*Original Article*

# Effect of *Curcuma comosa* on Uterine Smooth Muscle Contraction in Women with Adenomyosis

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***Objective:*** To analyze the relaxant effect of *Curcuma comosa* (Wan chak motluk) and explore its mechanism and its major compound, diarylheptanoid, on human myometrium with adenomyosis.

***Materials and Methods:*** An in vitro experimental study was performed in the Gynecologic Endocrinology Unit, Department of Obstetrics and Gynecology in Ramathibodi Hospital. Subjects were myometrial strips with histologically confirmed adenomyosis and collected from seventeen non-pregnant premenopausal women undergone hysterectomy. They were incubated in an organ bath system. Spontaneous activities and responses of myometrium to *Curcuma comosa* and diarylheptanoid were recorded under isometric conditions.

***Results:*** Log of the concentration required to achieve 50% of the maximal response (log IC50) for *Curcuma comosa* and diarylheptanoid were 1.504±1.797 and 1.487±5.334 µg/ml, respectively. Diarylheptanoid significantly decreased myometrial contraction in adenomyosis in a dose-dependent manner ($p = 0.04$). Pre-incubation with ICI (estrogen receptor antagonist), L-NAME (nitric oxide synthase inhibitor), or ODQ (guanylase cyclase inhibitor) did not exert significant changes in signal comparing to control (without pre-incubation).

***Conclusion:*** Diarylheptanoid provided relaxant effects on human myometrium with adenomyosis. However, these relaxant effects did not contribute through non-genomic estrogen receptor [ER], nitric oxide [NO] dependent guanylase cyclase pathway. Further study focusing on the molecular mechanism behind *Curcuma comosa* and diarylheptanoid is still needed. A major compound extracted from the Wan chak motluk could be an alternative treatment for dysmenorrhea and pelvic pain caused from adenomyosis.

***Keywords:*** *Curcuma comosa*, Diarylheptanoid, Adenomyosis, Myometrium

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Adenomyosis is characterized by the ectopic foci of the endometrial glandular structure in the myometrium leading to the diffusely enlarged uterus[1]. The frequency of adenomyosis at hysterectomy is approximately 20% to 30% and most prevalent in women aged between 35 and 50 years. The common symptoms include menorrhagia (50%), dysmenorrhea (30%), and metrorrhagia (20%). Frequency and severity of symptoms correlate with the extent and depth of the ectopic endometrium in the myometrium[2,3].

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Dysmenorrhea is associated with an elevated basal intrauterine pressure tone and altered amplitude, duration and frequency of uterine contraction[4]. Uterine contractility is regulated by sex steroid hormones and mediated through prostaglandins [PG], oxytocin, vasopressin, and neurotransmitters. Oxytocin receptor is significantly elevated in the myometrium of adenomyosis and its level is associated with severity of dysmenorrhea[5]. PGF2α is produced mainly in endometrium and regulated by oxytocin receptor, estrogen, and progesterone[6]. It stimulates uterine contractions and afferent nerve fibers causing pelvic pain[7].

*Curcuma comosa* Roxb. [*C. comosa*] or Wan chak motluk, a plant in the ginger (Zingiberaceae) family, has been widely used among Thai women[8] as

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a traditional remedy for many gynecologic problems including vaginal dryness, hot flushes[9], dysmenorrhea, and for uterine involution in postpartum period[10]. *C. comosa* exerts estrogenic like activities such as induction cornification of the vaginal epithelium and keratinization of the vagina mucosa. It has been also demonstrated to exert several non-estrogenic like pharmacologic actions in vitro such as inhibition of platelet aggregation, anti-oxidation, anti-inflammation, and anti-tumor promoting effects[7,8].

*C. comosa* is prepared in many forms including dried rhizome powder, crude extracts, and pure extract compound diarylheptanoid. Rhizome powder is prepared by cutting rhizomes of *C. comosa* into small pieces, drying in oven and ground to powder. The powder is extracted with n-hexane in an extractor, and after removal of the solvent, brown viscous oil crude extract is obtained. Diarylheptanoid is the major compound isolated from the crude extract by silica gel column chromatography[11]. A number of diarylheptanoids have been isolated from *C. comosa*. Of these, (3R)-1,7-diphenyl-(4E,6E)-4,6-heptadien-3-ol (hereafter diarylheptanoid) is the major substance[9,12]. The structure of diarylheptanoid is composed of two aromatic rings linked by a linear seven-carbon aliphatic chain[8] (Figure 1).

