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Bronchodilator effects of *Lignosus rhinocerotis* extract on rat isolated airways is linked to the blockage of calcium entry

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ABSTRACT

Background: *Lignosus rhinocerotis* (Cooke) Ryvarden is a popular medicinal mushroom used for centuries in Southeast Asia to treat asthma and chronic cough. The present study aimed to investigate the effect of this mushroom on airways patency.

Materials and methods: The composition of *L. rhinocerotis* TM02 cultivar was analyzed. Organ bath experiment was employed to study the bronchodilator effect of *Lignosus rhinocerotis* cold water extract (CWE) on rat isolated airways. Trachea and bronchus were removed from male Sprague-Dawley rats, cut into rings of 2 mm, pre-contracted with carbachol before adding CWE into the bath in increasing concentrations. To investigate the influence of incubation time, tissues were exposed to intervals of 5, 15 and 30 min between CWE concentrations after pre-contraction with carbachol in subsequent protocol. Next, tissues were pre-incubated with CWE before the addition of different contractile agents, carbachol and 5-hydroxytrptamine (5-HT). The bronchodilator effect of CWE was compared with salmeterol and ipratropium. In order to uncover the mechanism of action of CWE, the role of beta-adrenoceptor, potassium and calcium channels was investigated.

Results: Composition analysis of TM02 cultivar revealed the presence of β-glucans and derivatives of adenosine. The extract fully relaxed the trachea at 3.75 mg/ml ($p < 0.0001$) and bronchus at 2.5 mg/ml ($p < 0.0001$). It was observed that lower concentrations of CWE were able to fully relax both trachea and bronchus but at a longer incubation interval between concentrations. CWE pre-incubation significantly reduced the maximum responses of carbachol-induced contractions (in both trachea, $p = 0.0012$ and bronchus, $p = 0.001$), and 5-HT-induced contractions (in trachea, $p = 0.0048$ and bronchus, $p = 0.0014$). Ipratropium has demonstrated a significant relaxation effect in both trachea ($p = 0.0004$) and bronchus ($p = 0.0031$), whereas salmeterol has only affected the bronchus ($p = 0.0104$). The involvement of β_2-adrenoceptor and potassium channel in CWE-mediated airway relaxation is ruled out, but the bronchodilator effect was unequivocally affected by influx of calcium.

Conclusions: The bronchodilator effect of L. *rhinocerotis* on airways is mediated by calcium signalling pathway downstream of $G_{\alpha q}$-coupled protein receptors. The airway relaxation effect is both concentration- and incubation time-dependent. Our findings provide unequivocal evidence to support its traditional use to relieve asthma and cough.

Introduction

Asthma is characterized by chronic airway inflammation associated with airway remodelling and airway smooth muscle hypercontractility (Abramson et al., 2014). Patients with chronic cough and asthma are known to have compromised and obstructed airways making breathing

Abbreviations: *L. rhinocerotis*, *Lignosus rhinocerotis*; CWE, Cold water extract; 5-HT, 5-hydroxytrptamine; KCl, Potassium chloride; COPD, Chronic obstructive pulmonary disease; CRC, Concentration-response curve

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difficult. Airway narrowing occurs immediately during acute asthma attack which lasts about 1 h, and sometimes during late-phase asthmatic reaction 4–6 h later in more sensitive individuals. The late-phase airway compromise, which is thought to be induced by chronic inflammation, may last for h to days. On the other hand, chronic obstructive pulmonary disease (COPD) is a progressive disease associated with persistent airflow limitation and enhanced chronic inflammation in the airways and lungs. Both asthmatic and COPD patients often have clinical manifestation of coughing.

Evidence shows that activation of muscarinic receptors (neuronal and non-neuronal) by excessive release of acetylcholine in asthma and COPD contributes to bronchial smooth muscle contraction (bronchoconstriction) and mucus secretion (Erle and Sheppard, 2014). Similarly, 5-hydroxytrptamine (5-HT) has been linked to the pathophysiology of asthma through triggering the same pathway as cholinergic agonist. $G_{\alpha q}$-protein-coupled M_3 muscarinic receptor and 5-HT receptor, especially 5-HT_{2A} receptor subtype on airway smooth muscle, when activated respectively, results in 1, 4, 5-trisphosphate (IP_3) generation and mobilization of extracellular and intracellular Ca^{2+}. The increase in the intracellular Ca^{2+} level subsequently leads to airway smooth muscle contraction (Cazzolau et al., 1995; Erle and Sheppard, 2014). On the other hand, activation of G_s-protein-coupled β_2-adrenoceptor increases $3'-5'$-cyclic adenosine monophosphate (cAMP) level and activates Protein Kinase A (PKA), which traditionally phosphorylate myosin light chain kinase leading to airway smooth muscle relaxation. PKA also opens K^+ channels leading to hyperpolarisation of plasma membrane and subsequently limits extracellular Ca^{2+} entry. This second pathway further relaxes the smooth muscle (Billington et al., 2013).

