

Tocolytic Effect of White Pepper (*Piper nigrum* Linn.) on Isolated Rat Uterine Contraction *in vitro*

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Abstract

Piper nigrum Linn. (Pepper) is a very well-known spice used for different purposes such as human dietaries, medicine and preservative. In Thai traditional medicine, white pepper is a component of herbal formulas used to treat many disorders including symptoms of dysmenorrhea. It is of interest to investigate the effect of methanolic extract of white pepper (MEWP) on the isolated rat uterus contractions induced by various uterine stimulants and the possible mechanism(s) involved. Strips of uterus were prepared from estrogen-primed female Wistar rats, fixed in the organ bath filled with an appropriate physiological solution with carbogen bubbled continuously at 37 °C. The measurement of isometric tension was then performed using force transducer attached to a polygraph. MEWP was tested its effect on the uterine contraction and found the inhibitory effects on the contraction induced by oxytocin, PGF_{2α} and depolarizing solution. TEA inhibited the uterine relaxing effect of MEWP while glibenclamide had no effects when the uterus was induced to contract with oxytocin. Propranolol abolished the uterine relaxing effect of isoproterenol while it had no effects on the relaxing effect of MEWP. The results suggested that MEWP is likely to be a general uterine relaxant. The effects may be due to the interference on Ca²⁺ influx into the uterine muscle cells which may involve the opening of K_{Ca} channel. The effect was unlikely to occur via the K_{ATP}-channel and may not involve the interaction of MEWP on β-adrenergic receptor on the uterine muscle cells.

Keywords: *Piper nigrum*, Oxytocin, PGF_{2α}, Depolarizing solution, Uterine relaxation, Tetraethylammonium, Glibenclamide

Introduction

Piper nigrum Linn. (Pepper) is a very well-known spice of the Piperaceae family. It contains a pungent alkaloid piperine, found in the roots and fruits of *Piper nigrum* both black and white pepper grains [1]. Previous study has reported major components of white pepper essential oils which are limonene, β-caryophyllene, δ-3-carene, α-pinene and β-pinene [2]. *Piper nigrum* can be used for different purposes such as a spice in culinary recipes, medicine and preservative [3]. In Thai traditional medicine, white pepper is a component of herbal formulas used to treat symptoms of dysmenorrhea. The pepper is mostly in the form of ground white pepper. Although, it has been used for a number of years, the precise mechanism of action of white pepper for the treatment has not been reported.

Piper nigrum Linn. was reported to have anti-inflammatory activity probably by an inhibition on the expression of COX-2 protein and COX-2 mRNA [4], leading to an inhibition of prostaglandins (PGs) synthesis. Over production of PGs in the uterus during menstruation is believed to be the main cause of dysmenorrhea. Our preliminary study has shown that methanolic extract of white pepper had an ability to inhibit calcium chloride-induced contraction in isolated rat uterus using calcium-free high potassium chloride solution [5]. Thus, it is of interest to study further on the effect of white pepper on the uterus. The aim of the present study was to investigate the effect of methanolic extract of white pepper on the isolated rat uterus contractions induced by uterine stimulants and the possible mechanism(s) involved.

Materials and methods

Plant material preparations

White pepper (dry seed of ripe pepper fruits without husks) was obtained from local herbal drugstore in Songkhla Province, Thailand. The peppercorns were soaked in distilled water and only peppercorns with good quality were selected, air-dried in well ventilated area at room temperature and further dried until

dryness in hot air oven at 50 °C. The dried peppercorns were ground, and 1 kg of ground pepper was then used for extraction.