Many studies in vitro and in vivo demonstrated several similar estrogenic and non-estrogenic properties of *C. comosa* and diarylheptanoids such as improvement of learning and memory function, acceleration of human osteoblast formation and differentiation[8,11,13]; however, both of them did not exert exactly the same activity in every aspects[13-15]. The present study focusing on the relaxant effect of *C. comosa* and diarylheptanoid using rat aortic ring as a model demonstrated the enhancement of their relaxant effects inducing by acetylcholine through estrogen receptor [ER] and endothelium[16].

The aims of the present study were to study the effects of *C. comosa* and diarylheptanoid on human myometrium with adenomyosis. The authors tested their effects using myometrium containing adenomyosis pathology in organ bath chamber filling with physiologic solution. This organ bath recorded spontaneous myometrial contraction of adenomyosis prior to cumulative addition of *C. comosa* and diarylheptanoid. An inhibitor for nitric oxide synthase [NOS] NG-nitro-L-arginine methyl ester [L-NAME], an selective inhibitor for soluble guanylyl cyclase 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one [ODQ], and anti-estrogen fulvestrant (ICI 182,780) were pre-incubated to the tissue before adding of *C. comosa* and diarylheptanoid in order to test whether *C. comosa* and diarylheptanoid exhibit their relaxant effects through NOS, soluble guanylyl cyclase, or ER.

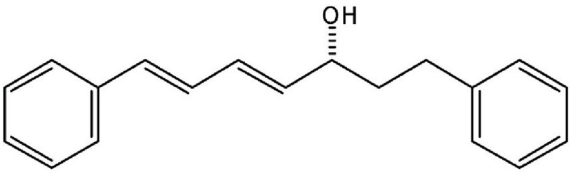


**Figure 1.** Structure of the diarylheptanoid or (3R)-1,7-diphenyl-(4E,6E)-4,6-heptadien-3-ol purified compound isolated from *C. comosa* rhizome[8].

## Materials and Methods

An in vitro experimental study was conducted between June 1, 2013 and May 31, 2015 at the Gynecologic Endocrinology unit, Department of Obstetrics and Gynecology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University. The present study was approved by the ethical clearance committee on human rights related to researches involving human subjects.

### *Study population*

The inclusion criteria were the myometriual tissues collected from pre-menopausal women undergoing abdominal hysterectomy or laparoscopic hysterectomy for dysmenorrhea with or without menorrhagia diagnosed as adenomyosis by transvaginal ultrasonography before surgery and not using pill or oral exogenous hormones for at least three months, or GnRHa or depot medroxyprogesterone acetate injection for at least six months before surgery. The ultrasonographic diagnostic criteria for adenomyosis includes a globular shape uterine enlargement, myometrial anterior-posterior asymmetry, poorly described areas in the myometrium, a heterogeneous myometrial echotexture, subendometrial echogenic linear striations, and intramyometrial cysts[10]. The authors excluded the subject if there was no endometrial glands and stroma histologically presented in the myometrium.

### *Tissue collection and preparation*

All women requiring hysterectomy for dysmenorrhea with diagnosis of adenomyosis were invited to participate in the present study and signed the informed consent before surgery. The myometrial tissues were collected immediately after hysterectomy. The tissue with gross features of adenomyosis included

the prominent trabeculated pattern, hypertrophic swirls of smooth muscle separating duller, and gray foci of endometrium were retrieved[12].

Tissue samples with a diameter of 1x1.5x1 centimeters were excised, immediately placed in physiologic salt solution, and transported on ice to the laboratory. The samples were then dissected and incubated in an organ bath chamber. The nearby second tissue sample was excised, placed in formalin, and later stained with hematoxylin and eosin dye (H&E staining). The myometrial strips were dissected vertically lengthwise with the diameter 1x1x10 millimeters.

### *Experiment*

One end of the myometrial strip was tied to a tissue holder and the other was tied to the transducer under 2 g resting tension. The myometrial strips were incubated in an organ bath chamber contained a Krebs-Henseleit solution pH 7.4, which was composed of (mM) NaCl (119.0), $NaHCO_3$ (25.0), glucose (11.1), $CaCl_2 \cdot 2H_2O$ (1.6), KCl (4.7), $KH_2PO_4$ (1.2), $MgSO_4 \cdot 7H_2O$ (1.2), and aerated with 95% $O_2$, and 5% $CO_2$ at 37°C. All strips were allowed to equilibrate for at least 30 minutes.

