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Bronchorelaxation Activity of *Coptosapelta Flavescens* Korth Root's Methanol Extract by *In-Vitro* Inhibition of Calcium Channel

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Abstract
The objective of this study was to examine the bronchorelaxation effect of the methanol extract of Coptosapelta flavescens Korth roots (MECFR) by inhibiting calcium channel on guinea pig bronchial rings. Both Experiment I and II used isolated bronchial rings incubated with 3 concentrations of MECFR, DMSO (negative control) and Verapamil (positive control) in a Ca^{2+}-free medium, given 60 mM KCl (I) or 2µM histamin (II), and contracted with cumulative dose of $CaCl_2$ to obtain $CaCl_2$ dose-response curve (DRC). All MECFR's (3 concentrations) DRCs shifted to the right, similar to Verapamil's, and with smaller E_{max} and pD_2 compared to the negative control's DRC for both experiments I and II, indicating that MECFR inhibits both extracellular Ca^{2+} influx and Ca^{2+} release, results in a decrease in intracellular Ca^{2+} levels. It can be concluded that MECFR has bronchorelaxation activity through inhibition of Ca^{2+} channel.

Keyword: *Calcium channel, Coptosapelta flavescens Korth's roots, bronchorelaxation.*

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1.0 INTRODUCTION

Coptosapelta flavescens Korth root (CFR) traditionally is used to treat coughing and shortness of breath (Darusman, 2004). The methanol extract of CFR (MECFR) has been scientifically proven to act as a bronchodilator by stimulating $beta_2$ adrenergic receptors, inhibiting cholinergic receptors and histamine receptors (Kosala, 2019). It has been presumed that the MECFR's bronchorelaxation activities are also contributed by other mechanisms, such as inhibition of calcium channel (Gao, et al, 2016).

The mechanism of smooth muscle contraction involves transduction of different signals, all of which have effects on the increase in levels of cytoplasmic calcium. Both extracellular Ca^{2+} influx through voltage-dependent calcium channel (VDCC) or intracellular Ca^{2+} release via receptor-operated calcium channels (ROCC) results in increased levels of cytoplasmic calcium (Cao, Zhang, J. He, L. He, & Xu, 2006). By inhibiting the extracellular Ca^{2+} influx or intracellular Ca^{2+} release, it is expected that the levels of cytoplasmic calcium will not increase and thus, no contraction will occur or contration will be decreased (Barrett, Boitano, Barman & Brooks, 2012). To determine if the MECFR bronchorelaxation activity is through the inhibition of calcium channel, this was tested by *in vitro* inhibition of extracellular Ca^{2+} influx and intracellular Ca^{2+} release, using guinea pigs' isolated bronchial rings.

2.0 MATERIALS AND METHODS

2.1 Materials

Coptosapelta flavescens Korth roots (CFR) were obtained from Paser Regency, East Kalimantan Province, and were identified by a taxonomist from the Mulawarman University Forestry faculty. All chemicals such as proanalytic methanol used for extraction, and the materials to make Kreb's Henseleit solution were purchased from pharmaceutical distributors in Surabaya. Carbogen gas was purchased from PT Murni Gas Raya in Samarinda.

Male guinea pigs aged 3-4 months weighing 350-450 g were obtained from the Pharmacology laboratory of School of Medicine, Mulawarman University. Ethical approval was obtained from the health ethics committee of School of Medicine, Mulawarman University. The bioassay equipment used to test the contractility of guinea pigs' isolated bronchial ring consists of a six-chamber isolated organ bath, a Power Lab/16SP digital recorder, an isometric transducer and an octal bridge amplifier.

2.2 Preparation of MECFR

The MECFR was prepared following the procedure of MECFR's preparation by Kosala, (2019). The concentrated MECFR obtained was stored in the refrigerator at 4°C until it was ready for use.

2.3 Preparation of Guinea Pig Bronchial Rings

The bronchial rings were prepared following the procedure of guinea pig bronchial ring's preparation by Kosala, (2019). The bronchial ring was placed on a tissue holder connected to an isometric transducer with a load of 1g, and a Power Lab recording device with AD Instrument Program Chart v.5 (Albuquerque et al., 2016).