Drugs and chemicals

Dimethyl sulfoxide (DMSO), estradiol benzoate, oxytocin, tetraethylammonium (TEA), (\pm)-isoproterenol hydrochloride and propranolol were obtained from Sigma-Aldrich chemical company (St. Louis, U.S.A). Dinoprost tromethamine (prostaglandin $F_{2\alpha}$) was purchased from Zoetis. Calcium chloride (CaCl_2) 10^{-2} M, isoproterenol 10^{-6} M, oxytocin 1 mU/mL, $\text{PGF}_{2\alpha}$ 10^{-6} M, propranolol 10^{-6} M, and TEA 10^{-3} M were prepared as stock solutions in 0.1 % ascorbic acid in distilled water. Estradiol benzoate (1 mg/mL) was prepared by dissolving in olive oil for injection intraperitoneally. Methanolic extract of white pepper (MEWP) was dissolved in a small amount of DMSO and the solution was adjusted with an appropriate amount of incubation medium to make a stock solution of 20 mg/mL for use in all experiments. The total amount of DMSO in the organ bath in all experiments was less than 0.5 % and no interference on the response of the uterus was observed. Stock solutions were kept as aliquots at -20 °C until use. MEWP stock solution was diluted with appropriate physiological solution which was freshly prepared for use only on the day of experiment. Sodium bicarbonate (NaHCO_3) and Sodium chloride (NaCl) were obtained from Merck&Co. Inc. Potassium chloride (KCl), calcium chloride (CaCl_2), magnesium chloride (MgCl_2) and glucose were purchased from Carlo Erba. The physiological solutions used throughout this study were prepared using these common salts and were prepared freshly for use on each day of experiment. The composition of physiological solutions was as follows: 1) De Jalon solution (in mM) 154.0 NaCl, 5.6 KCl, 0.3 CaCl_2 , 1.7 NaHCO_3 , 1.4 MgCl_2 and 5.55 glucose. 2) Locke-Ringer solution (in mM) 154.0 NaCl, 5.63 KCl, 2.16 CaCl_2 , 5.95 NaHCO_3 , 2.10 MgCl_2 and 5.55 Glucose and 3) Depolarizing solution (in mM) 103.3 NaCl, 56.3 KCl, 0.648 CaCl_2 , 5.95 NaHCO_3 and 2.77 Glucose.

Extraction procedures

The extraction of ground white pepper (1 kg) was performed by macerated in 2 L of absolute methanol at room temperature for 3 days and the maceration was repeated for 5 times with fresh methanol. All methanolic extracts collected from each extraction were then combined and evaporated until dryness under reduced pressure at 50 °C using Rotary Evaporator (Buchi®). Methanolic extract (6.42 %) were obtained and stored in an air tight container at 4 °C until use.

Experimental animals

Female Wistar rats age 2 - 3 months (weighing between 200 - 300 g) were purchased from The Southern Animal Facility, Animal house Division, Prince of Songkla University. All animal protocols were performed in accordance with the guidelines approved by Institutional Animals Care and Use Committee (IACUC), Prince of Songkla University (protocol number: MOE 0521.11/363). The rats were maintained under standard conditions in temperature and humidity-controlled environment (22 - 24 °C) with 12 h light/dark cycle. All rats received intraperitoneal injection with estradiol benzoate (100 $\mu\text{g}/\text{rat}$) 24 h prior to the experiment.

Isolated uterus preparation and contractile tension measurement

On the day of experiment, the rats were euthanized by cervical dislocation. The uterus was removed immediately and immersed in De Jalon-Ringer solution bubbled continuously with carbogen (95 % O_2 and 5 % CO_2). The tissues were cleared of fat, ovary and other connective tissues, and cut into strips of 1 cm. A uterine strip was then fixed in an organ bath containing an appropriate physiological solution bubbled continuously with carbogen with the temperature maintained at 37 °C throughout the experiments. The uterine strips were also connected to force displacement transducer attached to Grass polygraph by which isometric contraction of the uterus was recorded. The initial tension was adjusted to 2 g and the uterine strip was left to equilibrate with the solution for at least 1 h. During this period, the solution was changed periodically at 15 min interval with fresh solution.

Experimental procedures

Effect of propranolol on uterine relaxing effects of MEWP or isoproterenol

After equilibration period for 1 h, the uterine strips were stimulated to contract by substituting the physiological solution in the organ bath to depolarizing solution. When the tonic contraction induced by depolarizing solution was stable, cumulative concentrations of MEWP (10^{-6} - 10^{-3} gm/mL) or isoproterenol (10^{-10} - 10^{-5} M) were then added and the contractile responses were recorded. The uterine strips were then washed for many times with De Jalon-Ringer solution. The above procedures were then repeated but the

uterine strips were preincubated with propranolol (10^{-6} M) for 10 min before the addition of MEWP or isoproterenol. The control strips were also performed in parallel but the vehicle of MEWP or isoproterenol at the similar volume were added instead.