The contractile activity was recorded by isometric force transducer connected to bridge amplifier. The signal was then transferred to data acquisition system (AD instruments). After the equilibration period, the spontaneous contractions were recorded for 10 minutes. Cumulative additions of *C. comosa* crude extract and diarylheptanoid (0.001, 0.01, 0.1, 1, 10 ug/ml) were applied at 30-minute intervals. The contractile activity was measured, analyzed, and recorded following each concentration by Chart software version 4.2 (PowerLab, AD instruments).

In order to explore the mechanistic actions of *C. comosa* crude extract and diarylheptanoid, the authors pre-incubated the tissue with the following reagents before adding either *C. comosa* extract or diarhylheptanoid. L-NAME has been widely used to inhibit NOS. The ODQ is a specific inhibitor for soluble guanylyl cyclase. Fulvestrant (ICI 182,780) is an ER antagonist.

### *Chemical agents*

*C. comosa* extract and diarylheptanoid were provided by Professor Pawinee Piyachaturawat, Department of Physiology, Faculty of Science, Mahidol University, Bangkok, Thailand. The other substances were obtained from Sigma Chemicals (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide [DMSO].

### *Statistical analysis*

Contractile activity is calculated from the area under time-force curve by Graphpad Prism 4. The concentration-response curves were analyzed by fitting to the following equation:

$$Y = Y\ min + (Y\ max - Y\ min)/(1 + 10)^{(X\text{-}LogIC50)\ (hill\ slope)}$$

While Y is the observed response; Y max and Y min are the maximal and minimum contractile activity, respectively. Inhibitory concentration [IC] 50 is the drug concentration causing the half maximal inhibitory response.

Contractile activities among the three groups were compared by one-way ANOVA for each concentration. The *p*-value of 0.05 was considered to be statistically significant. The data were analyzed by IBM SPSS Statistics ver.17.0.

## Results

The characteristics of the participants are listed in Table 1. Mean age (SD) was 44.55±4.1 years. Patients with proliferative menstrual cycle were found more frequently than the ones with secretory endometrium. The uteri were removed by abdominal hysterectomy except one that was removed by laparoscopic hysterectomy. The authors recruited 17 patients with adenomyosis and none of them was excluded after H&E staining of tissue for histologic diagnosis (Figure 2). All of the myometrium revealed regularly spontaneous myometrial contraction, therefore, no uterine muscle contractant was added to the specimen (Figure 3).

### *Relaxant effect of diarylheptanoid on myometrial contraction with adenomyosis*

The isometric recording demonstrated a dose dependent relaxant effect of both *C. comosa* and diarylheptanoid on myometrial contraction when compared to control (Figure 4). However, only diarylheptanoid at concentration of 10 ug/ml decreased myometrial contraction statistically significantly ($p = 0.04$). *C. comosa* at the same concentration did

**Table 1.** Participant's characteristics

| Characteristics | n = 17 |
|---|---|
| Age (years), mean ± SD | 44.55±4.10 |
| Pathological phase of menstruation cycle, n (%) | |
| Proliferative | 12 (70.59) |
| Secretory | 5 (29.41) |
| Technique of hysterectomy | |
| Laparotomy | 16 (94.12) |
| Laparoscopy | 1 (5.88) |

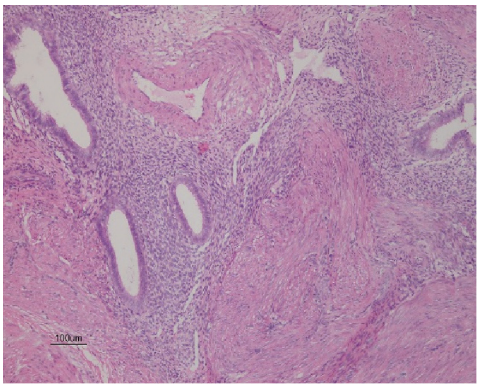

**Figure 2.** Histology confirmation for myometrium with adenomyosis. The myometrial tissue was stained with H&E dye.

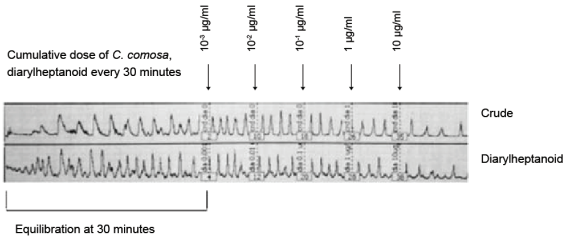


**Figure 3.** Mechanical recordings of myometrial contraction by isometric tension tranducer. Spontaneous myometrial contraction was shown on the left end of the tracing. The tissues were placed in physiologic solution for equilibrium about 30 minutes, and the cumulative dosages of crude extract of *C. comosa* and diarylheptanoid were then added.

