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Mechanism Identification of *Ficus Deltoidea* Aqueous Extract in Rat Uterine Contractions

(Pengenalpastian Mekanisme Ekstrak Akues *Ficus Deltoidea* dalam Kontraksi Uterus)

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ABSTRACT

Ficus deltoidea or 'mas cotek,' is a uterotonic herb traditionally consumed by women to improve menstrual circulation, assist labour, remove retained placenta and treat postpartum bleeding. The aim of the study was to elucidate the mechanism of F. deltoidea in uterine contraction. Crude extracts from 2 different variants of F. deltoidea were used in the study; F. deltoidea var. Deltoidea (FDD) and F. deltoidea var. Angustifolia (FDA). This study was conducted ex vivo on the strips of isolated rats uterus treated with either FDD or FDA aqueous extract with increasing concentrations ranging from 10 µg/ml until 1280 µg/ml at time intervals of 5 minutes between doses. The frequency and intensity of the uterine contractions were monitored via Powerlab software. Maximum contractions for both extracts were identified, recorded and the uterine strips samples at maximum contraction were selected and homogenized in order determine the role of prostaglandin F2α (PGF2α) in the mechanism of uterine contraction. Other than that, phosphorylated 42/44 (p42/44) of mitogen activated protein kinase (MAPK) expression was also detected via immunoblotting. The results showed that the maximum contraction induced by FDD was at the concentration of 320 µg/ml, whereas for FDA was at 960 µg/ml. Both FDD and FDA increased the intensity of uterine strips contractions and there were notable trend of increased PGF2α expression as well. Further analysis revealed that the uterine contractions involved the MAPK pathway through the phosphorylation of p42/44 protein. In conclusion, Ficus deltoidea of both variants have the ability to stimulate uterine contraction through the mechanism of MAPK pathway.

Keywords: Ficus deltoidea; uterine contraction; Mitogen Activated Protein Kinase (MAPKs); prostaglandin

ABSTRAK

Ficus deltoidea atau 'mas cotek,' adalah herba uterotonik yang digunakan secara tradisional oleh wanita untuk melancarkan peredaran haid, memudahkan proses bersalin, mengeluarkan plasenta tersekat dan merawat pendarahan selepas bersalin. Kajian ini dijalankan untuk menentukan mekanisme F. deltoidea dalam kontraksi otot uterus. Ekstrak kasar daripada 2 varian F. deltoidea digunakan dalam kajian ini; F. deltoidea var. Deltoidea (FDD) dan F. deltoidea var. Angustifolia (FDA). Kajian ini dilakukan secara ex vivo pada sampel uterus yang dikeluarkan daripada tikus, dipotong membentuk jaluran dan dirawat dengan ekstrak akues FDD atau FDA dengan peningkatan kepekatan dari 10 µg/ml hingga 1280 µg/ml dalam selang masa 5 minit antara dos. Kekerapan dan intensiti kontraksi tisu uterus dipantau melalui perisian Powerlab. Kontraksi maksimum bagi kedua-dua ekstrak telah dikenalpasti, direkodkan dan jaluran tisu uterus pada kontraksi maksimum tersebut dipilih dan dihomogenisasikan untuk menentukan peranan prostaglandin F2α (PGF2α) dalam mekanisme kontraksi uterus. Selain itu, pengekspresan protein terfosforilasi p42/44 daripada kumpulan mitogen activated protein kinase (MAPK) juga dikesan melalui kaedah pemblotan imuno. Hasil penyelidikan menunjukkan bahawa kontraksi maksimum yang dihasilkan oleh FDD adalah pada kepekatan 320 µg/ml, manakala untuk FDA pula adalah pada 960 µg/ml. Kedua-dua FDD dan FDA meningkatkan intensiti jaluran tisu uterus dan aktiviti kontraksi ini adalah selari dengan peningkatan PGF2α. Analisis selanjutnya menunjukkan bahawa kontraksi uterus melibatkan tapakjalan MAPK melalui fosforilasi protein 42/44. Sebagai kesimpulan, Ficus deltoidea kedua-dua varian berkeupayaan untuk merangsang kontraksi uterus melalui mekanisme tapak jalan MAPK.