Effect of tetraethylammonium (TEA) on MEWP-induced uterine relaxation

After equilibration period, the uterine strips were stimulated to contract by the addition of oxytocin (1 mU/mL). When steady rhythmic contraction of the uterus was observed, MEWP (10^{-6} - 3×10^{-4} gm/mL) were added cumulatively to the organ bath with or without 10-minute pretreatment with TEA (10^{-3} M). In the control treatments which were done in parallel, the vehicle of MEWP at the same volume as MEWP solution was added to the organ bath of both control preparations, with and without TEA (10^{-3} M) pretreatment for 10 min. The uterine responses to various concentrations of the MEWP and its vehicle (in the control treatment) in the absence or presence of TEA were then recorded.

Effect of glibenclamide on MEWP-induced uterine relaxation

After equilibration period for 1 h in Locke-Ringer solution, the uterine strips were stimulated to contract by 1 mU/mL of oxytocin. When the rhythmic contraction induced by oxytocin was steady, MEWP (3×10^{-4} - 10^{-1} mg/mL) were added cumulatively to the organ bath with or without 10 min glibenclamide (10^{-5} M) pretreatment. In the control treatments, the vehicle of MEWP at the same volume as MEWP solution was added at the same time points to the organ bath in the absence or presence of 10 min pretreatment with glibenclamide (10^{-5} M). All experiments were performed in parallel. The effect of various concentrations of the MEWP and its vehicle in the absence or presence of glibenclamide was then recorded.

Uterine relaxing effects of MEWP on $PGF_{2\alpha}$ -induced contraction

After the uterine strips were equilibrated in Locke-Ringer solution for 1 h, $PGF_{2\alpha}$ (10^{-5} M) was then added to the organ bath to stimulate the uterus to contract. When the rhythmic contraction induced by $PGF_{2\alpha}$ was stable, MEWP (3×10^{-4} - 10^{-1} mg/mL) was then added cumulatively to the organ bath. The uterine responses to various concentrations of MEWP were allowed to develop maximally before adding the next higher MEWP concentration. In control treatment which was performed in parallel, the vehicle of MEWP was added at the same volume in replacement of MEWP solution. Changes in force of contraction were then recorded.

Data analysis

Data were selected from the maximum uterine tension in the presence of each concentration of the uterine relaxant or vehicle which were then calculated as percentage of maximum tension induced by the uterine stimulants from the same uterine strip. Values in all figures were expressed as mean \pm SEM of 10 replicates. Repeated-measure analysis of variance (ANOVA) were used for statistical comparisons among treatments. The *p*-values of less than 0.05 were considered statistically significant. When there was a significant difference between group of means, Duncan's multiple range test or Dunnett's test was then performed for multiple comparison among means.

Results and discussion

The present study aimed to determine the uterine relaxing effect and the mechanism of MEWP action on the isolated rat uterus. White pepper has been reported to contain many active constituents including various lignans derivatives, phenolics, terpenes, chalcones, alkaloid, steroid and flavonoid [6]. The crude methanolic extract used in this study may contain these active compounds which may have diverse effects on various systems of the body.

Many subtypes of adrenoceptors that are present on uterine smooth muscle cells also play a role on uterine contraction and relaxation. It is reported that the β_2 -subtype are mainly β -adrenergic receptors existing on myometrium and the activation of β_2 -receptor bring about the uterine relaxation [7,8]. The present study has shown that isoproterenol, a nonspecific β -adrenergic receptor agonist, produced uterine relaxation in a concentration-dependent manner in KCl-depolarized uterus (**Figure 1(A)**). The pretreatment of the uterus with propranolol, a non-specific β -adrenergic blocker, was able to reduce this effect. This result is consistent with the known classical interaction of β -adrenergic agonist and antagonist on the uterine relaxation reported elsewhere [8]. MEWP alone caused a relaxation of the KCl-depolarized uterus similarly to the effect of isoproterenol. Although, propranolol was able to inhibit the effect of isoproterenol, it was

not able to abolish the relaxing effect of MEWP (**Figure 1(B)**). This result suggested that the relaxing effect of MEWP may be unrelated to the interaction with β_2 -adrenergic receptor.

