% reduction) and decreased mucosal myeloperoxidase (MPO) activity in a dose dependent manner (Cheng and Koo, 2000).

The anti-gastric ulcers effects of *Centella asiatica* water extract at the dose of 0.05 g/kg, 0.10 g/kg and 0.25 g/kg and asiaticoside at the dose of 1 mg/kg, 5 mg/kg and 10 mg/kg given orally for 3 days or 7 days have been demonstrated on acetic acid induced gastric ulcers (kissing ulcers) in rats. It was found that the extract was able to reduce the size of the ulcers at day 3 and 7 in a dose-dependent manner, with a concomitant attenuation of myeloperoxidase (MPO) activity at the ulcer tissues. Epithelial cell proliferation and angiogenesis were also promoted. In the presence of *Centella asiatica* water extract and asiaticoside were expression of basic fibroblast growth factor (bFGF), an important angiogenic factor, was also up-regulated in the ulcer tissues (Cheng et al., 2004).

The effects of orally given water extract of *Centella asiatica* (CE) at the dose of 0.10 g/kg and 0.25 g/kg and asiaticoside (AC) at the dose of 5 mg/kg and 10 mg/kg for 1 day or 3 days or 7 days have been demonstrated on acetic acid induced gastric ulcers in rats. It was found that the extract reduce the size of the ulcer at days 1, 3 and 7 in a dose-dependent manner, with a concomitant attenuation of iNOS activity at the ulcer tissues. The levels of nitrite and nitrate (NO_X^-), the stable end-products of nitric oxide (NO) in the gastric ulcer tissues were also decreased (Guo et al., 2004).

The healing effect of ethanolic extract of *Centella asiatica* at the dose of 800 mg/kg in rats for 10 days in both normal and dexamethasone-suppressed wound healing, using incision, excision, and dead space wounds models were found. The extract of *Centella asiatica* significantly increased the wound breaking strength, epithelization and the rate of wound contraction in incision wound. Wet and dry granulation tissue weights, granulation tissue breaking strength, and hydroxyproline

content in a dead space wound model also increased at statistically significant. The extract of *centella asiatica* had the effect of attenuating the known effects of dexamethasone healing in all wound models (Shetty et al., 2006).

The healing effects of asiaticoside at the concentration of 10 μM on human dermal fibroblast cells were demonstrated. It was found that asiaticoside induce type I collagen synthesis. In addition, the asiaticoside induced binding of Smad 3 and Smad 4, and the nuclear translocation of the Smad 3 and Smad 4 complex were also found. The results showed that asiaticoside can induce type I collagen synthesis via the activation of the TβRI kinase-independent Smad pathway (Lee et al., 2006).

The healing effects of the application of *Centella asiatica* extracts at the dose of 2 and 5×10^{-4}% (w/w) and asiaticoside at 10^{-8}, 10^{-10}, and 10^{-12}% (w/w) were layered on the burn wound surface in Balb/c mice for 21 days. The topical application of asiaticoside at low dose 10 pg, 1 ng, or 100 ng/wound area increased monocyte chemoattractant protein-1 (MCP-1), vascular endothelial growth factor (VEGF), and interleukin (IL)-1β levels in burn wound exudates (Kimura et al., 2008).

The clinical efficacy of *Centella asiatica* gels at the concentration of 0.50, 1.00 and 2.00% in C934P base compared to placebo and triamcinolone acetonide oral base in treatment of aphthous ulcers has been demonstrated in 87 subjects. It have been shown that 0.50, 1.00 and 2.00% *Centella asiatica* gels and triamcinolone acetonide decreased rate of lesion size greater than placebo in patients with aphthous ulcers (Wangrangsimakul, 1999).

Toxicological effects

Aqueous extract of *Centella asiatica* at the concentration of 5 mg/plate has been founded to lack cytotoxicity and mutagenicity on *Salmonella typhimurium* strains TA98 or TA100 with or without S9 mixture (Yen, 2001).

The acetone fraction of *Centella asiatica* extract at the concentration of 50 μg/mL did not induce cytotoxicity in normal human lymphocytes. Oral administration of the crude extract and the acetone fraction of *Centella asiatica* to normal and tumour

bearing mice at maximal concentration of 500 mg/mouse did not produce any toxic symptoms while the body weights of the mice were increased (Babu et al., 1995).

The sensitizing capacity effect of extract of *Centella asiatica* and its triterpenic constituents asiaticoside, asiatic acid and madecassic acid in guinea pigs, were found to be very weak sensitizers (Hausen, 1993).

Standardized extract of *Centella asiatica* (ECa 233)

Fluctuation of biologically active constituents normally exists in the crude extract of medicinal plants. ECa 233 is a white to off-white standardized extract of *Centella asiatica* containing triterpenoids at least 80% and the ratio of madecassoside and asiaticoside would always be kept at 1.5 ± 0.5 (Tantisira, 2009). Recently wound healing effects on burn wound was studied in normal and streptozotocin-induce diabetic rats. ECa 233 gel at concentration of 0.05%, 0.1% and 0.2% increased rate of wound contraction and blood flow (Wannarat et al., 2009). Acute toxicity study of ECa 233 was conducted by administration of 10.0 g/kg extract into mice. The extract at the given dose did not cause any toxic sign and death within the observation period of 14 days. Sub-chronic toxicity study of ECa 233 has been investigated on Wistar rats in four groups. Control group was orally given distilled water and three experimental groups were orally administrated with ECa 233 in distilled water at the dose of 10, 100 and 1000 mg/kg/day for 3 months. All ECa 233 treated groups showed no difference with regards to food consumption, body weight and health in compared to placebo group. Histopathological results of internal organs did not show any incidence in dose-dependent manner with the increasing dose of ECa 233. It has been demonstrated that ECa 233 was no acute and sub-chronic toxicity and safety (Tantisira, 2009).

CHAPTER III

MATERIALS AND METHODS

Materials

1. Instruments

- Digital camera (Pentax, China)

- An experimental kit containing cotton bud (Ambulance, Thailand), 70% ethyl alcohol (SIRIBUNCHA, Thailand), sterile gauze pad (Ambulance, Thailand), cotton balls (PHARMAHOF, Thailand), mirror, sterile calibrated ruler and one tube of 10 g oral paste containing either 0.10% triamcinolone or 0.00%, 0.05%, 0.10% and 0.20% ECa 233.

- A self-explanatory aphthous ulcer diary booklet for self-report of number of ulcer, size of ulcer, pain score, adverse effect, other drug application and time of application (Appendix A).

2. Drug and test substances

2.1 0.10% Triamcinolone acetonide (KENO®, T.O. CHEMICALS, Thailand)

2.2 ECa 233 was kindly supplied by Assistant Professor Dr. Chamnan Patarapanich and Associate Professor Suwanna Laungchonlatan Department of Food and Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University. Oral paste containing 0, 0.05, 0.10 and 0.20% of ECa 233 in hydrocarbon gel base or 0.10% triamcinolone acetonide (TA) were prepared by Medica Innova Pharma Co. Ltd. Bangkok Thailand. Study drugs were dispensed in similar coded collapsible tubes.

Methods

1. Study population

The protocol of a randomized, single-blind, placebo-controlled trial which was approved by the Ethical Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University was used in the present study. Participating subjects with the following inclusion and exclusion criteria were recruited by advertising to the general population during December 2008-August 2009. The Declaration of Helsinki was observed throughout the study.

Inclusion criteria

1. Male or female, 18 to 65 years of age.
2. A history of recurrent minor aphthous ulcers at least twice within one year.
3. Presentation of 1-2 minor aphthous ulcers with less than 48 hour of onset and less than 1 cm in diameter.
4. Ulcers must be present in an easy accessible location, for evaluation and treatment, including the buccal mucosa, labial mucosa and floor of the mouth.
5. Willing to participate and being able to complete the written consent form.
6. Normal liver and kidney function tests and normal complete blood count.

Exclusion criteria

1. Illness or diseases which might affect the study, including diabetes, active Infectious disease, behcet's disease and coeliac disease.
2. Any dental surgical procedure within two weeks before study entry or using an orthodontic brace or an orthodontic retainer that can contact the ulcer.
3. Using chewing tobacco products, smoking pipe or cigar and consumption of alcohol within six months prior to study entry.
4. History of anxiolytic, sedative drug or cocaine/heroin drug abuse.

5. Treatment of systemic steroids, NSAIDs, analgesic, immunosuppressant drug, antibiotic, oral topical medication within seven days prior to study entry.
6. Pregnancy or breast feeding.
7. On chemotherapy.
8. Abnormal haematological values.

2. Study groups

The present study was conducted in 2 phases; pilot and comparative studies. Assignment of eligible subjects into different groups of treatment was done randomly by block randomization. A drop-put subject in any group of treatment would be simply made up by additional recruitment of subject.

Pilot study was conducted in a small number of subjects to check the feasibility of the study and to identify an optimal concentration of the test substance (ECa 233) to be used in a large scale comparative study. Subjects were randomly assigned into 4 different groups of treatment of oral paste containing 0, 0.05, 0.10 and 0.20% of ECa 233 as following:

1. Subjects receiving topical placebo oral paste.
2. Subjects receiving topical oral paste containing 0.05% ECa 233.
3. Subjects receiving topical oral paste containing 0.10% ECa 233 and
4. Subjects receiving topical oral paste containing 0.20% ECa 233.

Data obtained in pilot study were used to estimate the size of population in comparative study by the method described by Cohen (1988).

In comparative study, efficacy of ECa 233 in a selected concentration based on the result of pilot study would be evaluated in comparison to 0.10% triamcinolone as a positive control and a plain oral paste serving as a negative control. Thus, subjects were randomly assigned into 3 experimental groups as following:

1. Subjects receiving topical placebo oral paste.
2. Subjects receiving topical oral paste containing 0.05% ECa 233 and
3. Subjects receiving topical oral paste containing 0.10% triamcinolone.

3. Application of test substances

A dry cotton bud was used to apply 0.5 cm of the oral paste and spreading on lesion surface. Subjects have to avoid eating or drinking for at least half and hour after the application. Subjects were instructed to apply the oral pasted for 3 times a day; after breakfast, after lunch and before bed time for 11 days. Time of application has to be recorded in a subject log diary.

4. Assessment of efficacy and safety

4.1 Estimation of ulcer size and number of MiRAU

Diameter of ulcer size was measured from edge to edge by a sterile calibrated ruler with millimeter markings (Figure 3). Two measurements crossed at 90 degrees were made. Cross-sectional area of the ulcer was calculated by a simple multiplication of the two measurements in mm (Khandwala et. al., 1997). Subjects were instructed to measure ulcer size before the application of oral paste at bed time and daily record of ulcer size has to be noted in subject log diary for 11 days. Time to complete healing of ulcer elapses from day 0 to the first day that the ulcer size was 0x0. In addition to the self assessment report, the number and ulcer size were cross-checked evaluated by the investigator on the follow-up visit on day 3 and 10.

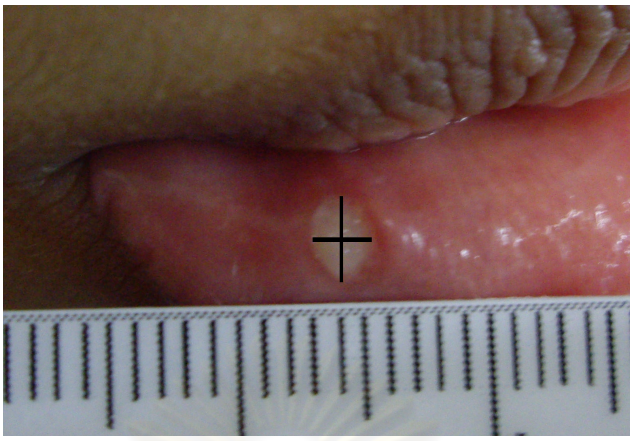


Figure 3 Method of measured diameter of ulcer size.

4.2 Estimation of pain score of MiRAU

Pain was induced by a touch of a dry cotton bud on the ulcer for 3 second and being assessed by a numeric Visual Analog Scale (VAS) consisting of a 0-10 scale horizontal line between no pain to unbearable pain (Figure 4). On day 0, an immediate relief of pain was measured before and at 10, 20 and 30 min after the application of oral paste. In addition, subjects were asked to record their pain score in response to a touch of cotton bud before the application of the oral paste at bed time for 11 days. Time to complete pain relief elapses from day 0 to the first day that the VAS score is evaluated by the investigator on the follow-up visit on day 3 and 10.

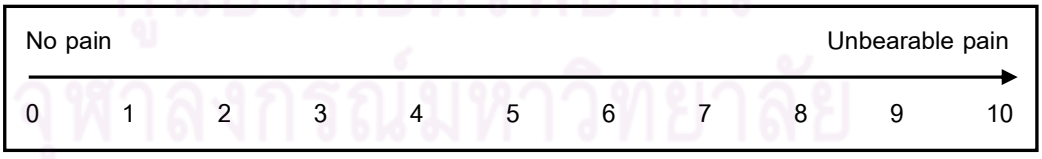


Figure 4 Numeric Visual Analog Scale (VAS).

4.3 Adverse effects

On the first application of oral paste on day 0, subject was observed for 30 min for any possible sign of acute hypersensitivity reaction and other adverse event. Interview on any unwanted effects was routinely carried out by the investigator on the follow-up visit on day 3 and 10. Subjects were instructed to record any unwanted effects such as burning upon application, mucosal changes, bitter taste and tingling sensation upon application that they might experience during 11 days of the trial. Phone number to call in case of serious adverse events due to the test oral paste was provided to all subjects.