Kata kunci: Ficus deltoidea; kontraksi otot uterus; Kumpulan Mitogen Activated Protein Kinase (MAPK); prostaglandin

INTRODUCTION

Labor is the physiological process by which a fetus is expelled from the uterus to the outside world and is defined as regular uterine contractions accompanied by cervical effacement and dilatation (Norwitz et al. 1999). Basically, the process involves two phases: birth preparation phase and the active birth phase (Garfield & Yallampalli 1993; Chwalisz & Garfield 1994, 1997). Maul et al. (2003) stated that active phase involves continued and coordinated uterine contractions.

To expedite the process of a smooth labor requires an increase in the coordination of uterine contractions along the connective tissue changes in the cervix that allows dilatation to occurs The changes are in line with simultaneous decline in progesterone level and increase in estrogen level (Bernal et al. 2003). During pregnancy, progesterone maintains the structure of the uterus by blocking the production of prostaglandins and inhibits gene expression of proteins associated with contraction in the myometrium (Challis et al. 2000; Norwitz et al. 2001). Meanwhile, the birth process involves a variety of hormones such as oxytocin and prostaglandins. In the early labor stage, oxytocin from the placenta will act directly on the myometrium to produce contractions and at the same time indirectly increases the production of prostaglandin, particularly prostaglandin F2α (Wilson et al. 1988). Increased prostaglandin synthesis is important for the progression of contractions during labor.

Women who experienced problems in uterine contraction may face difficulties during childbirth. To overcome this problem, uterotonic agents are often used to clinically facilitate delivery. Misoprostol or Cytotec, an analogue of prostaglandin E1, is an example of uterotonic drug. However, the usage often causes side effects to the mother such as hyperpyrexia (Dyer et al. 2010). Alternatively, Pitocin that resembles oxytocin activity is also used to induce uterine contractions during childbirth. The use of such synthetic drugs during labour can also provide adverse effects that are not favourable to women's health as well as to the infant. For instance, there was a higher relative risk of being diagnosed with depressive or anxiety disorder within the first year of postpartum and also incidents of jaundice in neonates (Kroll-Desrosiers et al. 2017; Garosi et al. 2016).

Taken together, the use of complementary and alternative medicine has become a favourable choice among women worldwide. Studies have shown that between 30 to 50% of adults in industrialized countries use some form of alternative medicine to prevent or treat health problems (Astin 1998). According to Eisenberg et al. (1998), 49% of women opted for alternative therapy and herbal therapy is a popular choice (Gibson 2001). There are many herbs that can be used to stimulate uterine contractions during labour. One of them is *Ficus deltoidea* and is also locally known as Mas Cotek. *Ficus deltoidea* has been scientifically proven to stimulate contractions of the uterus and is traditionally used by women to contract the uterus after delivery (Sulaiman et al. 2008). Two variants, *F. deltoidea* var. Deltoidea and *F. deltoidea* var. Angustifolia have been shown to stimulate uterine contractions (Umi Romaizatul Amiera et al. 2014). The mechanism of action of FDD and FDA in stimulating uterine contractions is yet to be investigated. Therefore, the aim of this study was to elucidate the uterotonic mechanism of FDD and FDA.

MATERIALS AND METHODS

PLANT MATERIAL

Ficus deltoidea leaves were obtained from Juaseh Tengah in Negeri Sembilan, Malaysia. The plant samples were taxonomically identified, authenticated and deposited at the Herbarium, Faculty of Science and Technology, Universiti Kebangsaan Malaysia with voucher number UKMB 29780 for *Ficus deltoidea* var. Angustifolia (FDA) and UKMB 29781 for *Ficus deltoidea* var. Deltoidea (FDD).

PREPARATION OF THE EXTRACT

After the leaves were cut into smaller pieces, the leaves were extracted with distilled water for 16 hours by using Soxhlet apparatus (Fisher Scientific, UK). The aqueous extract was filtered and freeze-dried (Labconco Corporation, USA) until it became lyophilized. The lyophilized powder was kept in an air-tight container and kept in the refrigerator at 4°C until needed.

