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Research Article

IN VITRO STUDY OF THE BRONCHORELAXATION EFFECT OF *COPTOSAPELTA FLAVESCENS* KORTH. ROOT'S EXTRACT AND FRACTIONS

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ABSTRACT

Objective: This study aimed to compare the bronchorelaxation effect of the methanol extract, hexane fraction, ethyl acetate fraction, and methanol fraction of *Coptosapelta flavescens* Korth. roots (CFRs) on the isolated bronchial rings of guinea pig.

Methods: Research design was experimental in which a 4-mm long bronchus of a male guinea pig was kept in an isolated organ bath containing Krebs-Henseleit solution at 37°C, pH 7.4, and flooded with carbogen gas. The bronchial ring was precontracted with methacholine and then given six doses of *C. flavescens* Korth. root's methanol extract (MECFR) solution cumulatively. The extract's solvent 10% dimethyl sulfoxide ethanol was used as negative control. This procedure was then repeated using CFRs hexane fraction (HFCFR) solution, CFRs ethyl acetate fraction (EAFCFR), and CFRs methanol fraction (MFCFR).

Results: The bronchorelaxation effect of MECFR, MFCFR, EAFCFR, and HFCFR is shown by their dose-response curves (DRCs) which are significantly different compared with the extracts solvent's DRC. The maximal efficacy (E_{max}) of MFCFR was the same ($p>0.05$) as the MECFRs E_{max}, but the EAFCFRs and HFCFRs E_{max} were smaller ($p<0.05$) than the MECFRs E_{max}.

Conclusion: The bronchorelaxation effect of the MECFR on the guinea pig's bronchial ring is similar to the CFRs methanol fraction, and is stronger than the CFRs ethyl acetate fraction and CFRs hexane fraction.

Keywords: Bronchorelaxation, *Coptosapelta flavescens* Korth. root, Extract, Fractions.

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INTRODUCTION

Coptosapelta flavescens Korth. is a liana plant from Rubiaceae family. In Kalimantan, it is known as Akar Tambolekar or Akar Merung [1] or Akar Manuran [2]. It is used in ethnobotany as herbs to overcome cough and shortness of breath [3]. Shortness of breath is a clinical symptom of bronchoconstriction that occurs in people with asthma or chronic obstructive pulmonary disease [4,5]. Therefore, it is presumed that *C. flavescens* Korth. root (CFR) has bronchorelaxation effect by relaxing the contracting bronchial smooth muscles. In a previously unpublished study, it has been shown that the CFRs methanol extract (MECFR) has a bronchorelaxation effect through the stimulation of the beta-2 adrenergic receptors. It has been known that MECFR contains secondary metabolites of saponins, polyphenols, triterpenoids, steroids, and anthraquinone; CFRs hexane fraction (HFCFR) contains anthraquinone and steroids; CFRs ethyl acetate fraction (EAFCFR) contains polyphenols, terpenoids, and anthraquinone; and CFRs methanol fraction (MFCFR) contains saponins, polyphenols, and terpenoids [6]. Secondary metabolites in plants contribute to the plants' pharmacological effects [7]. Thus, the secondary metabolites in CFR which are responsible for CFRs bronchorelaxation effect can be found by studying the bronchorelaxation effect of different CFR's fractions. While there were existing studies on MECFR bronchorelaxation effect on guinea pig's bronchial ring precontracted with methacholine, there are no data available on the bronchorelaxation effect of any CFRs fraction, whether polar or non-polar. Therefore, the aim of this study was to compare the bronchorelaxation effect of MECFR with CFRs hexane (non-polar) fraction, CFRs ethyl acetate (semi-polar) fraction, and CFRs methanol (polar) fraction. The study was conducted using the isolated bronchial ring of guinea pigs [8].

MATERIALS AND METHODS

Materials

C. flavescens Korth. roots (CFRs) were collected from Paser Regency of East Kalimantan Province. Identification was done by the taxonomist of the Forestry Faculty of Mulawarman University with an identification letter no. 09/UN17.4.3.08/LL/2014. The roots were sorted and washed in running water then cut into small pieces and dried in an oven at 60°C until the water content was <10%, then ground into powder. Methanol, n-hexane, and ethyl acetate for extraction were purchased from Sigma-Aldrich distributor in Surabaya. Ingredients to make Krebs-Henseleit solution such as NaCl, KCl, $MgSO_4$, Na_2HPO_4, KH_2PO_4, $CaCl_2$, and glucose were purchased from a distributor in Surabaya. Carbogen gas (95% O_2 gas and 5% CO_2 gas) was purchased from PT Murni Gas Raya in Samarinda.

Test animals were 3–4-month-old male guinea pigs weighing 350–400 g obtained from Pharmacology Laboratory, Faculty of Medicine of Mulawarman University, Samarinda; ethical approval was obtained from Medical Ethics Committee, Faculty of Medicine of Mulawarman University.