5. Study protocols

Upon enrollment, the study protocol was thoroughly explained to each individual subject by the investigator. Verbal and written informed consent was obtained from each individual subject. Eligible subjects are those whose baseline data were within the acceptance criteria. All baseline data were obtained on day 0, except for the haematological data of a complete blood count (CBC), fasting blood sugar, serum albumin, blood urea nitrogen (BUN), serum creatinine (Cr), serum aspartate transaminase (AST) and alanine transaminase (ALT) in which blood samples were taken on day 1 at the haematological laboratory of Faculty of Dentistry, Chulalongkorn University.

0n day 0, they were asked to complete questionnaire giving details of their medical history and illness according to the inclusion or exclusion criteria. Diagnosis of MiRAU on the basis of an appearance and location of ulceration as well as baseline values of initial size of ulcer, number of ulcers, initial pain score, erythema level and immediate pain relief was assessed by a dentist or an investigator under the supervision of the dentist. Photo of ulcer was taken by a digital camera. In accompany with the experimental kit, instruction on the application procedure of the oral paste, induction and measurement of pain using VAS score, record of medication used as well as

unwanted effects were given to each individual subjects. They were asked to complete the data in a log diary booklet for 11 days. Follow-up visit was scheduled on day 3 and day 10 to allow interim assessment of ulcer by the investigator. At the end of the trial on day 10, the oral paste remaining from the trial and the log diary booklet were collected back and questionnaires (Appendix D) to evaluate satisfaction/dissatisfaction to different aspects of the trials was completed by each subject. Protocol of treatment or evaluation was shown in Table 6.

Table 6 Study protocol.

Descriptions	Baseline	Treatment			
Time (day)	**0**	**1-2**	**3**	**4-9**	**10**
Consent form	x				
Initial history	x				
Intraoral examination	x				
Heamatology & Blood chemistry	x				
Photography of ulcers	x		x		x
Number, location & size of ulcer	x	x	x	x	x
New ulcers		x	x	x	x
Pain score (VAS)	x	x	x	x	x
Adverse effects		x	x	x	x
Time of oral paste application		x	x	x	x
Other drug application		x	x	x	x
Satisfaction questionnaire					x

Data and statistical analysis

Demographics data were summarized with descriptive statistics. The values were reported as mean ± standard error (SE). One-way ANOVA followed by LSD post test for pilot study and Bonferroni correction post test for comparative study was used to test the differences between and within group. The level of significance was established at a p value < 0.05. All data were analyzed using SPSS software (Ver. 17.0).

CHAPTER IV

RESULTS

1. Pilot study

1.1 Demographics

Twenty four subjects, 8 males and 16 females, enrolled in the study. Most of the subjects were university employees with average mean age of 31.96 ± 2.16. No significant difference of age was noted among different treatment groups. Most of them presented with single ulcer located mainly on non-keratinized labial and buccal mucosa. Initial ulcer size (mm^2) assessed by the subjects themselves was found to be 8.47 ± 1.09, 8.14 ± 2.42, 9.47 ± 1.00 and 9.39 ± 1.23 in placebo, 0.05%, 0.10% and 0.20% ECa 233 oral paste groups, respectively. Accordingly the corresponding initial ulcer size measured by the investigator was 8.47 ± 1.09, 8.14 ± 2.42, 9.32 ± 1.10 and 9.38 ± 1.23, respectively. Initial pain score (VAS) was found to be 6.83 ± 0.75, 7.17 ± 0.54, 7.00 ± 0.63 and 6.83 ± 0.40 in placebo, 0.05%, 0.10% and 0.20% ECa 233 oral paste groups, respectively. There were no statistically significant differences in the baseline characteristics of the four groups ($p > 0.05$).

As a result of the blinded randomization procedure, all treatment groups were well-matched with regards to age, baseline values of number and size of ulcers and pain score (Table 7).

Table 7 Subject demographics in pilot study.

Description	Percentage of ECa 233 (W/V)			
	0.00 (n= 6)	0.05 (n= 6)	0.10 (n= 6)	0.20 (n= 6)
Age (years)	34.17 ± 2.91	30.00 ± 3.92	27.00 ± 9.26	36.67 ± 6.30
Male/Female	4/2	6/0	1/5	5/1
Number of ulcer	1.17 ± 0.16	1.00 ± 0.00	1.17 ± 0.16	1.33 ± 0.21
Initial ulcer size (mm^2)	8.47 ± 1.09	8.14 ± 2.42	9.74 ± 1.00	9.39 ± 1.23
Initial pain score (VAS)	6.83 ± 0.75	7.17 ± 0.54	7.00 ± 0.63	6.83 ± 0.40

VAS, Visual analog scale.

1.2 Effects of Standardized extract of *Centella aisatica* ECa 233

1.2.1 Reduction of ulcer size

Mean ulcer size in the treatment groups of 0, 0.05, 0.10 and 0.20 % ECa 233 oral paste were gradually reduced over the course of the study of 11 days as shown in Figure 5. Mean ulcer size on day 3 reported by subjects was 9.45 ± 2.58, 2.49 ± 0.90, 7.75 ± 2.58 and 7.07 ± 1.75 in placebo, 0.05, 0.10 and 0.20 % ECa 233 oral paste groups, respectively. Corresponding mean ulcer size measured by investigator on day 3 was 9.39 ± 1.23, 2.58 ± 0.85, 7.68 ± 1.20 and 6.96 ± 1.35. The mean time to achieve complete healing of ulcer (ulcer size = 0) was 8.25 ± 0.85 days in placebo group whereas they were 6.00 ± 0.97, 7.60 ± 1.03 and 6.67 ± 0.67 days in 0.05, 0.10 and 0.20 % ECa 233 oral paste groups, respectively. Apparently, ulcer in all ECa 233 treated groups healed better than those in placebo group. In comparison to their respective baseline values on day 0, the mean ulcer size in 0.05, 0.10 and 0.20 % ECa 233 treated groups were initially found to be significant reduced on day 2, day 6, and day 4, respectively whereas significant reduction of mean ulcer size in placebo group was initially noted on day 9 ($p < 0.05$). Thus 0.05% ECa 233 oral paste seemed to reduce ulcer size better than did the other concentrations.

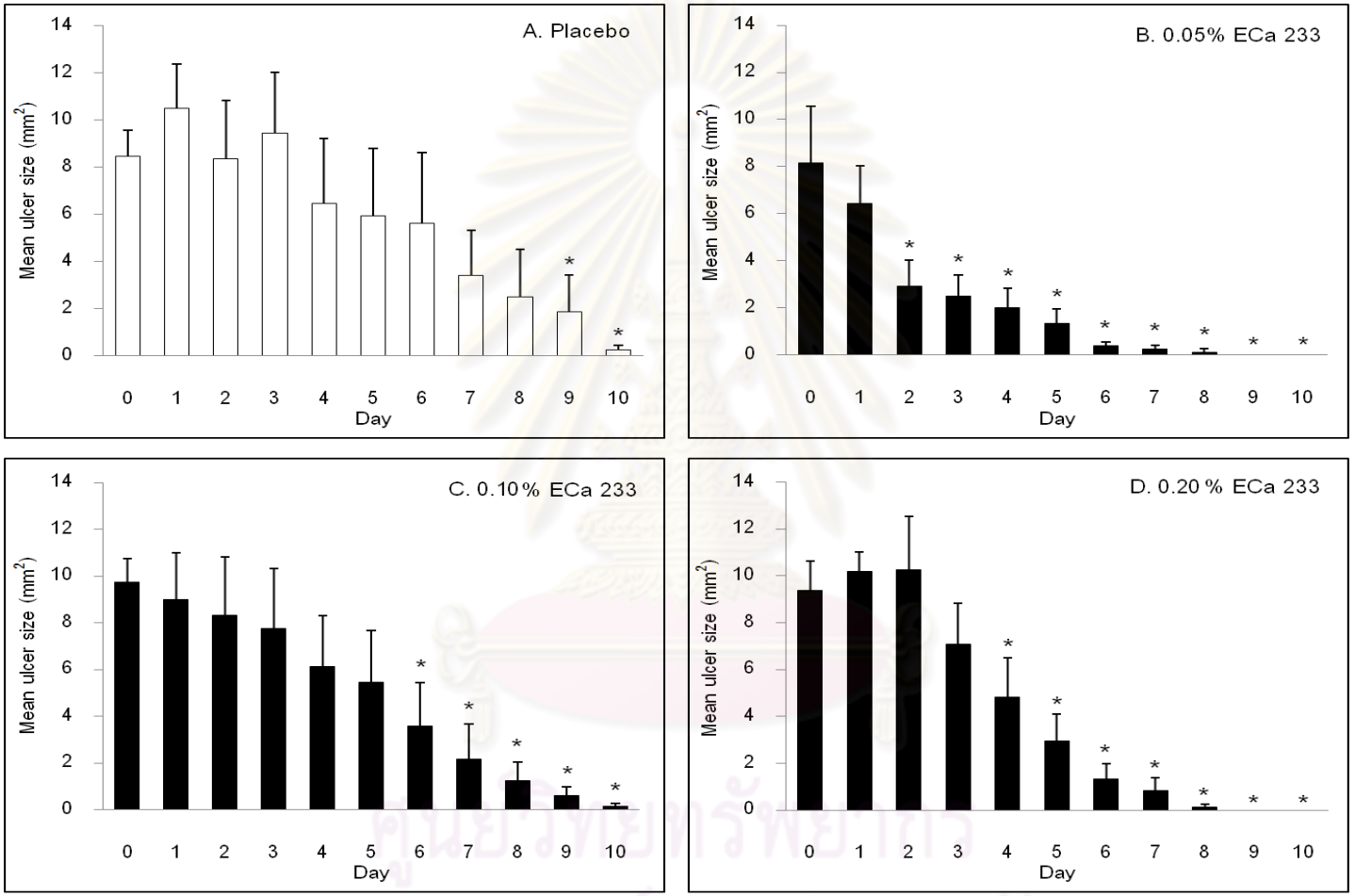


Figure 5 Mean ulcer size (mm^2) in different time course (day0-10) of placebo oral paste (A.); 0.05% ECa 233 oral paste (B.); 0.10% ECa 233 oral paste (C.) and 0.20% ECa 233 oral paste (D.). Values represent the mean ± SE of each group (n=6).

* denotes significant difference as compared to initial value at day 0 ($p < 0.05$).

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1.2.2 Reduction of pain score

According to the self-reported diary of daily pain score, the mean pain score in placebo, 0.05, 0.10 and 0.20 % ECa 233 oral paste groups were decreasing from their respective initial values to 5.00 ± 0.93, 1.67 ± 0.76, 5.17 ± 1.58 and 4.83 ± 1.25 on day 3. Similar results were observed by investigator mean pain score was 5.33 ± 0.88, 1.50 ± 0.56, 5.50 ± 1.06 and 5.00 ± 0.86 in placebo, 0.05, 0.10 and 0.20 % ECa 233 oral paste groups, respectively. The mean time to reach a complete pain relief (a VAS of 0) in the placebo group was 7.60 ± 0.81 days whereas they were 4.83 ± 0.75, 6.00 ± 1.05 and 5.00 ± 0.58 days in 0.05, 0.10 and 0.20 % ECa 233 oral paste groups, respectively. In agreement with a reduction of ulcer size, ECa 233 oral paste seemed to relieve ulcer pain better than did the placebo oral paste. Furthermore, significant reduction of pain score, in comparison to the pain score obtain on day 0 was initially reported on day 2, day 4 and day 3 in 0.05, 0.10 and 0.20% ECa 233 oral paste groups whereas significant reduction of pain in placebo group was demonstrated on day 5 ($p < 0.05$) (Figure 6). Comparatively, 0.05% ECa 233 oral paste seemed to reduce pain better than did the other concentrations.