ANIMAL

Non-pregnant Sprague Dawley rats (weight 200-250 g) were purchased from Laboratory Animal Research Unit, Faculty of Medicine, Universiti Kebangsaan Malaysia (UKM). The rats were supplied with standard laboratory pellet diet and water *ad libitum*. All animal procedures were approved by Universiti Kebangsaan Malaysia *Animal Ethics Committee* (UKMAEC No: FSK/BIOMED2011/NIHAYAH/403-NOV/403-NOV-2011-JUN2014.

UTERINE TISSUE EXPERIMENT

The protocols were obtained from Umi Romaizatul Amiera et al. (2014). In short, the non-pregnant female rats were injected with 0.2 mg/kg diethylstilbestrol (Sigma Aldrich, USA) 24 hours prior to experiment. This was done to induce the animals to be in estrus phase. Vaginal smear of the rats were done to confirm the stage of the estrous cycle. Once the rats were in estrus phase, the rats were killed and the uterine horns were taken out. The uterine horn was cleaned and cut into 2 cm strips. A strain gauge force transducer was sutured to the serosa of the uterine strip and mounted in 60 mL organ bath containing Tyrode solution aerated with 95% O_2, 5% CO_2 and temperature maintained at 37°C. The organ bath was then connected to a Powerlab system (ADInstruments, Australia). The transducer was previously calibrated to establish the association between the force applied to the transducer and gauge deflection with one gram corresponding weight. The uterine strip was allowed to stabilize for 30 min in the organ bath before the application of extracts.

EFFECT OF *FICUS DELTOIDEA* LEAVES AQUEOUS EXTRACT ON UTERINE CONTRACTION

This study was conducted ex vivo on uterine strip of rats treated with extracts of FDD and FDA at different concentrations ranging from 10 µg/ml to 1280 µg/ml at time interval of 5 minutes between doses. The frequency and intensity of uterine contractions were detected using Powerlab's Chart 5 software. Maximum contractions for both extracts were recorded.

PROSTAGLANDIN F2α LEVEL

Uterine samples collected at E_{max} were homogenized and processed accordingly. To determine the level of PGF2α in the samples, PGF2α high sensitivity ELISA kit from Enzo Life Sciences, Inc. (USA) was used. The procedures were performed according to the manufacturer's instructions. Absorbance was measured at λ 415 nm.

WESTERN BLOTTING

The protein concentration was determined via Bradford assay. Homogenate protein was subjected to SDS-PAGE and the gel was transferred to polyvinylidenedifluoride (PVDF) membrane. Blots were probed with phosphorylated p42/44 (p42/44) MAPK primary antibody conjugated with horse-radish peroxidase (HRP)(Cell Signalling Technology, USA). The transferred bands were visualized by enhanced chemiluminescence.

STATISTICAL ANALYSIS

Results were analyzed using Software Statistical Package for the Social Sciences (SPSS) version 20.0. Statistical significance was achieved when $p < 0.05$. Normality was checked, test Analysis of Variance (ANOVA) was used followed by post-hoc Dunnett test to compare the statistical difference between the groups. Data are presented as mean ± SEM.

RESULTS

EFFECT OF *FICUS DELTOIDEA* VAR. DELTOIDEA (FDD) AQUEOUS EXTRACT ON THE INTENSITY OF UTERINE CONTRACTIONS

The intensity of basal uterine contractions before the application of FDD extract was 0.135 ± 0.028 g. The intensity of the contractions, however, showed no significant increase ($p > 0.05$) following administration of the extract. Maximum uterine contractions or E_{max} (0.221 ± 0.039 g) was achieved at the concentration of 320 µg/ml (Figure 1).