2.4 The Effect of Extracellular Ca^{2+} Inhibition Assay

The bronchial ring in a Kreb's-Henseleit solution was equilibated for 60 minutes. After equilibration was achieved, the Kreb's solution was replaced with a Ca^{2+}-free Kreb's solution consisting of (mM) NaCl 50.58; KCl 50; $MgSO_4$ 3.1; KH_2PO_4 1.26; $NaHCO_3$ 23.8; Glucose 11.1, and EDTA 0.1 mM for 30 minutes to remove Ca^{2+} from the tissue. Then the bronchial ring was rinsed with Ca^{2+}-free Kreb's solution. Before the addition of 60 mM KCl, the bronchial ring was incubated with MECFR (5%; 7.5%; 10%) for 20 minutes, then contracted with the cumulative addition of $CaCl_2$ doses (10^{-5} - 10^{-2} M). The negative control was 1% DMSO and the positive control was Verapamil 1 µM (100 µl 10^{-4} M) (Gao, et al, 2016).

2.5 The Effect of Intracellular Ca^{2+} Inhibition Assay

After being stabilized in Ca^{2+}-free Kreb's solution for 30 minutes, the bronchial ring was pre-incubated with MECFR (6%; 10% and 15%) for 20 minutes, then added with 2 µM (20 µl 10^{-3} M) Histamine to stimulate the release of intracellular Ca^{2+} until maximum response was reached, then contracted with cumulative doses of $CaCl_2$ (Gao, et al, 2016).

2.6 Calculation of Percent Contraction

Percent contraction of bronchial rings was calculated with the following formula:

$$\%\ Contraction\ = \frac{Test\ contraction\ response(g) - Control\ contraction\ response(g)}{Maximum\ contraction(g)}\ x\ 100$$

Subsequently, the dose-response curve (DRC) was plotted from the concentration log vs the bronchial ring percent contraction for each treatment. From the DRC, E_{max}, EC_{50} and pD_2 (= -log EC_{50}) were obtained (Rang, Ritter, Flower & Henderson, 2016).

2.7 Statistical Analysis

Result data were presented as mean ± SEM. Percent contraction, E_{max} and pD_2 for each treatment were analyzed with one-way analysis of variance (ANOVA), where there was a statistically significant difference if $P < 0.05$.

3.0 RESULTS

3.1 The Effect of Extracellular Ca^{2+} Inhibition

Figure 1 shows the DRC of bronchial rings incubated with MECFR 5, 7.5 and 10%; 1% DMSO and 1 uM Verapamil, given KCL 60 mM, and contracted with cumulative dose of $CaCl_2$, marked as $CaCl_2$-MECFR5-KCl; $CaCl_2$-MECFR7.5-KCl; $CaCl_2$-MECFR10-KCl; $CaCl_2$-DMSO-KCl and $CaCl_2$-Ver-KCl. All MECFR (3 concentrations) DRCs as well as Verapamil DRC (calcium channel blocker) shifted to the right, accompanied with smaller E_{max} and pD_2 than the negative control's DRC; the greater the MECFR concentration the smaller the pD_2 value (Table 1). This showed that MECFR inhibits the increase of extracellular influx Ca^{2+} through KCl-induced VDCC; the greater the concentration of MECFR, the greater the resistance to VDCC, and thus, the smaller the potential for $CaCl_2$ to induce bronchial ring contraction.

3.2 The Effect of Intracellular Ca^{2+} Inhibition

Figure 2 shows the DRC of the bronchial rings incubated with MECFR 6, 10 and 15%; 1% DMSO and 1 uM Verapamil, added with 2 µM Histamine and contracted with a cumulative dose of $CaCl_2$. The curves were labeled as Ca-His-MECFR6; Ca-His-MECFR10; Ca-His-MECFR15; Ca-His-DMSO and Ca-His-Ver. All MECFR (3 concentrations) DRCs as well as Verapamil DRC shifted to the right, accompanied with smaller E_{max} and pD_2 than the negative control DRC; the greater the MECFR concentration the smaller the pD_2 value (Table 2). This showed that MECFR inhibits the release of Ca^{2+} from its storage in the histamine-induced reticulum sarcoplasm through ROCC;

the greater the concentration of MECFR, the greater the inhibition towards ROCCs and thus, the smaller the potential for $CaCl_2$ to induce bronchial ring contraction.

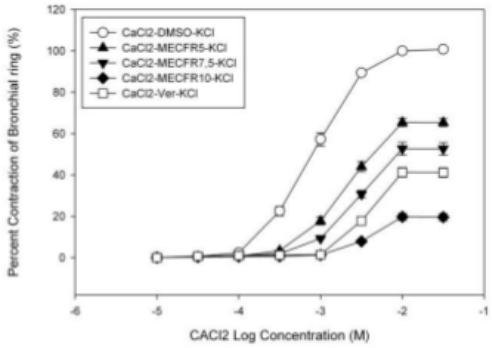

Figure 1. $CaCl_2$ DRCs on bronchial rings incubated with MECFR, given 60 mM KCl. n = 6.