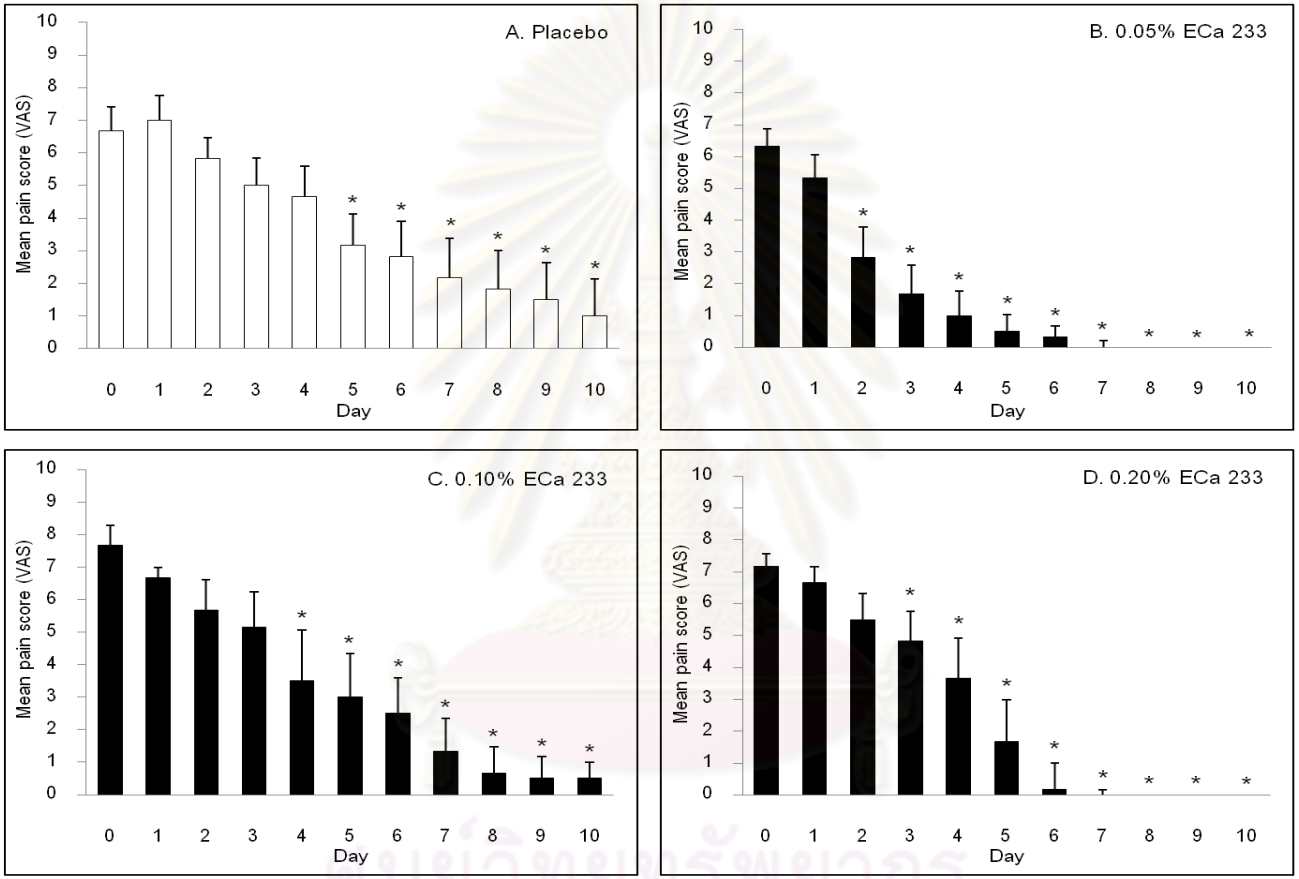


Figure 6 Mean pain scores in different time course (day0-10) of placebo oral paste (A.); 0.05% ECa 233 oral paste (B.); 0.10% ECa 233 oral paste (C.) and 0.20% ECa 233 oral paste (D.). Values represent the mean ± SE of each group (n=6).

* denotes significant difference as compared to initial value at day 0 ($p < 0.05$).

1.2.3 Reduction of erythema

Erythema levels was estimated by the investigator on day 0, day 3 and day 10. Initially almost all of the ulcers were surrounded by an inflammatory halo. As shown in Table 8, there were no differences on erythema levels across the four groups of treatment at day 0 (baseline). No improvement was observed in the placebo group at day 3 (2.17 ± 0.31) and the erythema still existed, though to a minor levels, at day 10 (0.33 ± 0.21). In contrast, all ECa 233 treated groups demonstrated a reduction of erythema at day 3 (1.00 ± 0.26, 1.83 ± 0.31 and 1.83 ± 0.31 in placebo, 0.05, 0.10 and 0.20% ECa 233 oral paste, respectively). Furthermore no sign of erythema could be visualized at day 10 (Table 8).

Table 8 Erythema levels of ulcer in different treatment groups.

Treatment	Erythema level* (mean ± SE)		
	Day 0	Day 3	Day 10
Placebo	2.17 ± 0.17	2.17 ± 0.31	0.33 ± 0.21
0.05% ECa 233	2.17 ± 0.17	1.00 ± 0.26	0.00 ± 0.00
0.10% ECa 233	2.17 ± 0.17	1.83 ± 0.31	0.00 ± 0.00
0.20% ECa 233	2.17 ± 0.17	1.83 ± 0.31	0.00 ± 0.00

* Erythema level : 0 = no erythema , 1 = light red/pink , 2 = red but not dark in color , 3 = very red, dark in color.

2. Selection of dose and size of samples for comparative study

Based on the observation that the reduction of size and pain of ulcer exhibited by 0.05, 0.10 and 0.20% of ECa 233 in pilot study were rather similar 0.05% ECa 233 was selected as an optimal. Concentration for further study in which the efficacy of ECa 233 would be evaluated in comparison to 0.1% triamcinolone. Sample size in comparative study was estimated by the following method (Cohen, 1998). By the fact that

k = Number of pilot study groups = 4

$u = k - 1 = 3$

f = required effect size = $\sigma m / \sigma = 0.34$

Note:

σ = Standard deviation (SD) of pilot study = 5.251

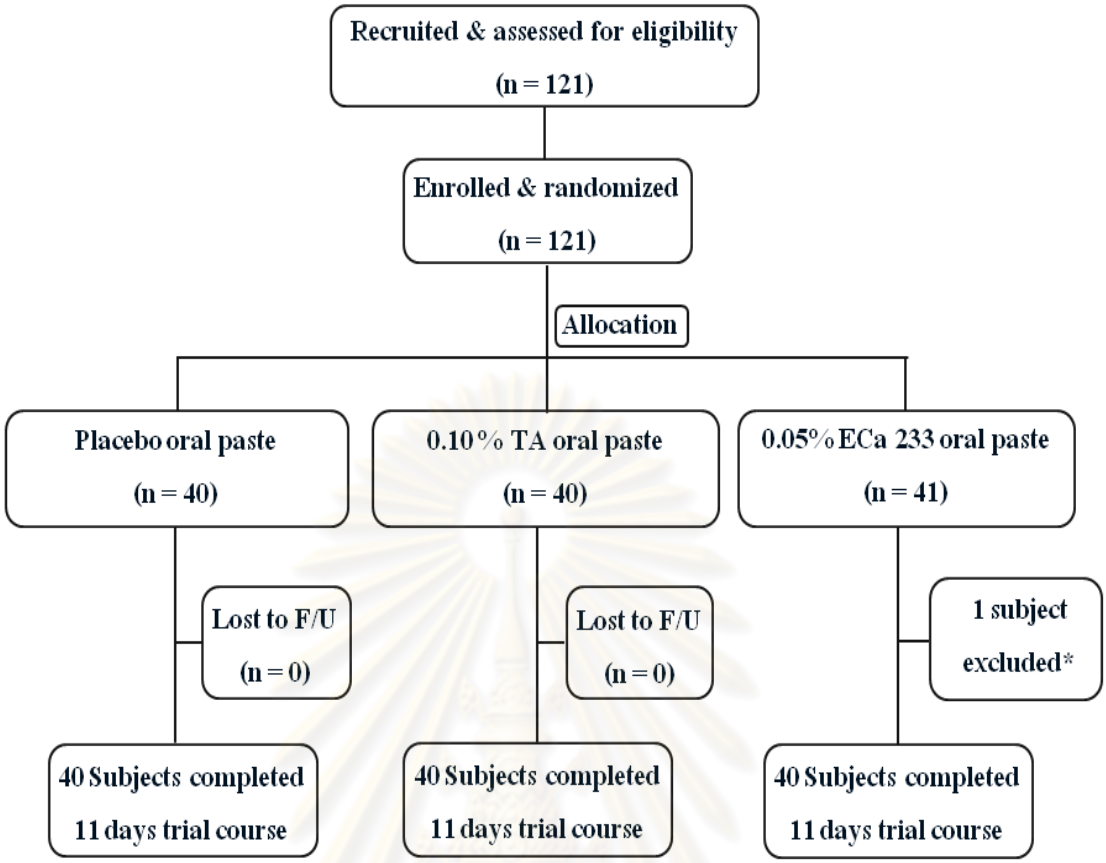
σm = Mean of standard deviation of pilot study = 1.774

Sample size per group in comparative study, where a level of significance of 5% and power of the test = 80% obtained for Cohen's tables (1988) was 25.

3. Comparative study

3.1 Demographics

Out of 121 subjects recruited, 120 subjects, 37 males and 83 females, completed the study (Figure 7). On entry to the trial, no subject had significant medical or surgical histories that may interfere with the conduct. No subject had clinically significant abnormal laboratory results (haematology, biochemistry) and no pregnancy. The mean age of subjects was found to be 21.78 ± 0.68, 22.40 ± 0.80 and 23.13 ± 1.07 years in placebo group, 0.05% ECa 233 oral paste group and 0.10% triamcinolone oral paste group, respectively. No significant difference in the mean age of the three groups. Most of them were university students presented with 1 or 2 ulcers located mainly on non-keratinized labial and buccal mucosa. The Initial ulcer size (mm^2) assessed by the subjects themselves was 8.14 ± 0.75, 7.80 ± 0.66 and 8.74 ± 0.76 and the Initial pain score (VAS) was 6.53 ± 0.30, 6.63 ± 0.26, and 6.75 ± 0.29 in placebo group, 0.05% ECa 233 oral paste group and 0.10% triamcinolone oral paste group, respectively. Accordingly the corresponding initial ulcer size measured by the investigator was 8.12 ± 0.72, 7.35 ± 0.64 and 8.57 ± 0.74, respectively. There were no statistically significant differences in the baseline characteristics of the three groups ($p > 0.05$). All treatment groups were well-matched with regards to age as well as baseline values of number, size and pain score of ulcers (Table 9).



* Taking NSIAD for the treatment of dysmenorrhea.

F/U = follow up.

Figure 7 Flow diagram of the clinical trial.

Table 9 Subject demographics in comparative study.

Description	Placebo (n= 40)	0.05% ECa 233 (n= 40)	0.10% triamcinolone (n= 40)
Age (years)	21.78 ± 0.68	22.40 ± 0.80	23.13 ± 1.07
Male/Female	15 / 25	6 / 34	16 / 24
Number of ulcer	1.20 ± 0.06	1.20 ± 0.06	1.23 ± 0.07
Initial ulcer size (mm^2)	8.14 ± 0.75	7.80 ± 0.66	8.74 ± 0.76
Initial pain score (VAS)	6.53 ± 0.30	6.63 ± 0.26	6.75 ± 0.29
Ulcer location (%)			
tongue/floor of mouth	0 (0)	1 (2.5)	2 (5)
buccal mucosa/ buccal vestibule	6 (15)	5 (12.5)	3 (7.5)
labial mucosa/ labial vestibule	34 (85)	34 (85)	35 (87.5)

VAS, Visual analog scale.

3.2 Effects of Standardized extract of *Centella aisatica* ECa 233

3.2.1 Reduction of ulcer size

Ulcer size measurements of "0 x 0 mm" were considered as being healed. Mean ulcer size in the treatment groups of 0.05% ECa 233 oral paste group and 0.10% triamcinolone oral paste group were gradually reduced over the course of the study of 11 days. In contrast mean ulcer size of placebo group increased at days 1 through 3 and, then graduate decreased until day 10 (Figure 8).

In comparison to placebo group, mean ulcer size in both subjects treated with 0.05% ECa 233 oral paste group and 0.10% triamcinolone oral paste group significantly decreased then those of placebo group during day 2-10 ($p < 0.05$). No significant difference in ulcer size was observed between 0.05% ECa 233 oral paste group and 0.10% triamcinolone oral paste group during the course of treatment ($p > 0.05$). On day 3 mean ulcer size in 0.05% ECa 233 and 0.10% triamcinolone oral paste groups was found to be 4.69 ± 0.64 and 5.32 ± 0.81, respectively, whereas it was 11.06 ± 1.04 in placebo group. Accordingly the corresponding mean ulcer size measured by the investigator on day 3 was 4.67 ± 0.68 and 5.22 ± 0.77 in 0.05% ECa 233 and 0.10% triamcinolone oral paste groups, respectively, whereas it was 10.44 ± 0.92 in placebo group.

In comparison to their respective baseline values on day 0, the mean ulcer size in both 0.05% ECa 233 oral paste group and 0.10% triamcinolone oral paste group were significantly reduced at days 3-10, whereas significant reduction of mean ulcer size in placebo group was noted at days 8-10 ($p < 0.05$). These results clearly supported the observation in the pilot study that 0.05% ECa 233 was effective in accelerating the healing of ulcer better than did the placebo. The new ulcers found in two subjects in placebo group and one subject in each 0.05% ECa 233 and 0.10% triamcinolone treated groups. There was no difference between any of the three groups (data not shown).