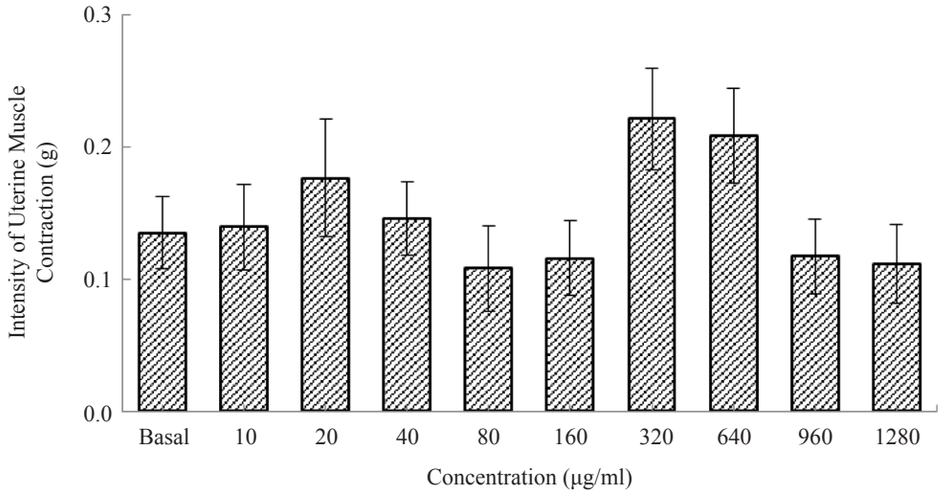

FIGURE 1. Intensity of the uterine contractions before (basal) and after treated with increasing concentration of *Ficus deltoidea* var. Deltoidea aqueous extract. The maximum uterine contraction or E_{max} for FDD was achieved at 320 µg/ml. Data was extracted from n = 6 strips and expressed as mean ± SEM

EFFECT OF *FICUS DELTOIDEA* VAR. ANGUSTIFOLIA (FDA) AQUEOUS EXTRACT ON THE INTENSITY OF UTERINE CONTRACTIONS

The intensity of basal uterine contractions before starting the treatment was 0.354 ± 0.092 g. The intensity of the contractions increased moderately following the administration of the extract in a dose-dependent manner. Maximum uterine contractions or E_{max} (0.613 ± 0.136 g) was achieved at the concentration of 960 µg/ml (Figure 2).

EFFECT OF *FICUS DELTOIDEA* AQUEOUS EXTRACT ON PROSTAGLANDIN F2α LEVEL IN MYOMETRIUM OF UTERUS

Based on the findings, there was no significant increase in PGF2α levels compared with basal contractions ($p > 0.05$)

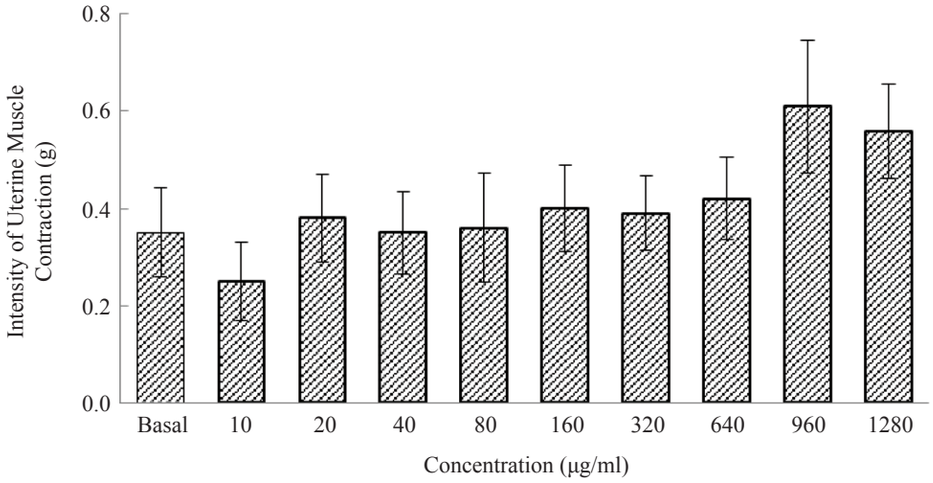

FIGURE 2. Intensity of the uterine contractions at basal and after treated with increasing concentration of *Ficus deltoidea* var. Angustifolia (FDA) aqueous extract at 5 minutes interval. Note that E_{max} for FDA was cumulatively achieved at 960 µg/ml. Data was extracted from n = 6 strips and expressed as mean ± SEM

(Figure 3). The level of PGF2α during basal contraction was 8.12 ± 1.32 pg/ml, FDD was 9.30 ± 0.23 pg/ml and FDA was 9.45 ± 0.20 pg/ml.