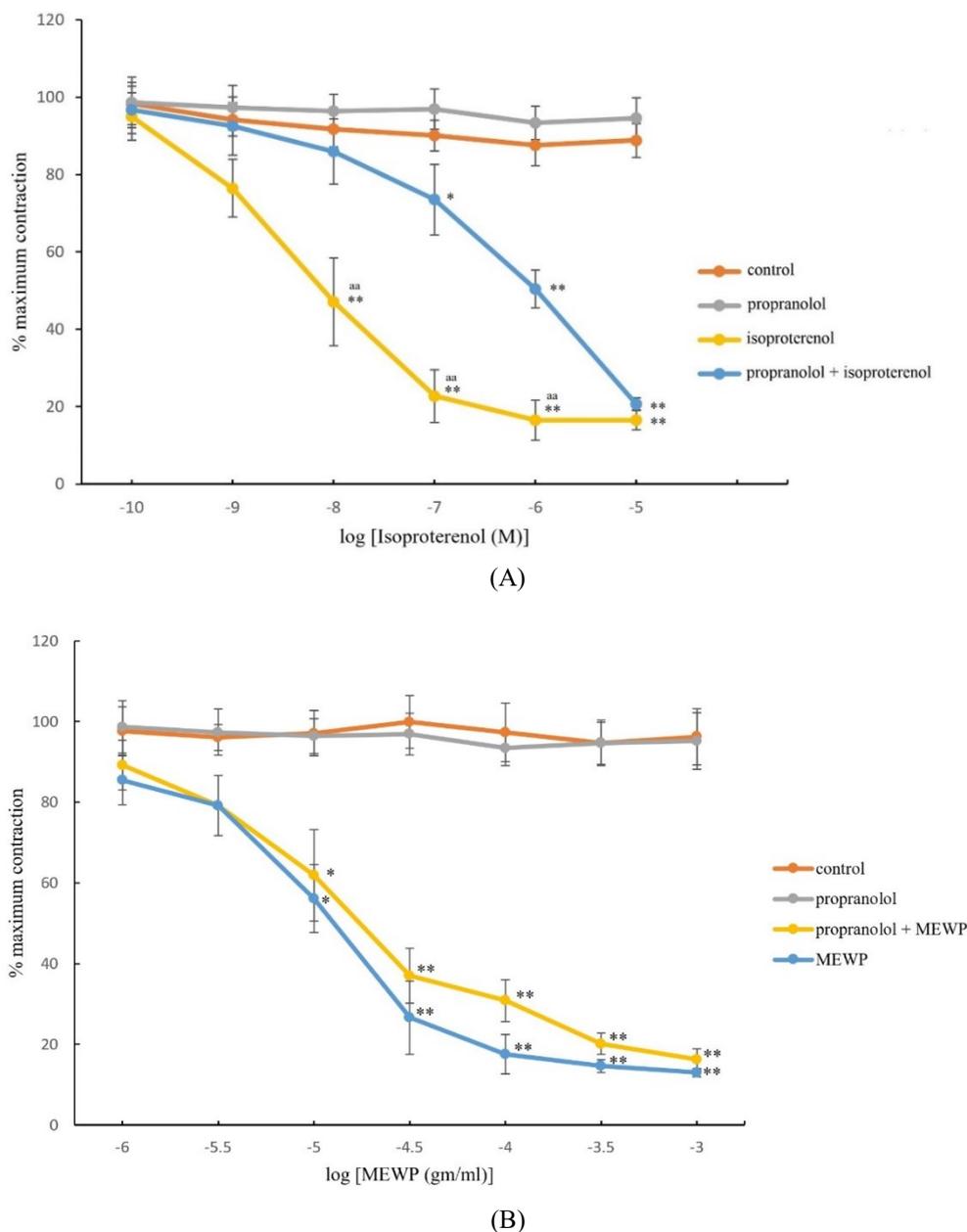


Figure 1 The relaxing effects of isoproterenol (A) or MEWP (B) in the presence or absence of propranolol (10^{-6} M) on isolated rat uterus precontracted with depolarizing solution. (*, $p < 0.05$, **, $p < 0.01$ as compared to the control.) (^a, $p < 0.05$, ^{aa}, $p < 0.01$ as compared among the comparing means which are in the same treatment group (extract or isoproterenol concentration)).

Our preliminary study showed a concentration-dependent effect of MEWP on the inhibition of uterine contraction induced by different concentration of CaCl_2 in Ca^{2+} - free - high KCl solution [5]. This study also demonstrated the concentration-dependent inhibition on the uterine contraction induced by depolarizing solution (**Figure 1(B)**). Under both conditions of high KCl concentration, KCl could change ion conductance which consequently activated the voltage-dependent calcium channel to open [9]. The addition of CaCl_2 to Ca^{2+} -free-high KCl solution and the presence of Ca^{2+} in depolarizing solution could cause the Ca^{2+} influx into the uterine muscle cell via opened Ca^{2+} channel and stimulated the uterus to

contract. MEWP used in our experiment was able to inhibit this effect. It is suggested that the uterine relaxing effect of the MEWP may be due to its direct action on the smooth muscle which interfere with Ca^{2+} influx through voltage dependent Ca^{2+} channel. The reduction in Ca^{2+} influx through the calcium channel may cause a reduction in the uterine contraction. In previous study reported elsewhere, similar contraction can be inhibited by verapamil, the Ca^{2+} channel antagonist [10].

Oxytocin and $\text{PGF}_{2\alpha}$ are known to cause a rise in intracellular Ca^{2+} from both Ca^{2+} influx via plasma membrane and Ca^{2+} release from internal storage (sarcoplasmic reticulum) [11]. The action of these agonists starts from their binding to an appropriate G-protein couple receptor on plasma membrane which results in an activation of phospholipase C. The enzyme then hydrolyses PIP_2 to IP_3 and DAG. IP_3 acts on its receptor on SR causing