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The essential oil from the twigs of *Cinnamomum cassia* Presl inhibits oxytocin-induced uterine contraction in vitro and in vivo

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ABSTRACT

Ethnopharmacological relevance: The twigs and bark of *Cinnamomum cassia* Presl (Lauraceae) are widely used in traditional Chinese medicine in the treatment of tumor, abdominal pain, dysmenorrhea, digestive system disease and inflammatory diseases. The aim of this study was to determine the inhibitory effect of the essential oil from the twigs of *Cinnamomum cassia* Presl (EOCC) on uterine contraction in vitro and in vivo.

Materials and methods: The Institute of Cancer Research (ICR) mouse uterine contraction was induced by oxytocin (OT) exposure following estradiol benzoate pretreatment. Mice were given the EOCC (60, 30, and 15 mg/kg) by gavage. The level of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in uterine tissue were determined according to specification of enzyme linked immunosorbent assay (ELISA) kit. Uterine tissue was collected for histopathological analysis (H & E). Myosin light chain 20 (MLC20), phosphorylation of myosin light chain 20 (p-MLC20) and cyclooxygenase-2 (COX-2) proteins in uterine tissue were assessed by Western Blot. Mouse isolated uterus strips were mounted in tissue organ baths containing Locke's solution. The contractile responses were recorded with Power Lab recording system. The effect of the EOCC on uterine contraction induced by OT, $PGF_{2\alpha}$, and acetylcholine (Ach) was observed. Myometrial cells were exposed to OT (7 μM) to induce Ca^{2+} release, and the effect of the EOCC (100, 50, and 25 μg/ml) on intracellular Ca^{2+} was analysed with fluorometry imaging.

Results: In vivo study demonstrated that the EOCC significantly reduced OT-induced writhing responses with a maximal inhibition of 66.5%. It also decreased the level of $PGF_{2\alpha}$ in OT-induced mice uterine tissue. Moreover, Western blot analysis showed that COX-2 and p-MLC20 expressions in uterine tissue of dysmenorrhea mice were significantly reduced. EOCC inhibited spontaneous uterus contractions in a dose-dependent manner, and the concentration of the EOCC giving 50% of maximal contraction (IC_{50}) value was 61.3 μg/ml. The IC_{50} values of the EOCC on OT, $PGF_{2\alpha}$, and Ach-induced contractions were 113.0 μg/ml, 94.7 μg/ml, and 61.5 μg/ml, respectively. Further in vitro studies indicated that the EOCC could restrain intracellular Ca^{2+} levels in favour of uterine relaxation.

Conclusion: Both in vivo and in vitro results suggest that the EOCC possesses significant spasmolytic effect on uterine contraction. Thus, the EOCC yields a possible therapeutic choice for the prevention and treatment of primary dysmenorrhea.

1. Introduction

Primary dysmenorrhea refers to menstrual cramping pain in the lower abdomen caused by enhanced uterine contractions, and it is a common gynecological complaint among adolescent girls (Wallace et al., 2010). Primary dysmenorrhea occurs in the absence of an identifiable pathological condition and characteristically begins at or shortly after menarche (Chen et al., 2014). Pain usually develops within

Abbreviations: EOCC, essential oil from the twigs of *Cinnamomum cassia* Presl; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; COX-2, cyclooxygenase-2; GC-MS, gas chromatography coupled with mass spectrometry; OT, oxytocin; Ach, acetylcholine; MLC20, myosin light chain 20; p-MLC20, phosphorylation of myosin light chain 20

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hours of the start of menstrual bleeding and may last 1–2 days (Banikarim et al., 2000). According to a survey done on women of reproductive age, the prevalence varies from 20% to 90% (Harel, 2006; Nguyen et al., 2015; Kazama et al., 2015). The women with primary dysmenorrhea usually suffer from menstruation-associated symptoms such as anxiety,depression, vomiting, nausea, and cold sweats (Dawood, 2006). The etiology of primary dysmenorrhea is due to increased uterine contraction caused by excessive production and release of uterine prostaglandins (PGs) (Pan et al., 2014). $PGF_{2\alpha}$ can cause contraction of the blood vessels and myometrium, which lead to tissue ischemia and pain (Ghoneim et al., 2015). PGs generate from arachidonic acid under the action of the cyclooxygenase (COX). High cyclooxygenase-2 (COX-2) expression leading to increased $PGF_{2\alpha}$ during menstruation is the mechanism most likely responsible for primary dysmenorrhea (Sales and Jabbour, 2003). Ca^{2+} signals with the myometrium play an important role in governing uterine contraction (Hsia et al., 2011). OT binds to myometrium cell membrane OT receptor to induce Ca^{2+} release (Barata et al., 2004). The increase in $[Ca^{2+}]i$ leads to formation of the Ca^{2+}-calmodulin complex which then activates myosin light-chain kinase (MLCK), resulting in the phosphorylation of myosin light chain 20 (MLC20) and myometrial contraction (Arrowsmith and Wray, 2014).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the main treatment in clinical medicine for dysmenorrheal. Ibuprofen nonspecifically inhibits both cyclooxygenase-1(COX-1) and COX-2 enzymes and relieves pain in women with primary dysmenorrhea (Ozgoli et al., 2009). Although NSAIDs successfully relieve the painful symptoms of primary dysmenorrhea, but they have many side effects that can affect the hepatic, digestive, cardiac and renal systems (Zahradnik et al., 2010; Hendrix et al., 2002). Therefore, traditional Chinese medicine may be a feasible alternative to improve primary dysmenorrhea.