Tools for bronchorelaxation assay were six chambers isolated organ bath, octal bridge amplifier, Power Lab/16SP digital recorder, isometric transducer, pH meter, tweezers, and surgical scissors.

Preparation of MECFR

Dried and ground simplicia of CFR (300 g) were macerated in 1.5 l of pro-analytic methanol solution and shaken continuously for 3 day. The filtrate was collected and the residue was macerated further in pro-analytic

methanol solution. This procedure was repeated until the resulting filtrate became colorless. All the collected filtrates were then evaporated using a 50°C vacuum rotavapor. The condensed mass obtained was heated in an oven (50°C) to obtain a dry extract with a moisture content of <10%.

Preparation of hexane, ethyl acetate, and methanol fractions of CFR
The CFR fractions were made using the same procedure as with MECFR, in a successive manner [9] by first using the non-polar solvent n-hexane. The concentrated extract obtained from the n-hexane filtrate was the HFCFR. The residue subsequently was aerated in the open air for 24 h to evaporate out the remaining n-hexane. Second, the semi-polar solvent ethyl acetate was added using the same procedure as above. The concentrate obtained from the ethyl acetate filtrate was the EAFCFR. The residue was then aerated in the open air to evaporate out the remaining ethyl acetate solvent, and subsequently, the polar solvent methanol was added according to the above procedure. The concentrated extract obtained from the methanol filtrate with water content < 10% was the MFCFR. Each of the MECFR, HFCFR, EAFCFR, and MFCFR was weighed to calculate their yields.

Guinea pig's bronchial preparation
Male guinea pigs (*Cavia porcellus*), 3–4 months old (350–400 g), were fasted for 12 h and subsequently anesthetized and sacrificed by cervical dislocation. Surgery was done by opening the abdomen and thorax until the lungs were visible. After dissecting the lungs and trachea, they were placed in a Petri dish containing Krebs-Henseleit solution. Attached fats and connective tissue were removed from the bronchial segments, and it was then cut into a length of 4 mm and put into an organ bath filled with 10 ml Krebs-Henseleit solution, maintained at 37°C, pH 7.4, and flooded with carbogen gas. Bronchial ring arrangement was in accordance with what has been described by Albuqueque [8]. One end of the bronchial ring was connected with a stainless steel tissue holder and the other end was mounted on an isometric transducer with a tone of 1 g and connected to a Power Lab recording device, AD Instrument with Program Chart v.5. Changes in bronchial contractility on the computer were recorded in g.

Bronchorelaxation effect of MECFR on bronchial ring assay
Before treatment, the bronchial ring was equilibrated in Krebs-Henseleit solution for 60 min, and every 15 min, the solution was replaced with fresh solution. After the equilibrium state had been achieved, single dose amounted to 20 µl of 10^{-3} M methacholine solution was administered and maximum contraction was reached. After plateau had been reached, the extract solvent 10% dimethyl sulfoxide (DMSO) ethanol was given cumulatively in increasing dosages every 100 s and then left undisturbed for 10 min. Subsequently, the solution was replaced with new Krebs-Henseleit solution for 3 times every 10 min. The treatment was continued again with precontraction using a single dose of 20 µl of 10^{-3} M methacholine solution until the maximum contraction was reached, and then, MECFR solution was given cumulatively in increasing dosages of six different concentrations every 100 s and then left undisturbed for 10 min.

Bronchorelaxation effect of HFCFR, EAFCFR, and MFCFR on bronchial ring assays
The procedure for the bronchorelaxation effect assay for each HFCFR, EAFCFR, and MFCFR was similar with the bronchorelaxation effect assay for MECFR on the isolated bronchial ring of guinea pig which was contracted with methacholine, using 10% DMSO ethanol solvent as the negative control (NC).

Calculation of the percentage of relaxation
The bronchial ring's percent of relaxation was calculated using the following equation:

$$\%\,\text{Relaxation} = \frac{\text{Relaxation response}\,(\text{g}) - \text{response of NC}\,(\text{g})}{\text{Max precontration response}\,(\text{g})} \times 100$$

Then, the dose-response curve (DRC) was made by plotting the negative log of extract or fraction concentration on X-axis and bronchial ring's percentage of relaxation on Y-axis. The maximum efficacy (E_{max}) was obtained from the maximum percentage of relaxation.

Statistical analysis
The data obtained were presented in the mean ± standard error of the mean (sem). Percentage of relaxation from all six concentrations and E_{max} from the DRC of MECFR, HFCFR, EAFCFR, MFCFR, and NC were analyzed with Sigma plot 12.5 one-way ANOVA, followed by the Tukey test. p=0.05 or less was considered to be statistically significant.