Ca^{2+} release from internal storage. In addition, oxytocin and $\text{PGF}_{2\alpha}$ induce an increase in intracellular Ca^{2+} which is mediated by Ca^{2+} influx via plasma membrane by an unknown mechanism. Our results revealed the inhibitory effect of MEWP on the contractile effect of oxytocin and $\text{PGF}_{2\alpha}$ (Figures (2) - (4)). It is suggested that the inhibitory pathway of MEWP may take place, at least in part, by an inhibition on Ca^{2+} influx via plasma membrane. However, their effects on the Ca^{2+} release from internal storage cannot be excluded.

To date, several types of K^+ channels have been demonstrated on plasma membrane of uterine smooth muscle cell, including voltage-dependent channels (K_V), large-conductance voltage and calcium-dependent (BK) channels, small-conductance calcium-dependent (SK) channels and ATP-sensitive (K_{ATP}) channels [11]. The most abundant of these channels include ATP-sensitive K^+ channel and Ca^{2+} -sensitive K^+ channel which play an important role on uterine contraction. The K^+ efflux through these channels in rat myometrium is the primary ionic current contributes to the maintenance of the resting membrane potential. The opening of K^+ channel by K^+ channel opener may cause increase in K^+ conductance and hyperpolarize the cell membrane. It was believed that the hyperpolarization triggered by K^+ efflux led to the closure of VOCs, thus preventing inward Ca^{2+} current and, eventually, cause the uterine relaxation. In this study, glibenclamide, a blocker of ATP-sensitive potassium channels [7,12], was not able to antagonize the uterine relaxation produced by MEWP (Figure 2). In contrast, such relaxation was significantly diminished by TEA, a K_{Ca} blocker (Figure 3). The lack of an effect of glibenclamide on the relaxation produced by MEWP suggested that the MEWP may not act by an activation on ATP-sensitive K^+ channel. The effect is likely to be mediated, at least in part, via an activation of Ca^{2+} -activated K^+ channel. The action of the MEWP on other subtypes of K^+ -channel, apart from ATP-sensitive K^+ channel, has yet to be determined.