The current mainstays of treatments for asthma are β_2-adrenoceptor agonist acting as bronchodilator and steroid to reduce inflammation. Despite that COPD is present with chronic airway inflammation, steroid is largely ineffective in attenuating inflammation in COPD and anticholinergic drug constitutes the first-line treatment for COPD. Both salmeterol (β_2-adrenoceptor agonist) and ipratropium (muscarinic antagonist) are used as bronchodilators for maintenance of asthma and COPD. Complications including medication non-compliance, tolerance towards long-term use of β_2-adrenoceptor agonists, and unfavourable side effects of steroid (Lavorini, 2013), which arise from the multiple therapies for asthma, have prompted the development of alternative therapeutic agents to tackle both airway obstruction and inflammation.

Lignosus rhinocerotis (Cooke) Ryvarden is one of the popular medicinal mushrooms consumed by the indigenous communities in Southeast Asia and Southern China for several centuries (Abdullah et al., 2013) . It belongs to the family of Polyporaceae (Basidiomycota) and consists of sclerotium and fruiting body with pileus and stipe (Lai et al., 2013). The sclerotial part was claimed to possess therapeutic properties (Chang and Lee, 2004).

Its history of usage dated back to 1664 when *L. rhinocerotis* was first recorded in the name of "*Lac Tygridis*" (meaning tiger's milk) where it was reported that local communities use it to treat incurable diseases (John et al., 1907). A recent survey (Lee et al., 2009) showed that *Lignosus* is the most common macrofungi used by majority of the indigenous communities in Peninsular Malaysia for medicinal purposes e.g. as a general tonic to treat asthma, cough, fever, cancer and to hasten wound healing. Based on ethnomycological surveys, asthma and cough treatment is the main traditional usage of *Lignosus* (Lau et al., 2015). Preparation methods for these medicinal purposes vary among indigenous communities, but it is mainly consumed orally, for instance, by eaten raw or decoction. There were limited scientific studies on *L. rhinocerotis* a decade ago mainly due to its scarcity. Only until 2009 when cultivation protocols of *L. rhinocerotis* TM02 cultivar on agar, solid and spawn media have been successfully established, the supply for pharmacological studies and therapeutic purpose was made possible to substantiate its traditional claims (Tan, 2009). Anti-proliferative, anti-inflammatory and anti-oxidative activities of *L. rhinocerotis* have been extensively studied (Lau et al., 2015).

Although *L. rhinocerotis* is highly regarded in treating asthma and cough, scant scientific evidence on airways is available. A recently published paper showed that *L. rhinocerotis* hot water extract possesses anti-asthmatic potential on its anti-inflammatory properties (Johnathan et al., 2016). Levels of several key inflammatory mediators in asthma were attenuated and inflammation in the lungs was suppressed in sensitized Sprague–Dawley rats, when compared to the untreated group. However, the study did not report any physiological evidence that the medicinal mushroom could relieve the obstructed airways. Therefore, we aimed to investigate the bronchodilator effects of *L. rhinocerotis* on rat isolated airways and uncover its mechanism of action.

Materials and methods

Cold water extraction

Cultivated ground sclerotial powder of *L. rhinocerotis* (TM02 cultivar) was provided by Ligno Biotech Sdn. Bhd. (Selangor, Malaysia). The mushroom was identified by the internal transcribed spacer (ITS) regions of their ribosomal RNA by Tan et al. (2010). The mushroom specimen voucher is deposited in the Royal Botanic Garden Kew, K(M) 177812. The preparation method for the cold water extract (CWE) was adopted from previous paper (Yap et al., 2013). Sclerotial powder was dissolved in purified water (Milli-Q quality) at a mass/volume ratio of 1:20 (g/ml), followed by 24 h stirring at 4–7 °C. The mixture was then filtered and freeze-dried. Extracts were kept at −80 °C for long-term storage. The percentage yield of the CWE was 10% (w/w). CWE was dissolved in purified water to make stock concentration of 100 mg/ml prior to the experiment.