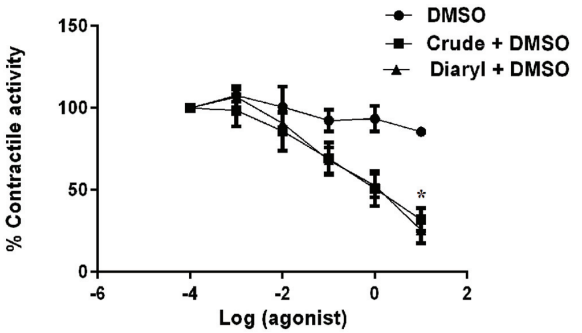


**Figure 4.** Effect of *C. comosa* crude extract and diarylheptanoid on myometrial contraction with adenomyosis compared with control (n = 17). Six doses of *C. comosa* crude extract and diarylheptanoid (0.0001, 0.001, 0.01, 0.1, 1, and 10 ug/ml) were cumulatively added into the organ bath and the contractile activity of myometrial was evaluated. Both *C. comosa* extract and diarylheptanoid decreased spontaneous uterine contraction of human myometrium with adenomyosis but only diarylheptaoid reached statistically significance at concentration of 10 ug/ml ($p$ = 0.04). Data are presented as mean ± SEM of log concentration (DMSO, dimethy sulfoxide; Crude, *C. comosa* crude extract; Diaryl, diarylheptanoid).

not lower myometrial contraction significantly ($p$ = 0.09). Means log IC 50 and SD for relaxant effect on myometrium of *C. comosa* and diarylheptanoid were 1.504±1.797 and 1.487±5.334, respectively.

### *Mechanistic effect of C. comosa extract and diarylheptanoid on myometrial contraction*

In order to study the mechanism of the relaxant effect of *C. comosa* extract and diarylheptanoid, the authors pre-incubated the tissues with L-NAME, ODQ, and fulvestrant (ICI 182,780) before adding *C. comosa* extract and diarylheptanoid. Three of the myometrial strips were pre-incubated with either 1) 100 uM L-NAME, 2) 1uM ODQ, or 3) 1uM ICI for 30 minutes before cumulative adding either *C. comosa* extract or diarylheptanoid. The other two strips were served as a negative control (for either *C. comosa* extract or diaryl-

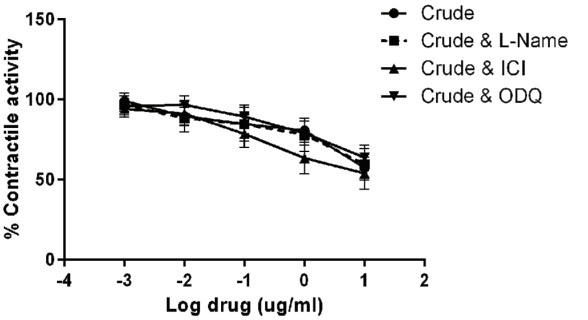


**Figure 5.** The graph compares myometrial contraction from *C. comosa* extract with pre-incubation substances at different concentrations. There were no significant difference in mean percentage contractile activity between *C. comosa* crude extract and the ones with pre-incubation with the agent ($p$>0.05). Data are presented as mean ± SEM.

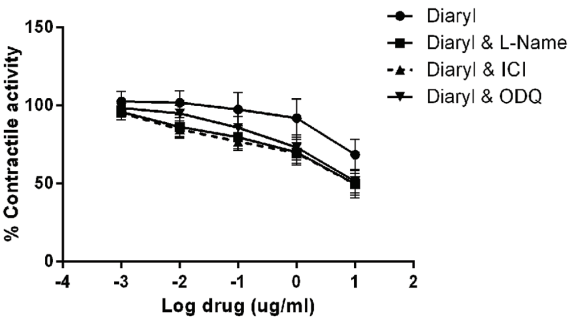


**Figure 6.** The graph compares myometrial contraction from diarylheptanoid with pre-incubation substance at different concentrations. There was no significant difference in mean percentage contractile activity between diarylheptanoid and the ones with pre-incubation with the agent ($p$<0.05). Data are presented as mean ± SEM.

heptanoid without pre-incubation). The contractile activity was measured and analyzed. The result showed no significant change in signal between the pre-incubation group and the negative control (without pre-incubation). The concentration response curve did not statistically change with each pre-incubation substance ($p$>0.05) (Figure 5, 6).