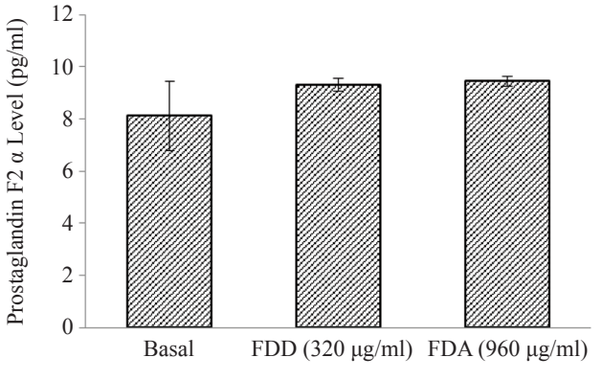

FIGURE 3. Level of PGF2α basal contractions (basal), at maximum uterine contractions (E_{max}) induced by FDD at 320 ug/ml) and FDA at 960 ug/ml. Data was extracted from n = 3 strips and expressed as mean ± SEM

EFFECT OF *FICUS DELTOIDEA* AQUEOUS EXTRACTS ON THE EXPRESSION OF PHOSPHORYLATED P42/44 (P42/44)

There was a notable increase in the density of phosphorylated p42/44 protein bands produced in FDD- and FDA-induced myometrium contractions compared to the basal contraction (Figure 4A). Further analysis revealed that, upon normalization to the β-actin expression, FDD extract caused a 2.4-fold increase in the relative intensity of phosphorylated p42/44 protein expression compared to the basal contractions ($p > 0.05$) (Figure 4B). However, FDA extract caused a significant 3.8-fold increase in the relative expression of phosphorylated p42/44 compared to the expression of the same protein at basal contractions ($p < 0.05$).

DISCUSSION

The study was carried out ex vivo, to assess concentration-response relationship of *Ficus deltoidea* var. Deltoidea (FDD) and *Ficus deltoidea* var. Angustifolia (FDA) aqueous extracts in contractile tissues of isolated rats' uterus. Based on the results obtained, the increased in the intensities of the uterine contraction were not significant following FDD and FDA extracts administrations ($p > 0.05$). However, there were trends of dose-dependent increase in the contraction intensities observed for FDA extract but not for FDD. Despite that, maximum contraction or E_{max} was achieved at 320 µg/ml for FDD, and 960 µg/ml for FDA. Umi Romaizatul Amiera et al. (2014) reported E_{max} concentrations of 640 µg/ml for FDD and 20 µg/ml for FDA, whereas Naguib & Vivi Noryati (2013) reported 2 mg/ml as the E_{max} concentration for FDA. The discrepancies might be due to the difference in the preparation of the extract itself as the methodology employed was not similar, thus, affecting the type, amount or concentration of constituents present in the extract. Despite the discrepancies in the concentrations that caused maximum contraction for the range of concentrations used, both FDD and FDD in the current study did show uterotonic activities similar to other reported *Ficus sp.* (Bafor et al. 2009; Watcho et al. 2011; Bafor et al. 2010; Naguib & Vivi Noryati 2013, Umi Romaizatul Amiera et al. 2014). There are several basis for the uterine-stimulating activities shown by both FDD and FDA. Phytochemical content presence in the plants contributes greatly to the reported biological functions. For instance, flavonoids and tannin compounds, as in the aqueous extracts of FDD and FDA, have been reported to contribute to the uterotonic effects (Calixto et al. 1986; Umi Romaizatul Amiera et al. 2014). Flavonoids are also a type of phytoestrogen which are similar to estradiol or synthetic estrogens (Kurzer & Xu 1997; Moon et al. 2006). Phytoestrogens can activate estrogen