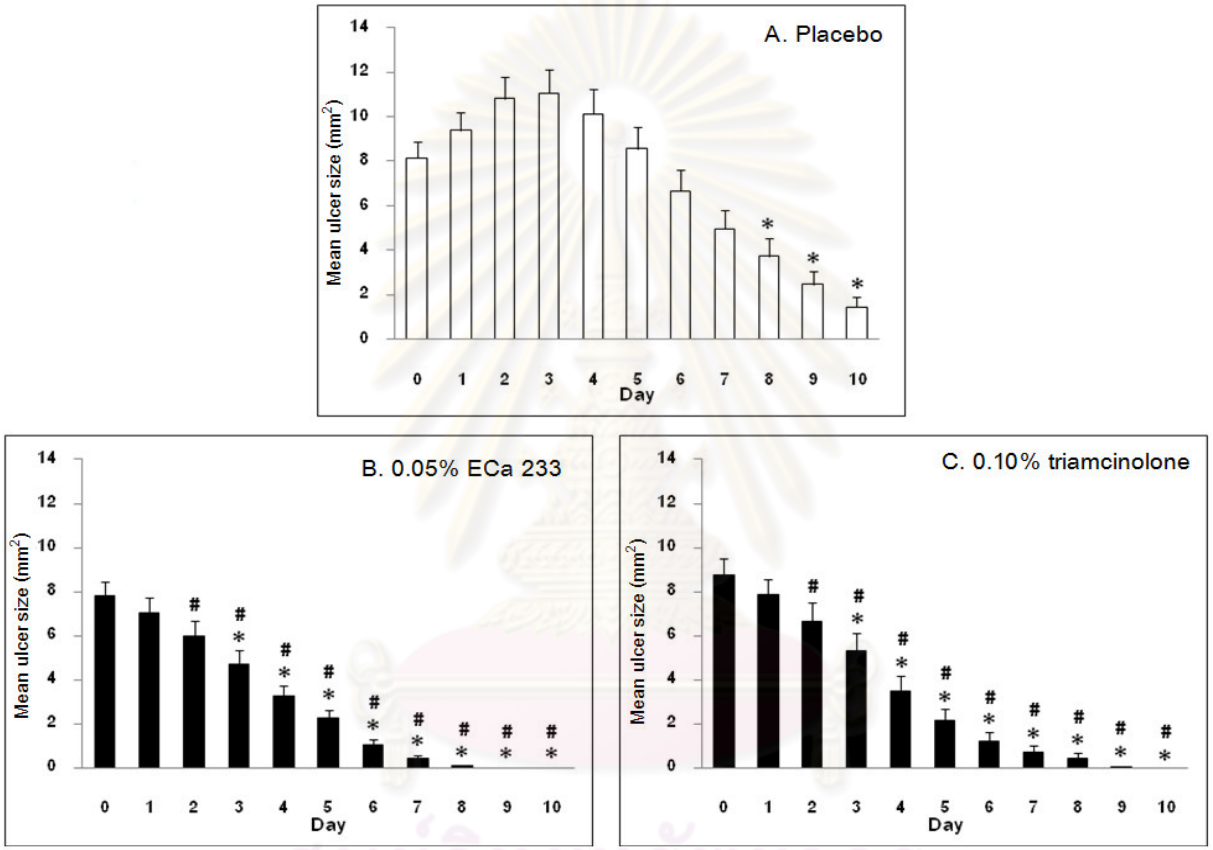


Figure 8 Mean ulcer size (mm^2) in different time course (day0-10) of placebo oral paste (A.); 0.05% ECa 233 oral paste (B.) and 0.10% triamcinolone oral paste (C.). Values represent the mean ± SE of each group (n=40).

* denotes significant difference as compared to initial value at day 0 ($p < 0.05$).

# denotes significant difference as compared to placebo oral paste group on the same day ($p < 0.05$).

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3.2.2 Reduction of pain score

3.2.2.1 Immediate relief of pain

On day 0, immediate relief of pain in response to touching by investigator was performed. Subjects were asked to record the level of pain induced by a touch of a dry cotton bud before and at 10, 20 and 30 min after the application of the test materials. Initial mean pain score before the application of test substances was 6.23 ± 0.26, 6.88 ± 0.22 and 6.98 ± 0.25 in placebo, 0.05% ECa 233 and 0.10% triamcinolone oral paste groups, respectively. Similar profile of immediate response was noted in all groups. Following the initial application, a rapid reduction of pain score which was sustained for 30 min was reported by subjects in placebo group as well as 0.05% ECa 233 or 0.10% triamcinolone treated groups (Figure 9).

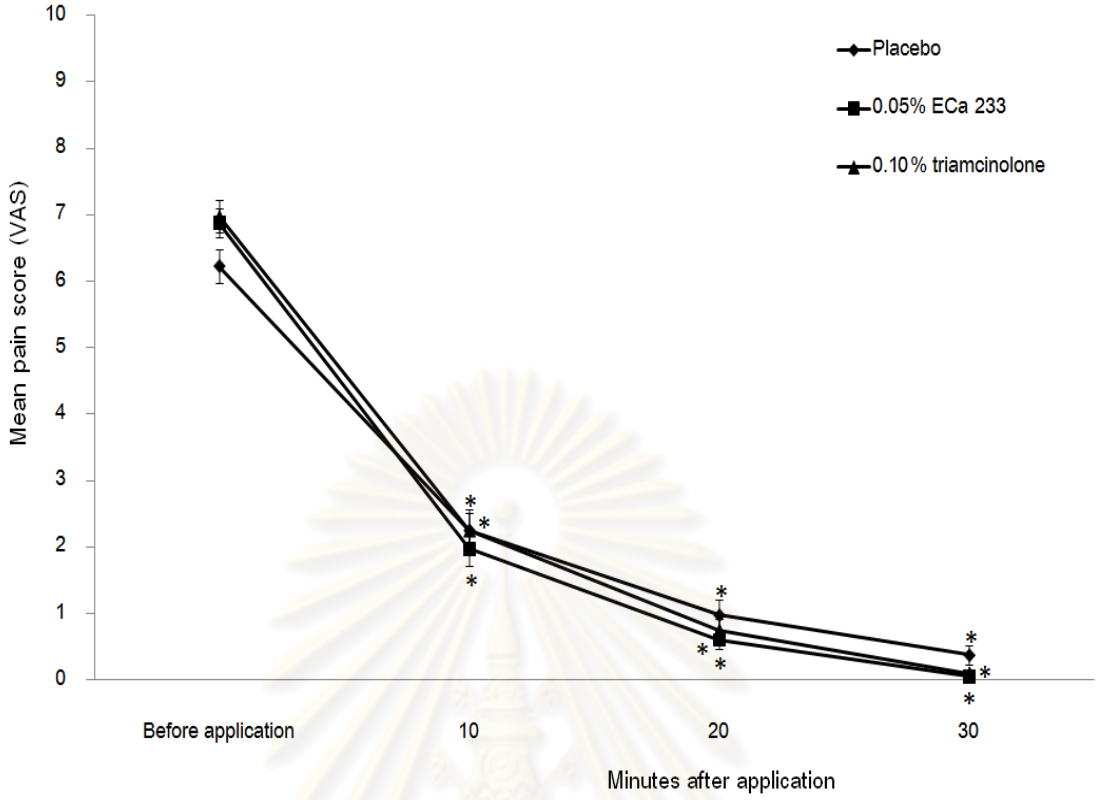

Figure 9 Baseline mean pain score before and after the application of placebo oral paste (A.); 0.05% ECa 233 oral paste (B.) and 0.10% triamcinolone oral paste (C.). Values represent the mean ± SE for each group (n=40).

* denotes significant difference as compared to initial value at minutes 0 ($p < 0.05$).

3.2.2.2 Relieving of pain during the course of treatment

With regards to the respective in 3.2.2.1, mean pain score in response to touching by the subject themselves agreed well with those performed by investigator. The baseline of mean pain score collected from the log diary booklet on day 0 was 6.53 ± 0.30, 6.63 ± 0.26 and 6.75 ± 0.29 in placebo, 0.05% ECa 233 and 0.10% triamcinolone treated groups, respectively. Furthermore comparative results were also obtained on day 3. As illustrated in Figure 10, a significant reduction of pain score in each group in reference to their respective initial values on day 0 was observed on day 5, day 1 and day 2 in placebo, 0.05% ECa 233 and 0.10% triamcinolone treated groups, respectively. Accordingly, pain score in placebo group during day 0-2 was rather similar and started to decline on day 3 and went on reduction until day 10. In contrast, a prompt decrease of pain score was observed on day 1 and gradually declined until completely resolved within the observation period in both 0.05% ECa 233 and 0.10% triamcinolone treated groups exhibiting statistical significant to those values in placebo group during day 2-10. However, no statistical significance was observed between 0.05% EC 233 and 0.10% triamcinolone treated groups.

Average mean time to reach a complete pain relief (a VAS of 0) in the placebo group was 8.74 ± 0.24 days whereas they were 6.23 ± 0.23 and 5.93 ± 0.26 days in 0.05% ECa 233 and 0.10% triamcinolone treated groups (Figure 11). Statistical significance was observed only between placebo group and 0.05% ECa 233 or 0.10% triamcinolone treated groups but not between the two of them (Figure 11).

Ulcers in all subjects receiving either 0.05% ECa 233 oral paste group or 0.10% triamcinolone oral paste group achieved complete heal within the observation period of 11 days. However complete healing of ulcer was observed only eighteen out of forty subjects in placebo group at the end of the trial. The mean time to complete healing was 8.65 ± 0.25 days in placebo group whereas they were 6.63 ± 0.23 and 6.58 ± 0.25 days in 0.05% ECa 233 and 0.10% triamcinolone treated groups, respectively. Apparently, ulcer in 0.05% ECa 233 and 0.10% triamcinolone treated groups significantly healed

faster than those in placebo group ($p < 0.05$) (Figure 12). No significant difference in time for complete healing was observed between 0.05% ECa 233 oral paste group and 0.10% triamcinolone oral paste group ($p > 0.05$). The result demonstrated that 0.05% ECa 233 oral paste decreased duration of ulcer and speed healing of ulcer.

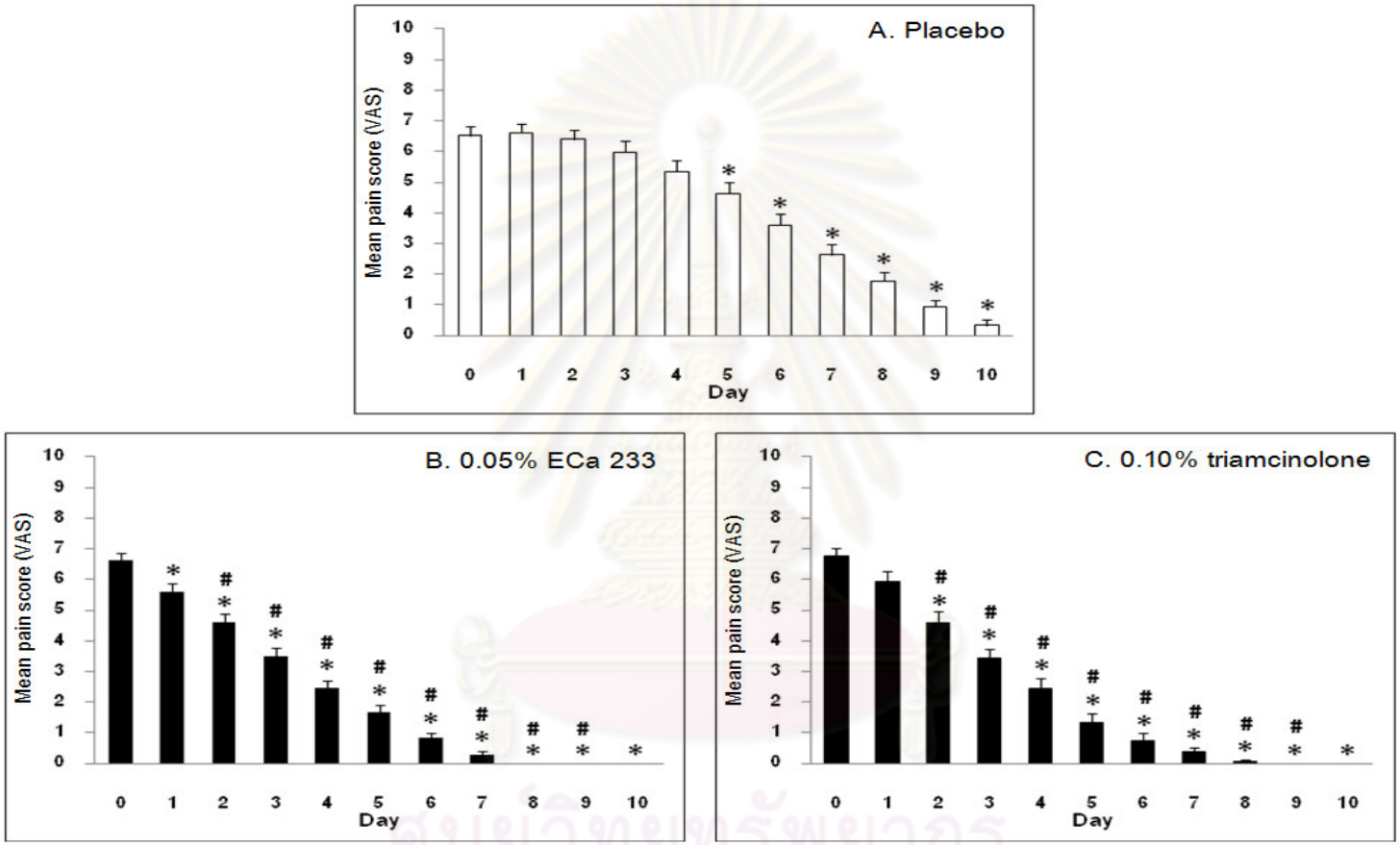

Figure 10 Mean pain scores in different time course (day0-10) of placebo oral paste (A.); 0.05% ECa 233 oral paste (B.) and 0.10% triamcinolone oral paste (C.). Values represent the mean ± SE of each group (n=40).

* denotes significant difference as compared to initial value at day 0 ($p < 0.05$).