Cinnamomum cassia Presl, an evergreen and aromatic tall tree belonging to the family Lauraceae, can be found in southern China. The twigs and the bark of *Cinnamomum cassia* Presl are commonly used as traditional Chinese medicine for treating dyspepsia (Nilius and Appendino, 2013), gastritis (Zhu et al., 1993), tumor (Jeon et al., 2009), dysmenorrhea (Jaafarpour et al., 2015; Kort et al., 2015) and inflammatory diseases (Jia et al., 2006; Kang et al., 2014). *Cinnamomum cassia* Presl has been studied to test its effectiveness in alleviating menstrual pain cited by the Chinese naturopathic medicine literature 4000 years ago (Qin et al., 2003; Cheng et al., 2000).

Previous studies examined the in vitro effect of cinnamon extract on different types of smooth muscle cells (Raffai et al., 2014; Yong et al., 2011; Yanaga et al., 2006). Alotaibi (2016) reported that cinnamon extract inhibited uterine contractility that might be due to cinnamon extract 's direct effect on L-type Ca^{2+} channels or Ca^{2+} release. Nonetheless, the molecular mechanisms of the EOCC for inhibition of uterine contraction remain largely unclear. In the present study, we aimed to investigate the underlying molecular mechanisms of the EOCC for inhibition of uterine contraction using relevant in vivo and in vitro experimental models.

2. Materials and methods

2.1. Chemicals and reagents

Estradiol benzoate injection (Ningbo second hormone factory, Ningbo, China, No. 20131116). OT injection (Henan Furen Pharmaceutical Co., Ltd., Zheng zhou, China, no. 1302031). $PGF_{2\alpha}$ (Santa Cruz Biotechnology, USA, sc201227A). Anti-COX-2 antibody (Abcam, England, ab1519). Anti-MLC20 antibody (Abcam, England, ab137063). Anti-myosin light chain (phospho s19) antibody (p-MLC20, Cell Signaling Technology, Inc, USA, 3675). GAPDH rabbit mAb (Cell Signaling Technology, Inc, USA, 5174 S). Goat anti-rabbit IgG-HRP (Abcam, England, ab6721). Goat anti-mouse IgG-HRP (Abcam, England, ab6789). Penicillin-Streptomycin (Gibco, 15140-122). CollagenaseII(Gibco). DMEM/F12 medium (Powder,Gibco, 12800-017). Fetal bovine serum (Gibco, 10099-141). Calcium 6-QF assay kit (MD). All other chemicals were analytical grade.

2.2. Preparation of the EOCC

The crude decoction piece of the twigs of *Cinnamomum cassia* Presl was purchased from Hexin Tang Raw Chinese Herbal Medicine Manufacturer, and their identities were authenticated in accordance with the Chinese Pharmacopoeia (2010 Edition). The voucher specimen (no. KPTCM1605032) was deposited at the Herbarium of the Jiangsu Kanion Pharmaceutical Co., Ltd., China. The twigs of *Cinnamomum cassia* Presl (10 kg) was distilled by a modified clevenger type apparatus for 6 h., and the essential oil (58.0 g; 0.58%) were separated. The EOCC was stored at 4 °C until chemical analysis and pharmacological experiments.

2.3. GC-MS analysis of the EOCC

For GC-MS analyses, an Agilent gas chromatograph coupled to mass spectrometer detector (7890GC-7000MS) was used. Compounds were separated on a Agilent HP-5MS (30 m × 0.25 mm × 0.25 μm) capillary column with the following temperature program: the program of column temperature was 80 °C for 3 min isothermally, from 80 to 160 °C at 20 °C/min for 6 min isothermally, and finally up to 250 °C for 24 min isothermally. Injector temperature was set at 240 °C. Nitrogen was used as the carrier gas at a constant flow rate of 1 ml/min, split ratio, 1:25. All mass spectra were acquired in EI mode (scan range *m/z* 50–500, ionization energy 70 eV). Major volatile compounds were identified by co-injection with authentic standards by comparing the retention times of the chromatographic peaks, and their MS fragmentation patterns with those of pure compounds, of the spectral database of the National Institute of Standards and Technology (NIST) MS. The chemical composition of the EOCC is presented in Table 1.

2.4. Animals

Female ICR mice (22–26 g of weight, 6–7 weeks old) were provided by Beijing Weitong Lihua experimental animal Co. Ltd., Beijing, China. All the animals were housed in plastic cages, under a 12 h light/dark cycle at constant room temperature (22 ± 2 °C) and humidity (60–80%) with water and food ad libitum. The animals were acclimatized for 5

Table 1
Constituents identified in the essential oil of the twigs of Cinnamomum cassia Presl by GC-MS analysis.

Compounds	Retention time (min)	Peak area (%)
Phenylethyl alcohol	5.533	0.32
(4-methoxycarbonylphenyl)3-Methoxybenzoate	5.765	0.19
Benzenepropanal	6.055	0.56
(Z) -Cinnamaldehyde	6.615	0.83
2,5-Octadecadiynoic acid, methyl ester	6.695	0.30
24,25-Dihydroxyvitamin D3	6.819	0.14
(E) -Cinnamaldehyde	7.072	79.39
10,13-Octadecadiynoic acid, methyl ester	7.362	0.21
α-Ylangene	8.116	0.29
Coumarin	8.842	0.70
3-Methoxycinnamaldehyde	8.971	0.31
α-Curcumene	9.331	1.10
Mitomycin derivative T 20	9.816	3.67
o-Methoxycinnamic aldehyde	10.068	3.63
Brassicasterol acetate	10.940	0.06
Deoxysericealactone	11.225	0.31
Ar-tumerone	12.946	4.68