## Discussion

The present study demonstrated that diaryl-heptanoid, a major compound of *C. comosa*, exhibited a significant relaxant effect on human myometrium with adenomyosis in a dose-dependent manner. Their relaxant effects did not mediate through non-genomic estrogen-ER, NOS, and nitric oxide [NO] dependent guanylyl cyclase pathways.

*C. comosa* crude extract is extracted from oven-dry rhizome powder using n-hexane solvent. It is composed of a variety of substances including major compound diarylheptanoid, labdane diterpene, and curcucomosin[15]. A pure compound substance, diarylheptanoid, is purified from *C. comosa* crude extract by column chromatography technique. Therefore, the property and activity of the crude extract and diarylheptanoid may not be the same. However, both of them exhibit similar estrogenic activities, for example, accelerating human osteoblast proliferation and differentiation and preventing bone loss in ovariectomized mice[11,17], lowering cholesterol and triglyceride in mice, and enhance vascular relaxation of aortic ring in rat[16]. From the present study in organ bath chamber, both crude extract and diarylheptanoid contributed the similar relaxant effect on adenomyometrium, however, only the relaxant effect of diarylheptanoid on myometrium reached statistical significance. Therefore, the major component, diarylheptanoid, seem to work on the myometrium with adenomyosis stronger than its crude extract. The relaxant effect of diarylheptanoid on myometrium is in line with the one on blood vessel demonstrated in rat aortic ring performed by Intapad et al[16]. However, the acute exposure of either of *C. comosa* crude extract or diarylheptanoid and the denudation of the endothelium on aortic ring did not induce relaxation. Therefore, longer incubation time of *C. comosa* crude extract and diarylheptanoid on the myometrium and intact-epithelium in myometrial tissue would exert stronger relaxant effect.

Myometrial contractility is controlled by several factors. Surprisingly, from the present study, myometrium with adenomyosis contributed spontaneous contraction, which is different from myometrium without pathology. This basal spontaneous contraction may be the cause of dysmenorrhea and pelvic pain. The mechanism of spontaneous contraction of adenomyosis is still unknown. Further study conducted to explore the mechanism is needed.

Intracellular calcium concentration is one of the important key regulators of myometrial contraction. Calcium entries inside the cell via the calcium ion-channel receptor. The release of intracellular calcium from endoplasmic reticulum enhancing the myometrium contraction. Several uterine contractants such as oxytocin, endothelin and PG bind to GTP-binding protein receptor (G protein) coupled receptor at cellular membrane, stimulate heterotrimeric G protein and then activate phospholipase C [PLC] enzyme. PLC catalyzes the production of inositol 1,4,5-trisphosphate [IP3] from phosphatidylinositide[18]. IP3 induce endoplasmic reticulum to release calcium. Myometrial relaxation is regulated by the increase in cAMP or cGMP. Some uterine relaxants such as beta-agonists and corticotrophin releasing hormone [CRH] bind to G protein coupled receptors, and then release Gαs protein. This protein activates adenylate cyclase enzyme to produce cAMP from ATP (Figure 7). NO activates soluble guanylate cyclase [sGC] to produce cGMP from GMP. cAMP and cGMP activate protein kinase [PK] the enzyme that regulates many proteins involve in calcium homeostasis[19]. In the present work, the authors tested the molecular action of *C. comosa* crude extract and diarylheptanoid through NO pathway