# denotes significant difference as compared to placebo oral paste group on the same day ($p < 0.05$).

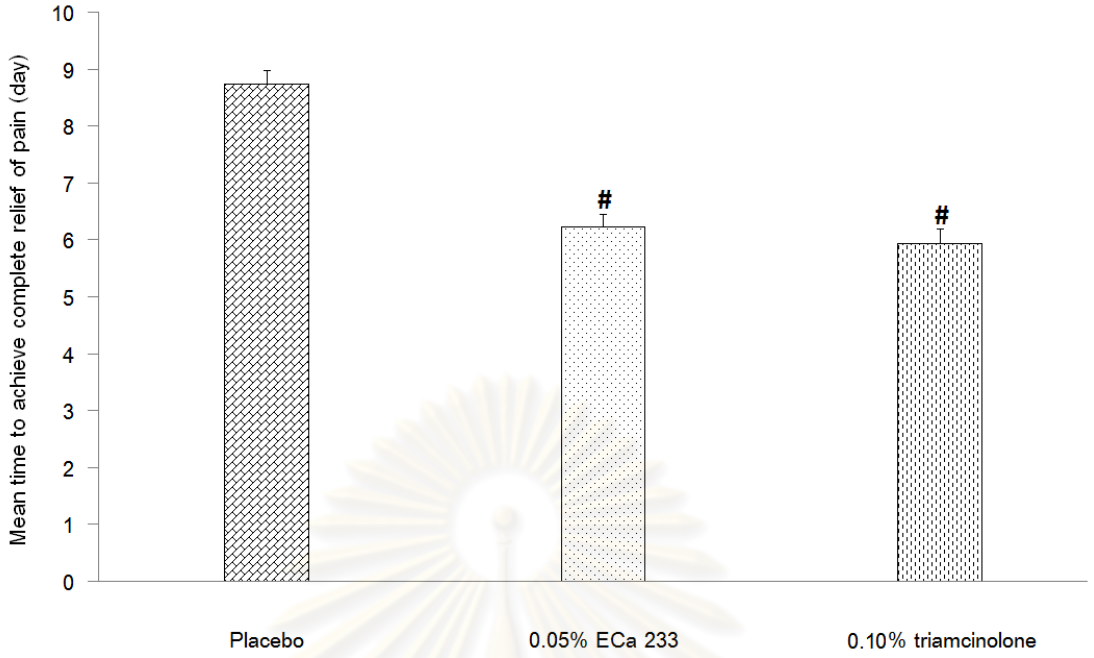

Figure 11 Mean time to achieve complete relief of pain (VAS=0) after receiving ▨ placebo oral paste; ⬚ 0.05% ECa 233 oral paste and ▩ 0.10% triamcinolone oral paste. Values represent the mean ± SE for each group (n=40).
# denotes significant difference as compared to placebo oral paste group ($p < 0.05$).

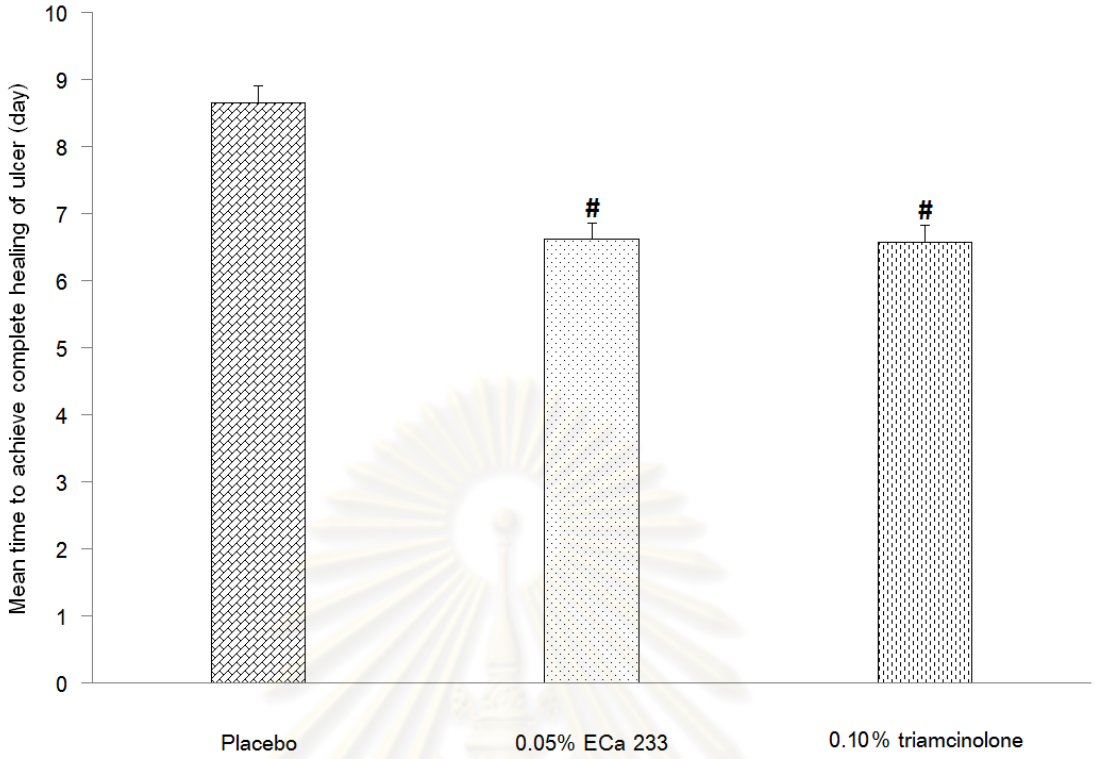

Figure 12 Mean time to achieve complete healing of ulcer across groups receiving placebo oral paste; 0.05% ECa 233 oral paste and 0.10% triamcinolone oral paste. Values represent the mean ± SE for each group (n=40).
# denotes significant difference as compared to placebo oral paste group ($p < 0.05$).

3.2.2.3 Reduction of erythema

Erythema levels were estimated by same investigator on day 0, day 3 and day 10 as shown in Figure 13. Initially almost all of the ulcers were surrounded by inflammatory halo. There were no differences on erythema levels across the three groups of treatment at day 0 (baseline). The placebo group, erythema still existed without improvement at day 3 (1.65 ± 0.08) although there were minor improvement at day 10 (0.48 ± 0.08). In contrast, both 0.05% ECa 233 oral paste group and 0.10% triamcinolone oral paste group demonstrated a reduction of erythema at day 3 (0.90 ± 0.09 and 0.88 ± 0.10, respectively) , furthermore no sign of erythema could be visualized at day 10 (0.05% ECa 233 and 0.10% triamcinolone treated groups were 0.00 ± 0.00).

In addition, erythema levels of both 0.05% ECa 233 oral paste group and 0.10% triamcinolone oral paste group on day 3 and 10 were significantly reduce compared to the result on day 0 ($p < 0.05$). No significant different in reduction of erythema level between these two groups ($p > 0.05$).

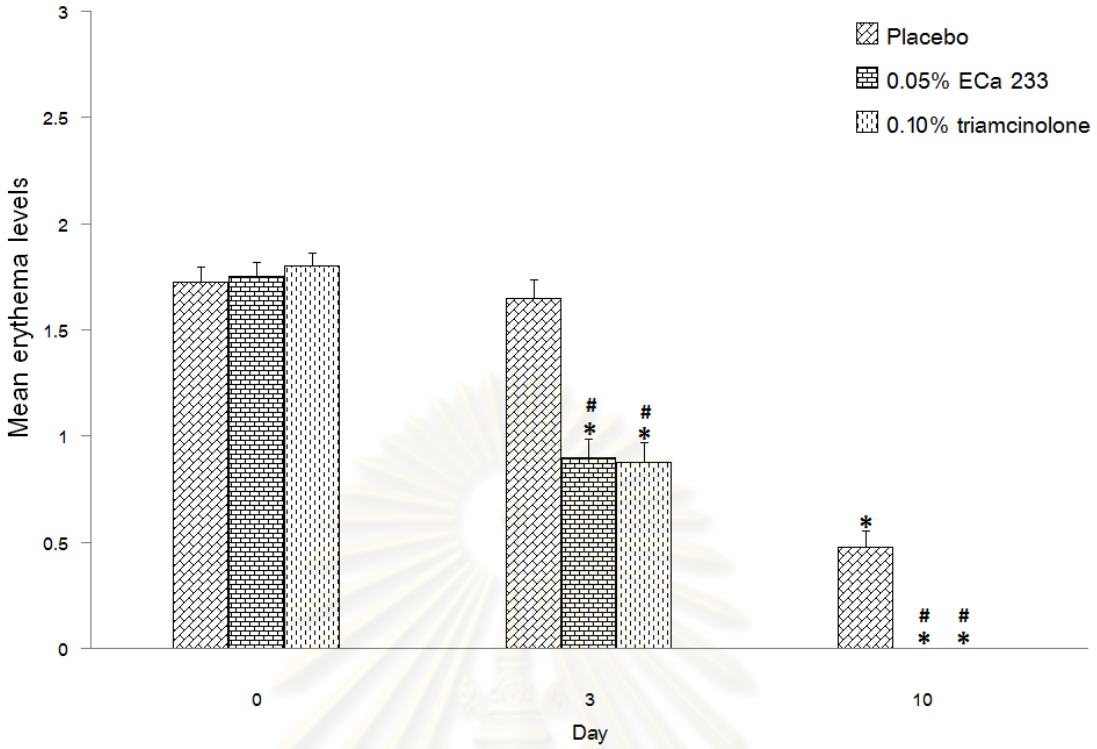

Figure 13 Mean erythema level of ulcer at day 0 (baseline) and after application at day 3 and 10 in the placebo oral paste; 0.05% ECa 233 oral paste and 0.10% triamcinolone oral paste. Values represent the mean ± SE for each group (n=40).

* denotes significant difference as compared to initial value at day 0 ($p < 0.05$).

\# denotes significant difference as compared to placebo oral paste group on the same day ($p < 0.05$).

3.2.2.4 Safety evaluation

Only one subject dropped out from the study because of taking antibiotic and non steroidal anti-inflammatory drug (NSAIDs) for an illness of pharyngitis and dysmenorhea. As the discontinuation rate is quite low (0.8%), we evaluated demographic and efficacy data without that one subject, which no influence to any interpretation or conclusion. No subject experienced any allergic reaction due to the treatment received. Throughout the course of treatment, no mucosal changes, no bitter taste, no pain or burning sensation or some other discomfort was reported to the investigators. Only one subject in 0.05% ECa 233 group reported to have a headache following the application of the 0.05% ECa 233 for 1-2 days, after which the symptom disappeared, which was considered by investigator as unrelated to study medication. A vast majority of subjects who were surveyed at the end of their treatment demonstrated a preference to the 0.05% ECa 233 oral paste as well as 0.10% triamcinolone which is a prototype in treatment of aphthous ulcers.

3.2.2.5 Compliance

No subject withdrew from the study prior to completing 11 days of treatment. All subjects used the study medication as directed and no patch applications were missed. Overall, subject compliance with data reporting were very good with 100% of subjects without missing data. Therefore, analyses of efficacy and safety were performed considering all subjects.

3.2.2.6 Subject acceptability

At the end of the trial on day 10, a series of questionnaire was unanimously answered by each subject. As shown in Table 10 a vast majority of subjects in each group were similarly and highly satisfied with the dosage form (Q3) and the convenience of application (Q4). Differences in satisfaction were observed between the placebo and 0.05% ECa 233 or 0.10% triamcinolone treatment groups with regards to efficacy and ability to relieve pain of the products used. About ¾ of the subjects in both 0.05% ECa 233 or 0.10% triamcinolone treated groups were highly satisfied with ability to relieve pain of the products of their treatment (Q1) whereas only 20% of patients in placebo group were highly satisfied. Similarly, while 80% and 82.50% of subjects in 0.05% ECa 233 and 0.10% triamcinolone treated groups were highly satisfied with the efficacy of the product (Q2) Only 12.5% of subjects in placebo group were highly satisfied with the efficacy of their products. Accordingly a global satisfaction of products (Q10) expressed by subjects in placebo, 0.05% ECa 233 and 0.10% triamcinolone treated groups were found to be 5%, 72.5% and 77.5% of very high satisfaction level and 82.50%, 22.50% and 15.00% of high level of satisfaction, respectively. Eighty percentage of subjects in 0.05% ECa 233 treated group and 85% of subjects in 0.10% triamcinolone treated groups were very likely to use the product again whereas only 12.5% of subjects in placebo treated group intended to do so. Taken all together, evaluation of the products by the subjects agreed well with the data on ulcer size and pain score previously mentioned.

Table 10 Satisfaction to subjects in different aspects of the treatment.

Descriptive	% Satisfaction level*														
	Placebo (n=40)					0.05% ECa 233 (n=40)					0.10% TA (n=40)				
	5	4	3	2	1	5	4	3	2	1	5	4	3	2	1
1. Satisfaction to pain relief	20.0	***50.0***	27.5	2.5	0.0	***77.5***	20.0	2.5	0.0	0.0	***75.0***	20.0	5.0	0.0	0.0
2. Satisfaction to effectiveness of product	12.5	***55.0***	22.5	10.0	0.0	***80.0***	12.5	7.5	0.0	0.0	***82.5***	12.5	5.0	0.0	0.0
3. Satisfaction with form of product	***82.5***	17.5	0.0	0.0	0.0	***87.5***	12.5	0.0	0.0	0.0	***85.0***	15.0	0.0	0.0	0.0
4. Satisfaction with easily use of product	***87.5***	12.5	0.0	0.0	0.0	***92.5***	7.5	0.0	0.0	0.0	***90.0***	10.0	0.0	0.0	0.0
5. Satisfaction of facilities and place in study	30.0	60.0	10.0	0.0	0.0	32.5	62.5	5.0	0.0	0.0	25.0	67.5	7.5	0.0	0.0
6. Satisfaction of studied time	10.0	72.5	17.5	0.0	0.0	5.0	77.5	17.5	0.0	0.0	7.5	70.0	22.5	0.0	0.0
7. Satisfaction of public relations and communication to study	10.0	65.0	25.0	0.0	0.0	17.5	70.0	12.5	0.0	0.0	22.5	60.0	17.5	0.0	0.0
8. Knowledge and skill gained from study	32.5	60.0	7.5	0.0	0.0	35.0	57.5	7.5	0.0	0.0	37.5	52.5	10.0	0.0	0.0
9. Advantage in participating in study	10.0	72.5	17.5	0.0	0.0	15.0	80.0	5.0	0.0	0.0	15.0	67.5	17.5	0.0	0.0
10. Satisfaction with overall in product	5.0	***82.5***	12.5	0.0	0.0	***72.5***	22.5	5.0	0.0	0.0	***77.5***	15.0	7.5	0.0	0.0

* Participated satisfaction questions were asked to subjects in divided 5 point scale between very highly satisfaction (5) to no satisfaction (1).