Table 1. E_{max} and pD_2 of bronchial rings contracted with $CaCl_2$ after incubation with MECFR and KCl

	DMSO (%)			Ver 1 µM (%)			MECFR5 (%)			MECFR7.5 (%)			MECFR10 (%)		
	Mean	±	SEM	Mean	±	SEM	Mean	±	SEM	Mean	±	SEM	Mean	±	SEM
E_{max}	100.78	±	0,467	41.30**	±	1.14	65.38**	±	0.981	52.71**	±	1.521	19.74**	±	0.858
pD_2	3.09	±	0,02	2.49**	±	0.002	2.70**	±	0.012	2.60**	±	0.003	2.49**	±	0.007

n=6, data were analyzed with ANOVA, ** indicates P< 0.001 compared to DMSO

Table 2. E_{max} and pD_2 of bronchial ring contracted with $CaCl_2$ after incubation with Histamine and MECFR

Log Conc	DMSO (%)			Ver 1 µM (%)			MECFR6 (%)			MECFR10 (%)			MECFR15 (%)		
	Mean	±	SEM	Mean	±	SEM	Mean	±	SEM	Mean	±	SEM	Mean	±	SEM
E_{max}	100.00	±	0.00	81.57**	±	2.13	91.15*	±	2.59	65.80**	±	1.20	33.89**	±	2.18
pD_2	3.110	±	0.016	2.79**	±	0.03	2.98*	±	0.012	2.87**	±	0.022	2.84**	±	0.053

n=6, data were analyzed with ANOVA, * means P< 0,05; ** means P<0,001 compared to DMSO

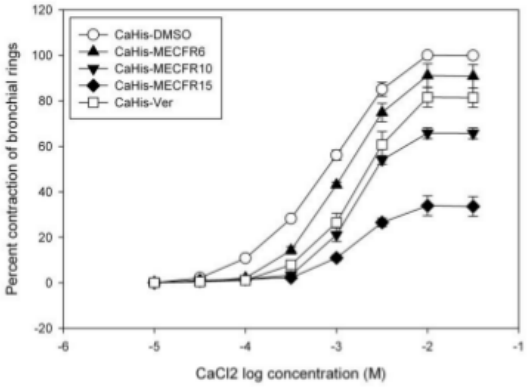

Figure 2. $CaCl_2$ DRCs on bronchial rings incubated with MECFR, given 2 µM Histamine. $n = 6$.

4.0 DISCUSSION

There are two types of calcium channels in the smooth muscle cells, namely VDCC and ROCC (Gao, et al, 2016). Contractions induced by high-dose K^+ are caused by membrane depolarization, activating VDCC and increasing the extracellular Ca^{2+} influx via VDCC. Meanwhile, the interaction of contractile agonist such as acetylcholine and histamine with its receptors induces the formation of inositol triphosphate (IP3) and diacylglycerol which activates protein kinase C. IP3 binds to its receptor (IP3R) in the sarcoplasmic reticulum, induces direct release of Ca^{2+} from its storage in the sarcoplasmic reticulum and induces initial contractions, evokes the influx of Ca^{2+} which is induced by the opening of VDCC and ROCC (Cao, et al, 2006; Barret, et al, 2012; Yang, et al, 2017).

The results of this study indicate that MECFR with concentration-dependent activity inhibits extracellular Ca^{2+} influx which are induced by high-dose KCl via VDCC, and inhibits the release of Ca^{2+} from its storage in the histamine-induced sarcoplasmic reticulum via ROCC. MECFR concentrations (6, 10 and 15%) which inhibit the intracellular Ca^{2+} release were greater than MECFR concentrations (5, 7.5 and 10%) which inhibit extracellular Ca^{2+} influx. This is thought to be due to the release of intracellular Ca^{2+} that is not only regulated by the IP3 receptor system (IP3R) but also by the ryanodine receptor system (RyRs) (Cao, et al, 2006).

In previous studies it has been shown that MECFR inhibits histamine-induced contraction (Kosala, 2019); this proves that MECFR inhibits the intracellular Ca^{2+} release via the IP3 receptor system. To prove that MECFR is also involved in inhibiting intracellular Ca^{2+} release regulated by the RyRs system, further research is needed.

5.0 CONCLUSION

Coptosapelta flavescens Korth root's methanol extract has bronchorelaxation effect by inhibiting the calcium channel.

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