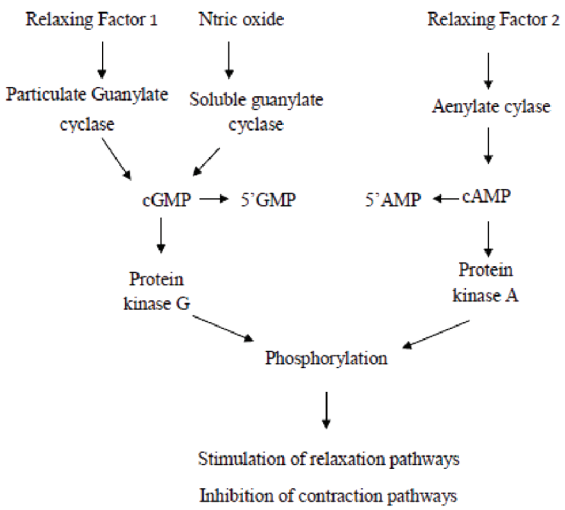


**Figure 7.** The relaxation mechanism of uterine myometrium[19] (GMP, guanosine monophosphate; cGMP, cyclic GMP; AMP, adenosine monophosphate; cAMP, cyclic AMP).

by pre-incubating tissues with NOS inhibitor L-NAME and specific soluble guanylyl cyclase inhibitor ODQ. ODQ is a highly selective and irreversible inhibitor. It binds competitively with NO; therefore, it is specific for NO pathway. The present study showed that *C. comosa* crude extract and diarylheptanoid decreased adenomyometrial contractility, which was in line with a study in rat model. However, the revealed mechanism of relaxant effect from the present study was different from the rat aortic study. The authors found that the relaxant effect of *C. comosa* crude extract and diarylheptanoid did not mediate through NO pathway. Neither did they mediate through estrogen-ER pathway. However, the genomic activity of steroid hormone occurs after at least two hours after E2 stimulation. Therefore, the investigation of mechanistic effect from the present study was done only for non-genomic steroid pathway.

The present study could not demonstrate the mechanistic action of *C. comosa* extract and diarylheptanoid on relaxation of myometrium in contrast to the rat aortic study. They demonstrated the enhancing effect mediated through the ER-Akt-enos pathway. They concluded that the endothelial lining vasculature serves as an important modulator of the underlying smooth muscle tone through the formation and release of several vasoactive mediators including NO[16]. A possible explanation for this different outcome would be because of the differences of the model species, rat versus human.

Several factors regulate the myometrial contractility. Although the present study tested some of the possible mechanisms of relaxant effect for *C. comosa* crude extract and diarylheptanoid, the explanation is still unclear. Further study using other agents to explore the mechanism of relaxant effect of *C. comosa* crude extract and diarylheptanoid is necessary, for example, oxytocin antagonist, PG inhibitor, and calcium channel blocker, to name some.

## Strength

The lack of scientific and clinical data of herb is the major obstacle for clinician for clinical use. The present study is a translational study that provides the scientific basic of the well-known Thai herb, Wan chak motluk, effect on the relaxation of myometrium in adenomyosis. The present study can uncover clinically relevant phenomenon and then would lead to the clinical utility of the herb in clinic.

Moreover, the present study is the pioneer study that clarify the beneficial effect of Wan chak motluk in terms of relaxing the myometrial contraction. This data would enhance the clinical use of this herb for treatment of the dysmenorrheal symptom.

## Limitation

The mechanistic action of diarylheptanoid for relaxant of myometrial contraction could not be demonstrated. Many questions persist and need to be answered. The clinical response of the herb regarding dysmenorrheal and pelvic pain in adenomyosis should be observed by clinical trial.

## Conclusion

Diarylheptanoid, a major compound of *C. comosa*, provide a relaxant effect on human myometrium with adenomyosis. However, these relaxant effects did not contribute through non-genomic ER, NO dependent guanylase cyclase pathway. The finding from the present study would be beneficial for women with dysmenorrhea, specifically dysmenorrhea caused by adenomyosis. Wan chak motluk would be alternative of treatment for patients with dysmenorrhea. More study focusing on the molecular mechanism behind *C. comosa* and diarylheptanoid is needed.

## What is already known on this topic?

Wan chak motluk is very well-known by Thai women to cure many symptoms such as leucorrhea, dysmenorrheal, pelvic pain, and even uterine involution. However, no study was ever performed to test the effect of the herbs.

*C. comosa* crude extract and its major compound diarylheptanoid exerted the relaxant effect on endothelium-intact aortic ring in rat model. The mechanism of action mediated through many pathways such as NOS and partially ER-estrogen activity.

## What this study adds?

The present study confirm the role of Wan chak motluk for one of the common gynecologic symptom, dysmenorrhea. Diarylheptanoid from Wan chak motluk improve the myometrial contraction of adenomyosis. This may improve the symptoms in the patients with adenomyosis.

## Acknowledgment

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## Potential conflicts of interest

The authors declare no conflict of interest.

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