CHAPTER V

DISCUSSION AND CONCLUSION

Recurrent aphthous ulceration (RAU) remains a difficult disorder to treat. As the precise aetiology is still unknown, most of the treatments are directed towards lessening the painful symptoms and decrease duration of the ulcers. Patients may seek advice from a variety of sources regarding appropriate therapy and special medication or agents to treat RAU. The most common topical agents suggested as the first line of treatment for RAU included glucocorticoids, antibiotics, and local analgesics. Topical glucocorticoids such as triamcinolone acetate, dexamethasone, betamethasone, and fluocinonide can effectively relief the symptoms; however, there is a possibility that the contact of glucorticoids with oral mucosa may cause local immunosuppression (Macphee et al., 1968; Scully et al., 2002; Femiano et al., 2003; Greenberg and Pinto, 2003). Topical antibiotics such as tetracycline, and penicillin G potassium have some clinical efficacy for the treatment of RAU. However, adverse effects, such as allergic reactions, bitter taste and tooth discoloration, may hurt patient compliance (Kerr et al., 2003; Preshaw et al., 2007; Gorsky et al., 2008). For local analgesics it has been demonstrated that 3% viscous lidocaine could not enhance healing of the ulcers (Saxen et al., 1997). Therefore, it is a major field of investigation in oral medicine to search for topical agents that can be easily applied with higher efficacy and lower side effect to treat RAU.

As RAU is an inflammatory lesion of the mucosal lining of the mouth, control of the inflammation or factors influencing the inflammation process could be one among several modalities of treatments (Shashy and Ridley, 2000). Anti-inflammatory drug, particularly topical corticosteroids is recommended as the first line therapy for the treatment of RAU (Yoke et al., 2006). *Centella asiatica* has long been claimed for its beneficial effects on aphthous ulceration which could be partly accounted by its anti-inflammatory effects (Liu et al., 2008; Yun et al., 2008). In line with them, our dose-finding study (n = 6) has demonstrated that an oral paste of ECa 233, a standardized extract of *Centella asiatica* containing triterpenoids at least 80% and the ratio between

madecassoside and asiaticoside is within 1.5 ± 0.5, was able to accelerate healing process of MiRAU. Ulcer size and pain score in subjects receiving oral paste containing ECa 233 in the concentration of 0.05, 0.10 and 0.20% were apparently improved faster than those being treated with a plain placebo. Average mean time to reach a complete healing was found to be 8.25 ± 0.85, 6.00 ± 0.97, 7.60 ± 1.03 and 6.67 ± 0.67days for 0 (control), 0.05, 0.10 and 0.20% ECa 233 treated groups, respectively. Similar to previous report in which no significance was observed in healing rate exerted by C934P base containing 0.50, 1.00 and 2.00% of *Centella asiatica* extract (Wangrangsimakul, 1999). The results observed hereby did not demonstrate significant difference among concentration used. Thus, the lowest concentration tested (0.05% ECa 233) was selected for further comparative study in which triamcinolone 0.10%, a corticosteroid commonly used in the treatment of MiRAU, was used as a comparator.

A randomized, single blind, placebo controlled trial was again conducted in a larger population of subjects (n = 40 in each group) using the same protocol and the same criteria of inclusion and exclusion as it was done in the dose-finding study. As a result of a randomization, subjects in different treatment groups designated as control or placebo, 0.10% triamcinolone and 0.05% ECa 233 treated groups were well matched in terms of demographic data including age, number of ulcer, initial ulcer size and initial pain score (Table 11).

According to Roger (1977) who divided RAU into 4 stages namely premonitory, preulcerative, ulcerative and healing. Preulcerative stage was characterized by painful circular or oval ulcer surrounded by an erythematous halo. Subsequently, ulcer reached ulcerative stage in which ulcers were enlarged and usually attained the maximum size at 4-6 days after the onset. By criteria of inclusion used in the present study, eligible subjects were only those presented with MiRAU no longer than 48 hours of onset. Therefore, they were all in preulcerative stage. However, an increase in ulcer size and erythema indicating ulcerative stage was clearly observed only in the placebo group in which an increase in ulcer size was clearly noted on day 3, and gradually declined to a complete heal within 8.65 ± 0.25 days. In contrast, ulcer size in either 0.10% triamcinolone or 0.05% ECa 233 treated groups showed no increment but simply

declined from the very first day after an application of the test substance and achieved complete heal at day 6.58 ± 0.25 and 6.63 ± 0.23 respectively.

Reduction of ulcer size by triamcinolone oral paste has been previously reported by several investigators. Anti-inflammatory and anti-immunologic properties resulting in a decrease of certain inflammatory cytokines such as IL-6 were claimed to account for the effects noted (Rhodus and Bereuter 1998; Xia et al., 2006). Based on the results in the present study, the ulcers in 0.05% ECa 233 or 0.10% triamcinolone treated group seemed to go directly from proulcerative stage to healing stage without an intermediate of ulcerative stage as observed in placebo group. Anti-inflammatory effect of *C. asiatica* and its major constituents such as asiaticoside or madecassoside have been previously reported in animal models (Cheng et al., 2004; Liu et al., 2008; Li et al., 2009). Based on the finding that ECa 233 apparently reduced the erythema level, symptom indicating inflammation, it is likely that ECa 233 may possess anti-inflammatory effect which could be beneficial to ulcer healing in the same manner as triamcinolone. Anti-inflammatory as well as antibacterial effects have been proposed to account for ulcer healing effects of an herbal extract from flowers of *M. chamomilla* (Sahba and Mohammadalipour, 2005). Antibacterial activity of *C. asiatica* has been reported by Ullah et al. (2009), however, ECa 233 demonstrated negative effect against *Staphylococcus aureus* (Sriubolmas, 2009). Therefore ulcer healing effect of ECa 233 should be explained by other mechanisms than its effect on bacteria. Stimulation of growth factor and epithelial proliferation (Kimura et al., 2008), promotion of collagen synthesis and angiogenesis (Cheng et al., 2004) demonstrated by *C. asiatica* are all likely to participate in the effects of ECa 233 observed and they should be further clarified.

Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage (Natah et al., 2004). The oral mucosa is extensively innervated by nociceptive, polymodal, mechanoreceptive, thermoreceptive and chemoreceptive sensory fibers to provide a wealth of sensory formation. Pain in aphthous ulcers derived from inflammatory sensitization of small diameter afferent nerve ending that form a plexus extend upward, into the epithelial layer. Therefore, the aphthous ulceration produces a superficial, focal, inflammatory lesion that is directly

associated with sensory nerve endings (Saxen et al., 1997). In RAU, ulcer associated pain is mostly troublesome as it may severely interfere with eating, speaking, swallowing and decreasing quality of life (QOL) (Meng et al., 2009). Palliation of pain symptom was one of three major goals of treatment; control the pain of the ulcer, promote ulcer healing and prevent recurrence (Shashy and Ridley, 2000). Visual analog scale (VAS), a scale 0-10 horizontal line between no pain to unbearable pain, is widely used to determine the severity of pain (Saxen et. al., 1997; Ylikontiola et al., 1997; Meng et al., 2009; Porter et al., 2009). In accordance with previous investigations of 0.2% hyaluronic acid and 3.0% diclofenac (Saxen et al., 1997; Ylikontiola et al., 1997), an initial evaluation of immediate relief of pain in day 0 demonstrated that application of placebo exhibited pain relieving profile in 30 min similar to those of 0.05% ECa 233 or 0.10% triamcinolone. Plausible explanation could be a psychic response to a sense of being treated or a mechanical barrier formed by excipients in the oral paste protecting the ulcer from local stimuli.

During the course of treatment, a significant reduction of VAS score in comparison to its own initial value was detected in placebo treated group on day 5 whereas the corresponding values in 0.05% ECa 233 and 0.10% triamcinolone were on day 1 and 2, respectively, indicating faster relieving of pain by the active treatments. Significant difference from their placebo counterpart was observed on day 2 in both treatment groups but not between them. Ulcer associated pain was completely relieved (VAS = 0) on 6.23 ± 0.23 and 5.93 ± 0.26 days in 0.05% ECa 233 and 0.10% triamcinolone treated groups, respectively, whereas a longer time (8.74 ± 0.24 days) was needed by placebo group. ECa 233 is more effective than placebo and as effective as triamcinolone in relieving aphthous associated pain. Analgesic, local anesthetic or anti-inflammatory effects are likely to account for reduction of pain score observed. Among them, local anesthetic is an unlikely one since there no scientific report ever indicated a membrane stabilizing effect of *C. asiatica* whereas some anti-inflammatory and analgesic effects of the plant have been reported (Somchit et al., 2004). Further study is needed to shed light on underlying mechanism.

Based on the results obtained from log diaries of subjected enrolled, a randomize, single-blind, placebo controlled clinical trial in the present study

demonstrates that 0.05% ECa 233 is as effective as 0.10% triamcinolone with regards to their effects on ulcer size, pain relieving and time to complete healing. Together with subjective evaluation by a set of questionnaire to be completed by each individual at the end of the trial, a beneficial effect of ECa 233 in the treatment of MiRAU has been clearly demonstrated. Further investigation on the prevention of recurrence should be further carried out to make a complete perspective of the treatment.

In consideration to advantages of ECa 233 as a white to off-white titrated extract of *C. asiatica*, a herb that can be easily grown in Thailand, with a consistency of known biomarkers and clinically-proved pharmacological properties in parallel with a very good safety profile, attempt should be made to bring ECa 233 into an alternative in the management of MiRAU.

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APPENDICES

APPENDIX A

วันที่.....

สมุดบันทึกข้อมูลแผลร้อนในวันที่ 0-10 (Day 0-10)

1. ตารางเวลาที่ทายา (ให้ลงเวลาในตารางเมื่อทายาแล้ว)

เวลา	เช้า	กลางวัน	ก่อนนอน
เวลาที่ทายา			

2. ผลข้างเคียงที่เกิดขึ้นจากการใช้ยาป้ายปากที่ได้รับ (ถ้ามี)

.....

.....

3. ยาอื่นๆที่ใช้ (โปรดระบุชื่อยา)

ชื่อยา	1	2	3
จำนวนเม็ด			
เวลาที่ใช้			

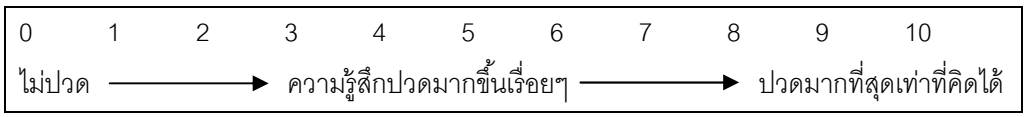
บันทึกก่อนนอน

4. จำนวนและขนาดของแผลร้อนใน : ก่อนทายาในช่วงก่อนนอน

จำนวนของแผลร้อนในก่อนนอน (ก่อนทายา)	ขนาดของแผลร้อนในก่อนนอน (ก่อนทายา) (mm.)
1.	
2.	

5. ระดับความปวด (Pain score)

การประเมินความปวด



ตารางบันทึกระดับความปวด : ก่อนนอน

ก่อนทายา	หลังทายา (30 นาที)

APPENDIX B

Case Record Form

วันที่.....เดือน.....พ.ศ.....

ลำดับที่.....

Code:……..

เรื่อง : การเข้าร่วมโครงการวิจัยผลของสมุนไพรบัวบกในการรักษาแผลร้อนใน

คำชี้แจง : โปรดทำเครื่องหมาย ✓ ลงในช่องที่ตรงกับข้อมูลของท่าน

- ส่วนทั่วไป

เพศ ☐ ชาย ☐ หญิง

อายุ.....ปี

น้ำหนัก.....กิโลกรัม ส่วนสูง..... เซ็นติเมตร

ภูมิลำเนา จังหวัด ..... เบอร์โทรศัพท์……..

ระดับการศึกษา ☐ ต่ำกว่าปริญญาตรี ☐ ปริญญาตรี ☐ สูงกว่าปริญญาตรี

อาชีพ.....

สถานภาพ ☐ โสด ☐ สมรส ☐ หย่าร้าง ☐ อื่นๆ.....

- ส่วนสำคัญของเรื่องที่จะวิจัย

1. ท่านเคยเป็นแผลร้อนในหรือไม่

☐ ไม่เคย ☐ เคย.....ครั้ง/ปี

2. ท่านเริ่มเป็นแผลร้อนในครั้งนี้มานานเท่าใด

☐ $\leq$ 48 ชั่วโมง ☐ > 48 ชั่วโมง

3. การสูบบุหรี่

☐ ไม่สูบบุหรี่ ☐ สูบบุหรี่ จำนวน ..... มวน/วัน

4. การดื่มสุรา

☐ ไม่ดื่มสุรา ☐ ดื่มสุรา

5. จำนวนอาหารที่รับประทาน.....มื้อ/วัน

6. รสชาติอาหารที่ชอบรับประทานเป็นประจำ

☐ เผ็ด ☐ หวาน ☐ เปรี้ยว ☐ เค็ม ☐ อื่นๆ.....

7. โรคประจำตัว

.....

8. โรคทางพันธุกรรม/ประวัติเจ็บป่วยของบุคคลในครอบครัว

.....

9. ประวัติการเจ็บป่วย/การนอนโรงพยาบาล/การผ่าตัด/ทันตกรรม

☐ ไม่เคย

☐ เคย .....

10. ประวัติการแพ้ยา ☐ ไม่แพ้ยา ☐ แพ้ยา .....