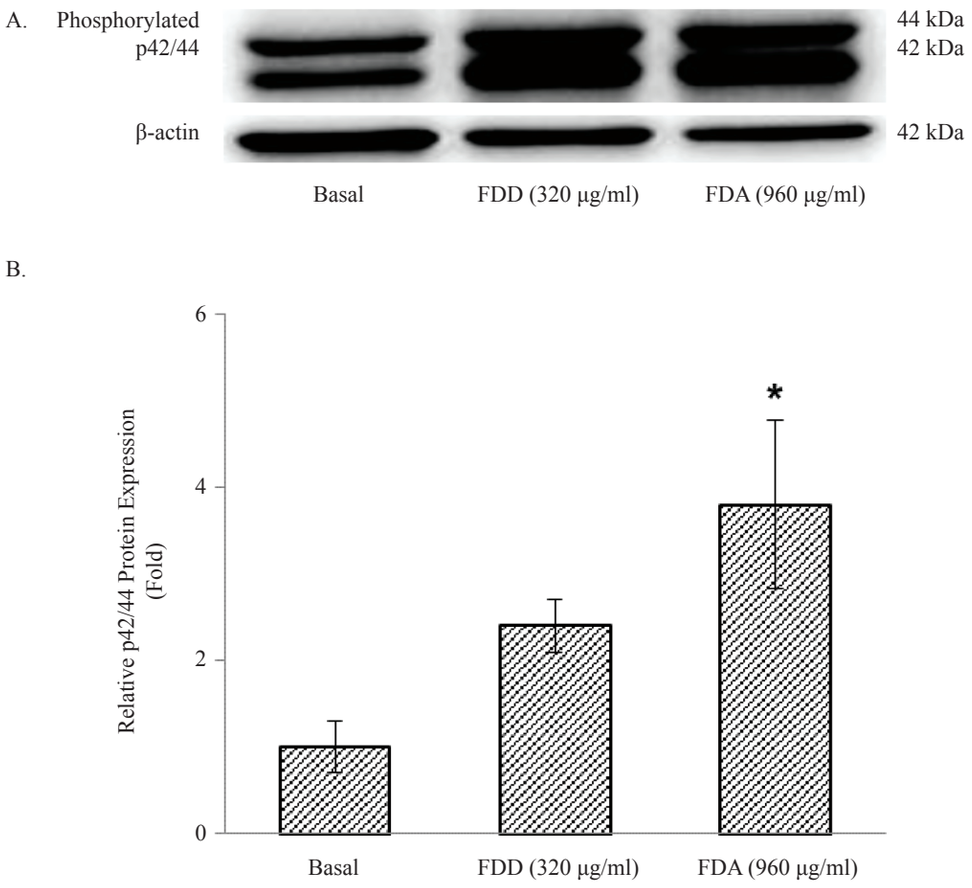

FIGURE 4. The intensity of phosphorylated p42/44 protein bands in the uterine strips at 3 different statuses; Basal, FDD (320 µg/ml), FDA (960 µg/ml). Note the increase in the band intensities of p42/44 at the maximum uterine contractions when treated with FDD and FDA extracts compared to the p42/44 expression at basal contractions (Figure 4A). At maximum contraction, administration of FDA extract caused a significant 3.8-fold increase in the expression of p42/44 protein expression compared to the basal contractions (Figure 4B). Data was extracted from n = 3 strips and expressed as mean ± SEM

receptors presence in the nucleus to produce estrogenic activities including uterine contractions (Anderson et al. 1999). Based on previous studies, *Radix trichosanthis* was found to amplify spontaneous uterine contractions *in vivo* and increased uterine muscle response to PGF2α and oxytocin in pseudopregnant rabbits (Chen & Chu 1993). It is believed that all phytoestrogens are able to influence the frequency and intensity of myometrial contractions, and depending on the concentrations may sometimes cause weak contractions or show weak estrogenic activity (Setchell & Cassidy, 1999; Picherit et al. 2000).