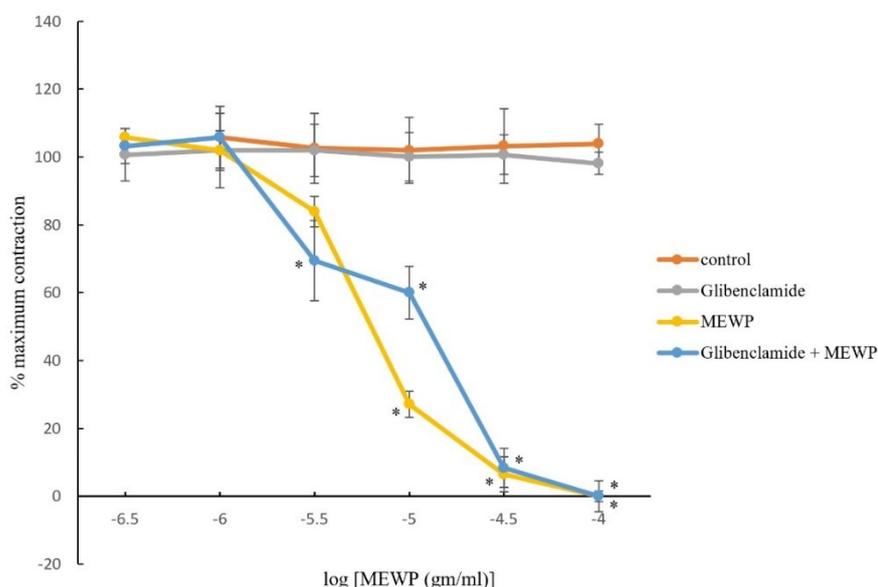


Figure 2 The concentration-response relationship of MEWP and uterine contraction in the presence and absence of glibenclamide (10^{-5} M) in isolated rat uterus precontracted with oxytocin (1 mU/mL). (*, $p < 0.05$, **, $p < 0.01$ as compared to their respective controls.)

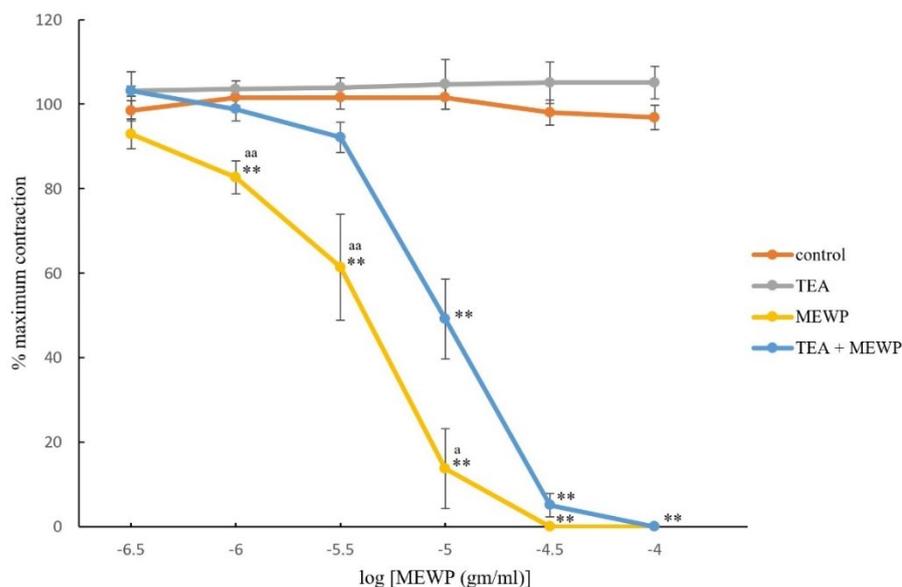


Figure 3 The concentration-response relationship of MEWP and uterine contraction in the presence and absence of TEA (10^{-3} M) in isolated rat uterus precontracted with oxytocin (1 mU/mL). (*, $p < 0.05$, **, $p < 0.01$ as compared to their respective control). (^a, $p < 0.05$, ^{aa}, $p < 0.01$ as compared to the same treatment group in the absence of TEA).