11. ในช่วง 6 เดือน ถึงปัจจุบัน ยาที่ใช้ประจำ

☐ ไม่มี

☐ มี ได้แก่ .....

12. ระดับความเครียด

☐ น้อย ☐ ปานกลาง ☐ มาก

13. วิธีการผ่อนคลายความเครียด

.....

14. การนอนหลับ พักผ่อน จำนวน ..... ชั่วโมง/วัน

15. การออกกำลังกาย

☐ ไม่เคย ☐ นานๆ ครั้ง ☐ สม่ำเสมอ

16. การตั้งครรภ์

☐ ไม่ตั้งครรภ์

☐ ตั้งครรภ์

☐ อื่นๆ เช่น ให้นมบุตร .....

ข้อคิดเห็น/ข้อเสนอแนะอื่นๆ

.....

.....

APPENDIX C

Table 11 Cohen (1988)- Sample size required to detect a difference in means for 2, 3, 4 or 5 groups.

n to detect f by F test at $\alpha = .05$ for $u = 1, 2, 3, 4$

$u = 1$

	f											
Power	.05	.10	.15	.20	.25	.30	.35	.40	.50	.60	.70	.80
.10	84	22	10	6	5	4	3	3	2	–	–	–
.50	769	193	86	49	32	22	17	13	9	7	5	4
.70	1235	310	138	78	50	35	26	20	13	10	7	6
.80	1571	393	175	99	64	46	33	26	17	12	9	7
.90	2102	526	234	132	85	59	44	34	22	16	12	9
.95	2600	651	290	163	105	73	54	42	27	19	14	11
.99	3675	920	409	231	148	103	76	58	38	27	20	15

$u = 2$

	f											
Power	.05	.10	.15	.20	.25	.30	.35	.40	.50	.60	.70	.80
.10	84	22	10	6	5	4	3	3	2	–	–	–
.50	662	166	74	42	27	19	15	11	8	6	5	4
.70	1028	258	115	65	42	29	22	17	11	8	6	5
.80	1286	322	144	81	52	36	27	21	14	10	8	6
.90	1682	421	188	106	68	48	35	27	18	13	10	8
.95	2080	615	230	130	83	58	43	33	22	15	12	9
.99	2855	714	318	179	115	80	59	46	29	21	16	12

$u = 3$

	f											
Power	.05	.10	.15	.20	.25	.30	.35	.40	.50	.60	.70	.80
.10	79	21	10	6	4	3	3	2	2	–	–	–
.50	577	145	65	37	24	16	13	10	7	5	4	3
.70	881	221	99	56	36	25	19	15	10	7	6	5
.80	1098	274	123	69	46	31	23	18	12	9	7	5
.90	1415	354	158	89	58	40	30	23	15	11	8	7
.95	1718	430	192	108	70	49	36	28	18	13	10	8
.99	2353	589	262	148	95	66	49	38	24	17	13	10

$u = 4$

	f											
Power	.05	.10	.15	.20	.25	.30	.35	.40	.50	.60	.70	.80
.10	74	19	8	6	4	3	2	2	–	–	–	–
.50	514	129	58	33	21	16	11	9	6	5	4	3
.70	776	195	87	49	32	22	17	13	9	6	5	4
.80	956	240	107	61	39	27	20	16	10	8	6	5
.90	1231	309	138	78	50	35	26	20	13	10	7	6
.95	1486	372	166	94	60	42	31	24	16	11	9	7
.99	2021	506	225	127	82	57	42	33	21	15	11	9

APPENDIX D

วันที่.....เดือน.....พ.ศ.....

แบบสอบถามความพึงพอใจ

เรื่อง : ความพึงพอใจของผู้เข้าร่วมโครงการวิจัยผลของสมุนไพรบัวบกในการรักษาแผลร้อนใน

คำชี้แจง : โปรดทำเครื่องหมาย ✓ ลงในช่องที่ตรงกับระดับความพึงพอใจของท่าน

ประเด็นคำถาม	ระดับความพึงพอใจของท่าน				
	มากที่สุด (5)	มาก (4)	ปานกลาง (3)	น้อย (2)	น้อยที่สุด (1)
1. การประชาสัมพันธ์ / การประกาศเข้าร่วมโครงการวิจัย					
2. สถานที่และสิ่งอำนวยความสะดวกอื่น ๆ ในการวิจัย					
3.ความรู้และทักษะในการดูแลรักษาแผลร้อนที่ได้รับ					
4.ความสะดวกรวดเร็วและง่ายต่อการใช้ยาทาบัวบก					
5. ความเหมาะสมของรูปแบบยาทาบัวบก					
6. การลดความปวดของแผลร้อนในจากการใช้ยาทาบัวบก					
7. ประสิทธิภาพของยาทาบัวบกในการรักษาแผลร้อนใน					
8. ความเหมาะสมของระยะเวลาในการเข้าร่วมการวิจัย					
9.ประโยชน์ที่ท่านได้รับ คุ้มค่ากับเวลาที่เสียไปในการเข้าร่วมการวิจัย					
10. ความพึงพอใจต่อผลิตภัณฑ์ยาทาบัวบก					

11. หากท่านเป็นแผลร้อนในในภายหน้า ท่านจะเลือกใช้ยาทาบัวบกหรือไม่

☐ ใช้แน่นอน ☐ อาจจะใช้ ☐ ไม่แน่ใจ ☐ ไม่ใช้

ข้อคิดเห็น /ข้อเสนอแนะ

.....

.....

- ขอขอบคุณที่กรุณาสละเวลาในการตอบแบบสอบถาม-

Table 12 Satisfaction data in comparative study.

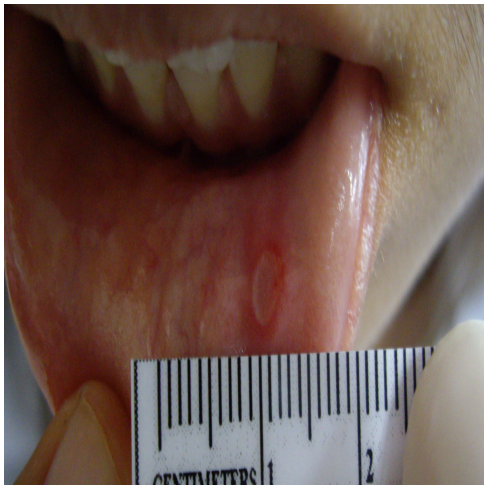
ประเด็นคำถาม	Placebo oral paste (n=40)					0.05% ECa 233 oral paste (n=40)					0.10% TA oral paste (n=40)				
	มากที่สุด (5)	มาก (4)	ปานกลาง (3)	น้อย (2)	น้อยที่สุด (1)	มากที่สุด (5)	มาก (4)	ปานกลาง (3)	น้อย (2)	น้อยที่สุด (1)	มากที่สุด (5)	มาก (4)	ปานกลาง (3)	น้อย (2)	น้อยที่สุด (1)
1. การประชาสัมพันธ์ / การประกาศเข้าร่วมโครงการวิจัย	4	26	10	0	0	7	28	5	0	0	9	24	7	0	0
2. สถานที่และสิ่งอำนวยความสะดวกอื่น ๆ ในการวิจัย	12	24	4	0	0	13	25	2	0	0	10	27	3	0	0
3.ความรู้และทักษะในการดูแลรักษาแผลร้อนที่ได้รับ	13	24	3	0	0	14	23	3	0	0	15	21	4	0	0
4.ความสะดวกรวดเร็วและง่ายต่อการใช้ยาทาบัวบก	35	5	0	0	0	37	3	0	0	0	36	4	0	0	0
5. ความเหมาะสมของรูปแบบยาทาบัวบก	33	7	0	0	0	35	5	0	0	0	34	6	0	0	0
6. การลดความปวดของแผลร้อนในจากการใช้ยาทาบัวบก	8	20	11	1	0	31	8	1	0	0	30	8	2	0	0
7. ประสิทธิภาพของยาทาบัวบกในการรักษาแผลร้อใน	5	22	9	4	0	32	5	3	0	0	33	5	2	0	0
8. ความเหมาะสมของระยะเวลาในการเข้าร่วมการวิจัย	4	29	7	0	0	2	31	7	0	0	3	28	9	0	0
9.ประโยชน์ที่ท่านได้รับ คุ้มค่ากับเวลาที่เสียไปในการเข้าร่วมการวิจัย	4	29	7	0	0	6	32	2	0	0	6	27	7	0	0
10. ความพึงพอใจต่อผลิตภัณฑ์ยาทาบัวบก	2	33	5	0	0	29	9	2	0	0	31	6	3	0	0

APPENDIX E

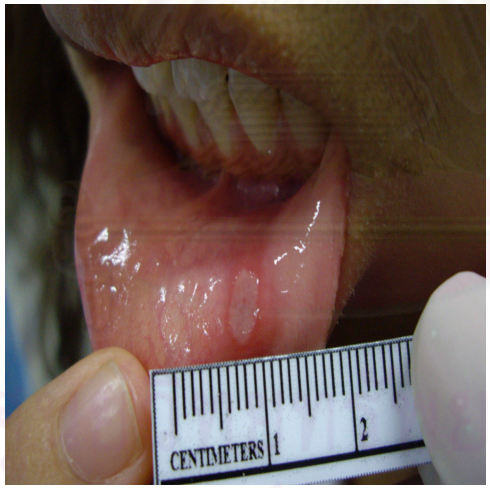
The figures showed sample of subjects with minor recurrent aphthous ulceration each treatment group.

Pilot study

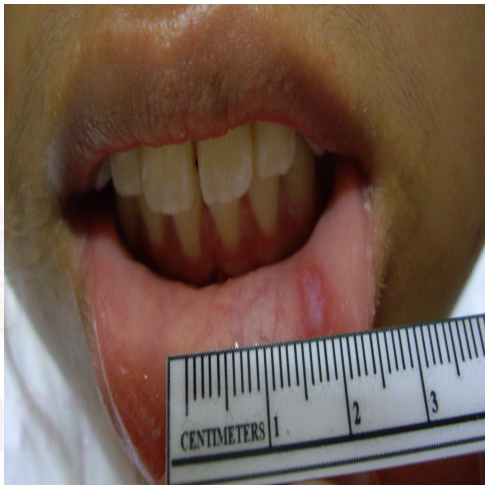
1. The subjects receive topical placebo oral paste.



Day 0 (A.)



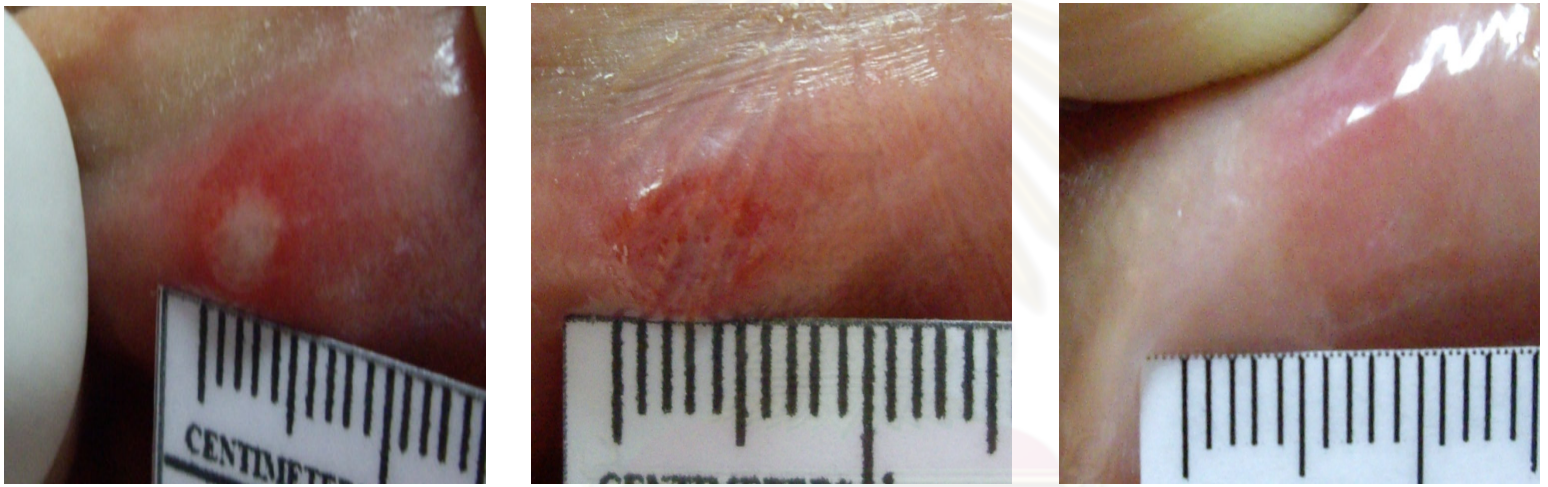
Day 3 (B.)



Day 10 (C.)

Figure 14 The study, subjects with MiRAU and receive topical placebo oral paste in day 0 (A.), day 3 (B.), and day 10 (C.)