In the current study, the level of prostaglandin F2-alpha (PGF2α) was investigated to determine the involvement of PGF2α in uterine muscle contractions induced by FDD and FDA extracts. PGF2α is the second most potent uterotonin after oxytocin (Naguib & Vivi Noryati 2013) The current results showed that PGF2α was also expressed in non-induced uterine tissues, suggesting its function in regulating spontaneous basal contractions and other endometrial functions (Kupittayanant et al. 2014; Blesson & Sahlin 2014). Though not significant, slight increase in the level of PGF2α upon maximum contractions (E_{max}) induced by FDD and FDD extracts suggest the involvement of prostaglandin in uterine muscle contraction. Naguib & Vivi Noryati (2013) reported that FDA-induced contraction was mediated via uterotonic receptors that include muscarinic, oxytocin and PGF2α receptors. In the current study, the level quantitated was PGF2α protein and not the receptors. Therefore it is possible that uterine muscle contractions induced by FDD and FDA stimulate endogenous myometrial synthesis of PGF2α that later bound to PGF2α receptors to activate downstream signalling (Naguib & Vivi Noryati 2013). Oxytocin is also able to stimulate endometrial secretion of endogenous PGF2α to effectively increase the uterine contractions by promoting a more forceful contraction (Carnahan et al. 1996; Dittrich et al. 2009, Arrowsmith et al. 2010). Other than that, estrogen also has been reported to increase PGF2α production that in turn has direct stimulation on uterine contractions (Egarter & Husslein 1982). In the estrus phase, estrogen level is high. Thus, the capacity to synthesize and secrete uterine PGF2α is increased during this period due to increased level of estrogen and decrease level of progesterone. Estrogen regulates expression of endometrial prostaglandin G/H

synthase 1 (PGHS-11), also known as cyclooxygenase-1 (COX-1), thereby stimulating the synthesis of PGF2α by the uterus (Barcikowski et al. 1974).

PGF2α activates MAPKs through stimulation of G proteins, either $G\alpha_{q/11}$ or $G\alpha_{i}$. According to Harbon et al. (1984), PGF2α binds to prostaglandin receptor (FP) through stimulation of $G\alpha_{q/11}$ which then produces rapid intracellular signal transduction and stimulates phospholipase C (PLC). PLC catalyzes the hydrolysis of phosphatidylinositol biphosphate (PIP_2). Subsequently PIP_2 produces inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DG). Next, DG activates protein kinase C (PKC) and undergoes several steps to activate Mitogen Activated Protein Kinases (MAPKs) through phosphorylation of p42/44 protein. Ohmichi et al. (1997) stated that PGF2α has been reported to activate MAPK in rat myometrium cell cultures. Based on the results of the current study, there was an increase in the density of phosphorylated p42/44 protein bands produced in the uterus treated with FDD and FDA compared to the basal contractions. Phytochemical screening of the leaves for both plants revealed the presence of terpenoids in FDA aqueous extract (Umi Romaizatul Amiera et al. 2014). This compound also has been implicated as chemical stimuli that can trigger smooth muscle contraction (Rezaeizadeh et al. 2016; Sommer et al. 2017). However, the exact role of terpenoids and its association with increased expression level of phosphorylated p42/44 MAPK protein is not known. Studies by Watts (1996) stated that p42/44 play a major role in regulating smooth muscle contraction in rats. Previous studies have suggested that p42/44 stimulate smooth muscle contraction through a thin filament regulatory pathway by phosphorylation of caldesmon (CAD), regulatory proteins that play a role in smooth muscle contraction (Adam et al. 1995; Horowitz et al. 1996; Morgan & Gangopadhyay 2001).

The results of the current study showed that FDD and FDA aqueous extracts both increased uterine contractions of rats *ex vivo*. Both FDD and FDA caused increased level of PGF2α during uterine contractions. Both *Ficus deltoidea* aqueous extract increased the contractions of the uterus through MAPK pathway with increased phosphorylated p42/44 expression compared to normal contractions.

CONCLUSION

Ficus deltoidea-induced uterotonic effect is related to the release of prostaglandin and contraction of the myometrial cells through phosphorylated p42/44 MAP kinase activation. These findings provide scientific basis to the ethnic use of *F. deltoidea* for its uterotonic properties.

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