RESULTS

Yield from methanol extract, hexane fraction, ethyl acetate fraction and methanol fraction of CFR
Table 1 shows the MECFR yield from 300 g of CFR simplicia and HFCFR, EAFCFR, and MFCFR yield from 667 g of CFR simplicia. The largest yield was obtained from MECFR (21.94%), while the least yield was from hexane fraction (HFCFR) and ethyl acetate fraction (EAFCFR) at only 4%.

Bronchorelaxation effect of MFCFR, EAFCFR, HFCFR, and MECFR on guinea pig's bronchial rings precontracted with methacholine
Fig. 1 shows the relaxation effect on bronchial rings precontracted with 2 µM of methacholine; then, each was given MFCFR, EAFCFR, HFCFR, MECFR, and the extract's solvent, 10% DMSO ethanol, at cumulative doses. DRC of MFCFR, EAFCFR, HFCFR, and MECFR on Fig. 1 showed that as the concentration of extract or fractions became larger, the bronchial ring's percentage of relaxation increased. The percentage of relaxation for each of the fractions and extract was larger and significantly different ($p<0.05$*) when compared to the NC, the extract's solvent; this showed that MFCFR, EAFCFR, HFCFR, and MECFR had bronchorelaxation effect and they were not influenced by the extract's solvent.

Fig. 1 also shows the maximum efficacy (E_{max}) of MFCFR, EAFCFR, HFCFR, and MECFR. The E_{max} of MFCFR (−136.63±14.31%) was not significantly different ($p>0.05$) compared with the E_{max} of MECFR (−146.48±21.03%). The E_{max} of EAFCFR (−76.74±4.68%) and HFCFR (−80.01±6.68%) was smaller and significantly different compared with the E_{max} of MECFR with $p<0.05$**. The E_{max} of EAFCFR was similar to the E_{max} of HFCFR ($p>0.05$). This showed that the bronchorelaxation effect of EAFCFR and HFCFR was similar but were weaker compared with MECFR and MFCFR. It could also be interpreted that among the extract and fractions, the bronchorelaxation activity of MECFR and MFCFR was the strongest.

DISCUSSION

The study's result shows that the bronchorelaxation effect of MECFR and MFCFR is of similar strength. In another unpublished study, MECFR has bronchorelaxation effect through stimulation of the beta-2 adrenergic receptors; MECFR acts as beta-2 agonist which stimulates beta-2 adrenoceptors which are part of the G-protein-coupled receptors group with G_s alpha subunit. Beta-2 adrenoceptor coupled with G_s protein stimulates adenylyl cyclase and produces second messenger cyclic adenosine monophosphate (cAMP) [10]. In the airways, cAMP will reduce intracellular Ca^{2+} concentration and activates protein kinase A resulting in the inactivation of myosin light-chain kinase (MLCK) and activation of ML-chain phosphatase (MLCP). MLCP activation will prevent the binding of actin and myosin which leads to smooth muscle relaxation. The decrease in intracellular Ca^{2+}

Table 1: Yield from CFR extract and fractions

Extract/fraction name	Obtained weight (g)	Yield (%)
MECFR	65.84	21.94
HFCFR	26.72	4.00
EAFCFR	27.29	4.09
MFCFR	79.80	11.96

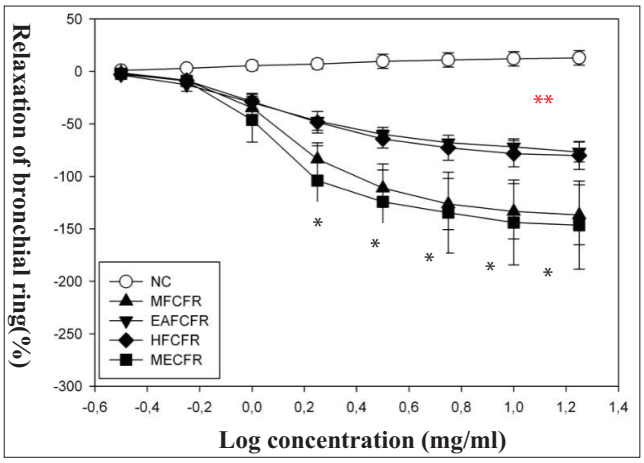

Fig. 1: The DRC of MFCFR, EAFCFR, HFCFR, and MECFR on guinea pig's bronchial ring precontracted with methacholine. Data presented as mean±standard error of mean from n=6. *p<0.05 significantly different compared to negative control. **p<0.05 significantly different compared to MECFR

concentrations also causes a reduction in Ca-calmodulin binding such that contraction does not occur [11,12,13]. Other mechanisms that can affect bronchorelaxation are non-adrenergic non-cholinergic receptor pathway inhibitor, calcium channel antagonist, potassium channel activator, and epithelium-derived relaxing factors such as nitrite oxide and prostaglandin E2 [13,14].