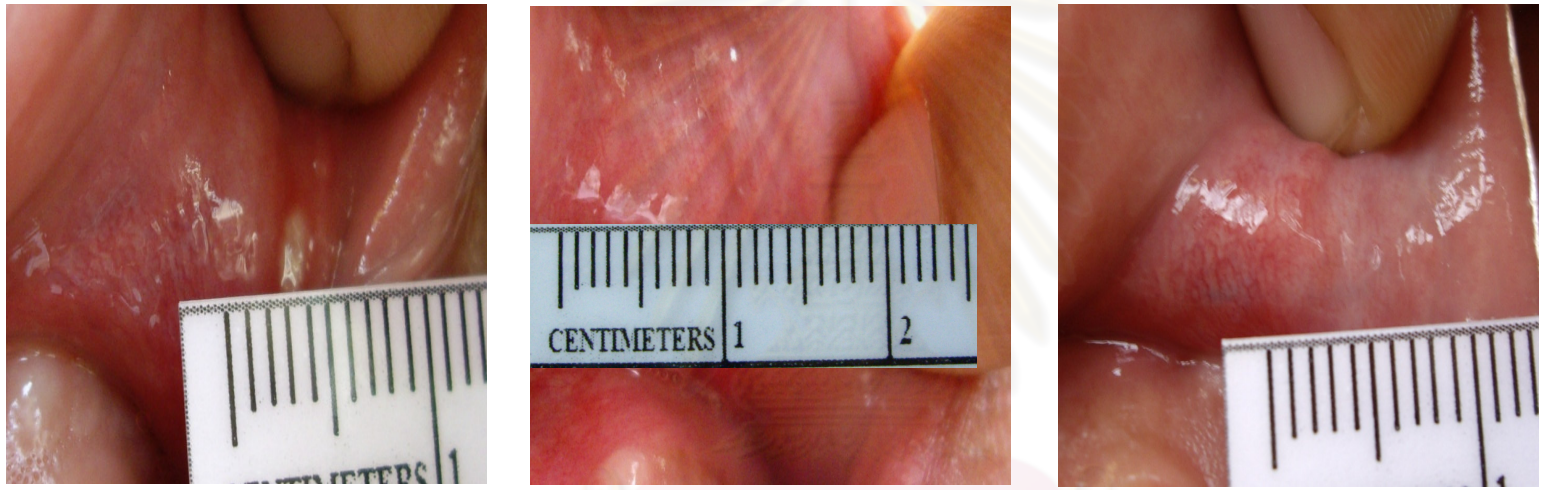
2. The subjects receive topical 0.05% ECa 233 oral paste.



Day 0 (A.) Day 3 (B.) Day 10 (C.)

Figure 15 The pilot study, subjects with MiRAU and receive topical 0.05% ECa 233 oral paste in day 0 (A.), day 3 (B.) and day 10 (C.).

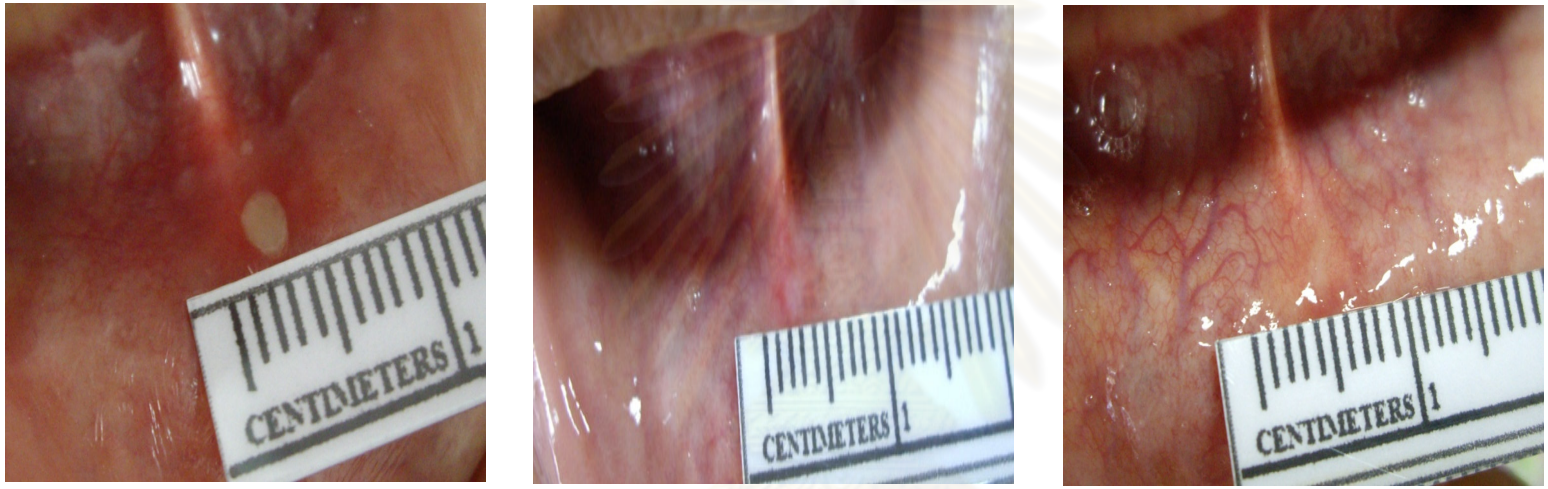
3. The subjects receive topical 0.10% ECa 233 oral paste.



Day 0 (A.) Day 3 (B.) Day 10 (C.)

Figure 16 The pilot study, subjects with MiRAU and receive topical 0.10% ECa 233 oral paste in day 0 (A.), day 3 (B.) and day 10 (C.).

4. The subjects receive topical 0.20% ECa 233 oral paste.

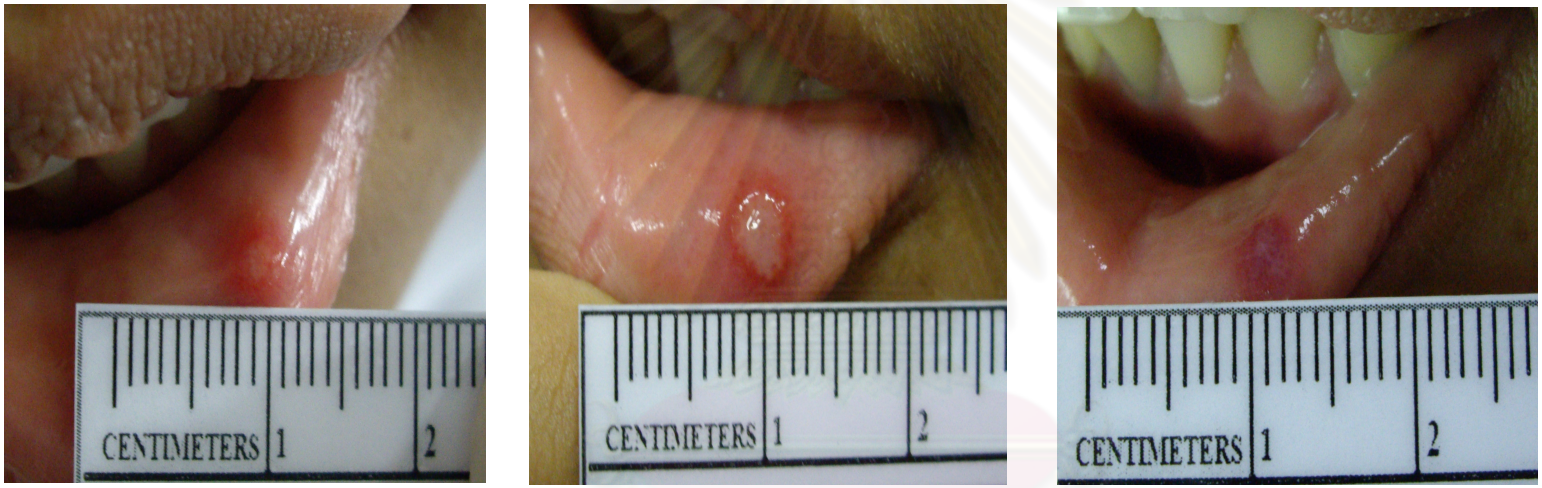


Day 0 (A.) Day 3 (B.) Day 10 (C.)

Figure 17 The pilot study, subjects with MiRAU and receive topical 0.20% ECa 233 oral paste in day 0 (A.), day 3 (B.) and day 10 (C.).

Comparative study

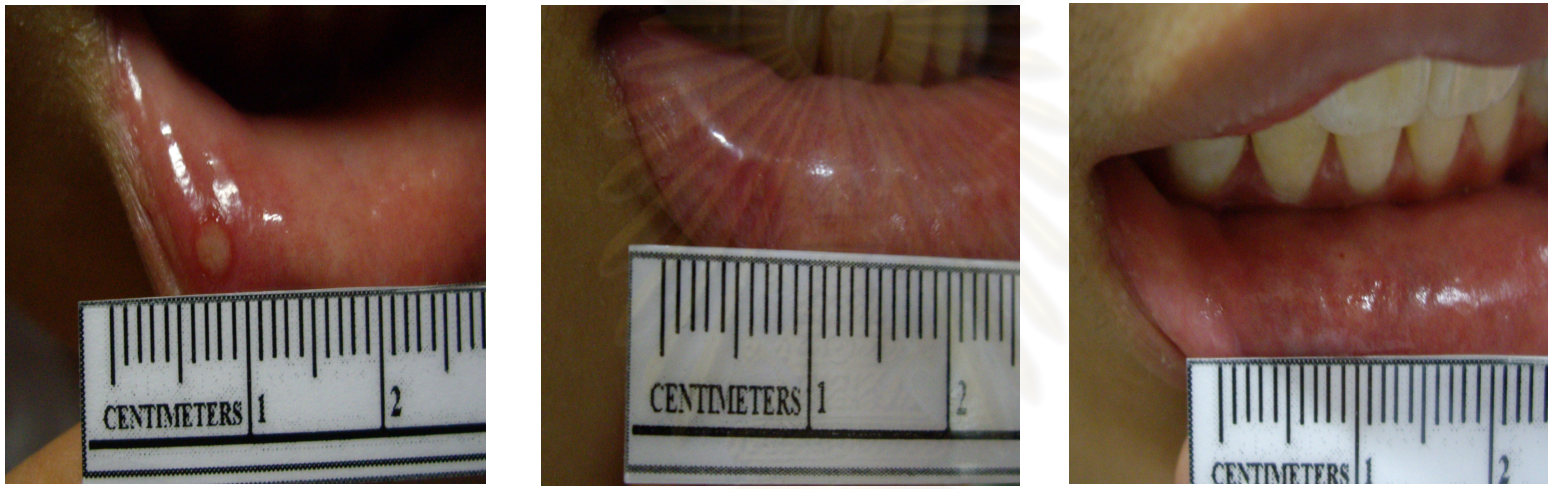
1. The subjects receive topical placebo oral paste.



Day 0 (A.) Day 3 (B.) Day 10 (C.)

Figure 18 The comparative study, subjects with MiRAU and receive topical placebo oral paste in day 0 (A.), day 3 (B.) and day 10 (C.).

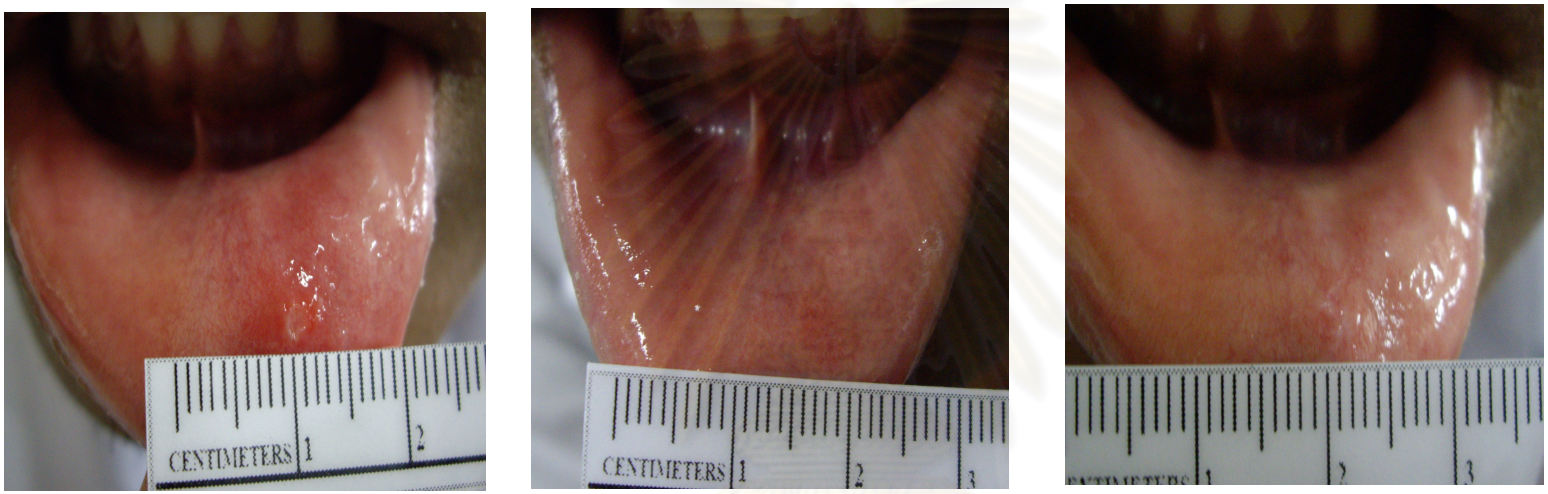
2. The subjects receive topical 0.05% ECa 233 oral paste.



Day 0 (A.) Day 3 (B.) Day 10 (C.)

Figure 19 The comparative study, subjects with MiRAU and receive topical 0.05% ECa 233 oral paste in day 0 (A.), day 3 (B.) and day 10 (C.).

3. The subjects receive topical 0.10% triamcinolone oral paste.



Day 0 (A.) Day 3 (B.) Day 10 (C.)

Figure 20 The comparative study, subjects with MiRAU and receive topical 0.10% triamcinolone oral paste in day 0 (A.), day 3 (B.) and day 10 (C.).

BIOGRAPHY

Name	Miss. Chalandakorn Ruengprasertkit
Birth date	January 17, 1983
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