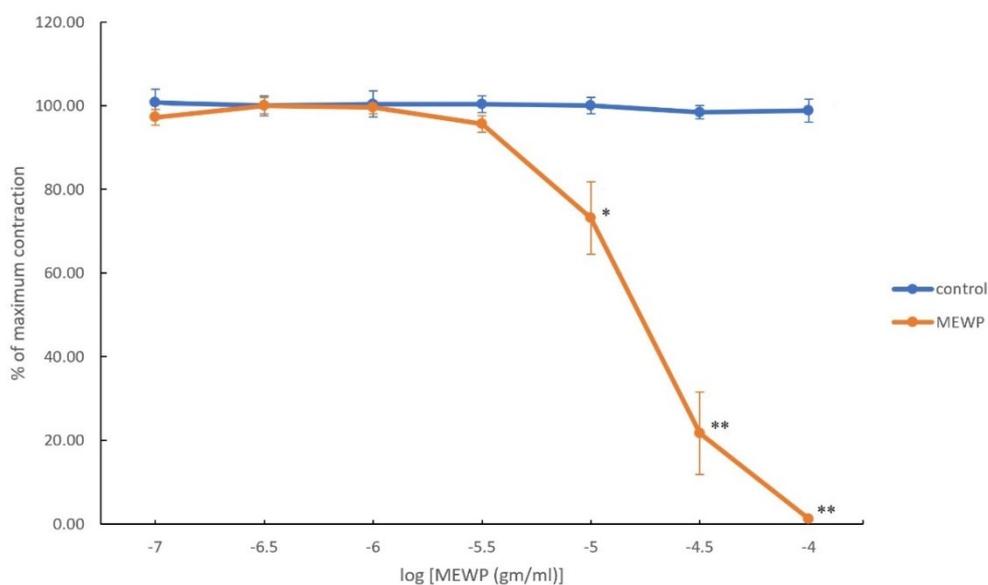


Figure 4 The concentration-response relationship of MEWP and the contractile response of the uterus in $PGF_{2\alpha}$ (10^{-5} M)-induced uterine contraction. (*, $p < 0.05$, **, $p < 0.01$ as compared to the control).

The results of this study demonstrated that MEWP possessed tocolytic effect on the isolated rat uterus which was induced to contract by various uterine stimulants such as depolarizing (high KCl) solution (**Figure 1(B)**), oxytocin (**Figures 2 and 3**) and $PGF_{2\alpha}$ (**Figure 4**) in a concentration-dependent manner. These results suggested that MEWP may act as a general uterine relaxant. It is very well known that over production of prostaglandins, particularly $PGF_{2\alpha}$, in the uterus at the beginning of menstruation is the main cause of dysmenorrhea [13]. MEWP has been shown to diminish contraction of the uterus induced by $PGF_{2\alpha}$. This result supports the role of white pepper to relieve the painful of dysmenorrhea.

Conclusions

In summary, the results of this study show the uterine relaxing effects of MEWP from the dried seed of *Piper Nigrum* in estrogen-primed rat myometrium. The relaxing effect of MEWP is unlikely to be mediated by an activation of β_2 -adrenergic receptor on the uterine muscle cells. The inhibitory effect of the extract may involve an inhibition of a rise in intracellular Ca^{2+} from both Ca^{2+} influx via plasma membrane of the uterine smooth muscle cell and probably Ca^{2+} release from internal storage. The effect of MEWP may be mediated mainly through a reduction on Ca^{2+} influx, possibly through voltage dependent Ca^{2+} channel. The effect may involve with the opening of K_{Ca} channel, which may arise from direct action of MEWP on the channel or be a consequence of an increase in $[Ca^{2+}]_i$. The uterine relaxing effect of white pepper strongly supports the role of white pepper to treat dysmenorrhea in Thai traditional medicine.

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