Research's result also shows that MECFR and MFCFR have stronger bronchorelaxation effect on bronchial ring precontracted with methacholine, compared with HFCFR and EAFCFR. This result correlates with the active compound or secondary metabolite content in each extract and fraction. It has been known that MFCFR contains polyphenol, terpenoid, and saponin; EAFCFR contains polyphenol, terpenoid, and anthraquinone; and HFCFR contains steroids and anthraquinone [6,15]. From other researches, it has been shown that polyphenol, terpenoid, saponin, and steroid in other plant's extracts have some effects on the receptor related to the airway smooth muscle relaxation. Examples are as follows: Thymoquinone, a terpenoid widely contained in the essential oil of *Nigella sativa*, protects guinea pigs against histamine-induced bronchospasm. The water extract of *N. sativa* relaxes the tracheal smooth muscle of guinea pig through beta-2 adrenoceptor stimulation [16]. ɣ-sitosterol, found in *Mimosa pudica* L., can relax the smooth muscles of histamine-induced tracheal rings of a guinea pig [17]. Quercetin, a polyphenol (flavonoid group) which is found abundantly in fruits and vegetables, has been reported to have relaxation effects on human's bronchial smooth muscle through the inhibition of acetylcholine and histamine-induced bronchial contraction. It also has effects in the relaxation of guinea pig's bronchus by increasing the rate of cAMP formation such that cAMP-dependent protein kinase is activated and inhibits MLCK through phosphorylation, resulting in the reduction in contraction [7].

There are other studies which have shown plant extracts containing other secondary metabolites in the presence of either polyphenol, terpenoid, saponin, or steroid, having the effect of relaxing airway smooth muscle such as the raw extracts of *Spinacia oleracea* L. seeds which contain phenol, saponin, steroid, and tannin compounds have bronchodilation effect on rabbit trachea samples which were contracted with carbachol and high dosages of K^+ [18]. The ethanol extracts of *Limonia acidissima* Linn. pulp which contain flavonoids, glycosides, saponins, tannins, alkaloids, polyphenol, and sterols have bronchodilatory and antiasthmatic effect on the tracheal chains of guinea pig [19]. Marmin, a coumarin derivative isolated from stem bark and the root of *Aegle marmelos* Corr., has the effect to inhibit the guinea pig tracheal chain contraction induced by a series of histamine concentration in a competitive manner [20]. The stem of *Albizia lebbeck* Benth., *Fabaceae*, which contains tannin, catechin, triterpenoid, and albiziasaponin A, B, and C, has activities in the protection against histamine- and acetylcholine-induced bronchoconstriction on some of the test animal models, as they have exhibited relaxation of smooth muscles, antihistamine, and antispasmodic activities. The extract of *Balanite saegyptiaca* Delile. and *Zygophyllaceae (Balanitaceae*) fruits, which contains steroidal saponin, coumarin, organic acid, balagyptin, balanitin-3 (spirostanol glycoside), balanitin-6 and 7, and other diosgenyl saponin and flavonoid, exhibits bronchodilation effect due to the presence of steroidal saponin [21].

Therefore, similarly applied to this research, the secondary metabolite in MECFR such as polyphenol, saponin, triterpenoid, and steroid has bronchorelaxation activities. The difference in strength of bronchorelaxation effects shown by the MFCFR, EAFCFR, and HFCFR could be affected by the type and quantity of the secondary metabolites in each fraction. From the extraction and fractionation result, EAFCFR and HFCFR yield were the smallest at only 4% while MFCFR yield was 11.9% and MECFR has the highest yield at 21.9% (Table 1). Consequently, the quantities of secondary metabolites in the EAFCFR and HFCFR were lower compared with MFCFR and MECFR which also can affect bronchorelaxation effect.

Polyphenol, saponin, and terpenoid are similar types of the secondary metabolites within MFCFR and MECFR which have stronger bronchorelaxation effect than EAFCFR and HFCFR. Therefore, it can be postulated that the secondary metabolites in CFR responsible for the bronchorelaxation effects are polyphenol, saponin, and terpenoid.

CONCLUSION

The bronchorelaxation effect of the MECFR on guinea pig's bronchial ring is of similar strength with its methanol fraction and of higher efficacy than its ethyl acetate fraction and hexane fraction.

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AUTHORS' CONTRIBUTIONS

Khemasili Kosala contributed in the design experiments, data collection and analysis, and manuscript writing; Moch. Aris Widodo was involved in research planning and supervision; Sanarto Santoso contributed in technical advisory and manuscript revision; and Setyawati Soeharto was involved in data analysis and interpretation.

CONFLICTS OF INTEREST

Authors declare that there are no conflicts of interest.

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