Composition analysis of TM02 cultivar

Alpha-glucan and beta-glucan analysis of TM02 freeze-dried powder was performed using Megazyme glucan test kit, with pre-treatments using chitinase and alpha-amylase (Sigma–Aldrich, USA). 3′-deoxyadenosine and adenosine analysis was adopted from the method by Furuya et al. (1983). Polysaccharide analysis was performed following hydrolysis in sealed tubes with 2 MTFA at 100 °C for 5 h. The hydrolyzates were evaporated to dryness. The residue was repeatedly evaporated with methanol until the acid had been completely removed. The molar ratios of neutral sugars were determined by converting them to their alditol acetates which were separated and quantified by gas chromatography on a DB-225 fused silica capillary column at 210 °C (15 m × 0.25 mm, J&W Scientific). Standard reference materials were obtained from Sigma–Aldrich, USA. Glycoprotein analysis was performed on pre- and post-amylase digested sample (control sample digested with placebo). Free carbohydrate was isolated by dialysis; resultant dialysate was dehydrated and subjected to elemental nitrogen content determined on a Flash 4000 Nitrogen/Protein analyzer. Sample pyrolized at 950 °C over palladium/copper catalyst in combustion gases carried by dry helium stream was analyzed by gas chromatography with thermal conductivity detection. Calibration curve from EDTA nitrogen was used for quantification. Authentic reference materials were obtained from Thermo Scientific/CE Inc.

Tissues preparation

Ethics approval was obtained from the University of Nottingham's Animal Welfare and Ethics Review Body (UNMC#2kn) and the research complied with the European guidelines for laboratory animal use and care (86/609/EEC). The experiments were conducted with male Sprague–Dawley rats (240–480 g; 2–3 months old). Animals were purchased from University Putra Malaysia and sacrificed on the day of experiment by carbon dioxide or cervical dislocation under anaesthesia with diethyl ether. Trachea and bronchus were removed, excised into

2 mm rings, and immediately immersed in standard cold Krebs solution (pH 7.4). All the methods used in this study were adopted from Loong et al. (2015). Changes in muscle tension responses produced by tissue contraction and relaxation were detected by force transducer (MLTF050/ST, ADInstruments, US). Data was recorded by a PowerLab data acquisition system (LabChart v7.3.4) and a computer (Hewlett–Packard, US). Muscle tension was expressed in unit of milliNewton (mN). Tissues were left to equilibrate to bath condition for 30 min after the application of tension of 9.8 mN. Following equilibration, all tissues were exposed twice to 60 mM potassium chloride (KCl) to assess tissue viability and to provide reference contracture for subsequent data analysis.

Experimental protocol

Effect of the CWE and incubation time on carbachol-induced contraction

The tone of the airways segments was induced with carbachol (1 μM) to obtain a stable submaximal contraction size between 70% and 80% of the maximum carbachol contraction. Upon establishing stable contractions, cumulative concentration-response curves (CRC) to CWE at time intervals of 5, 15 or 30 min between concentrations were constructed. CRC to adenosine, ipratropium (muscarinic antagonist) or salmeterol (β_2-adrenoceptor agonist) were constructed in different tissues pre-contracted with carbachol. Vehicle control, either purified water or the appropriate concentration of DMSO, was carried out in all the following protocols. Only one CRC was obtained per tissue. The degree of relaxation was measured as a fraction of the contraction achieved by 1 μM of carbachol and has been expressed as a percentage of carbachol-induced tone.

Effect of CWE pre-incubation on carbachol and 5-hydroxytryptamine (5-HT)-induced contractions

CWE at a final concentration of 3.5 mg/ml (this concentration was chosen to ensure 100% relaxation in all the airway segments) was added into the bath at least 30 min before construction of cumulative CRC to carbachol and 5-HT for both tracheal and bronchial rings. Adenosine (0.1 mM) was added into the bath 30 min before construction of cumulative CRC to carbachol for both tracheal and bronchial rings. The degree of contraction was measured as a fraction of the contraction from basal tension and has been expressed as a percentage of 60 mM KCl-induced tone.

Elucidation of the bronchodilation effect of CWE in airway segments

Non selective beta-adrenoceptor antagonist, propranolol (30 μM) or voltage-gated potassium channel blocker, tetraethylammonium chloride (TEA) (10 mM) was added into the bath 30 min before addition of carbachol (1 μM) to obtain a stable submaximal contraction. Upon establishing stable contractions, cumulative CRC to CWE at 15 min interval between concentrations were constructed. The degree of relaxation was measured as a fraction of the contraction achieved by 1 μM carbachol and has been expressed as a percentage of carbachol-induced tone.

To create a calcium-free environment, airway segments were immersed in Krebs solution without Ca^{2+}. Tissues were initially pre-incubated with CWE 3.5 mg/ml, nifedipine 10 μM or DMSO 0.1% v/v as vehicle control for 30 min, followed by introduction of increasing concentrations of calcium chloride to cause contraction. Nifedipine, the L-type calcium channel blocker, serves as a positive control. The degree of contraction was measured as a fraction of the contraction from basal tension and has been expressed as a percentage of 60 mM KCl-induced tone.

Statistical analysis

Data were analysed and graphs were drawn using PRISM v6.0 (Graph pad software). All data were expressed as mean ± standard error of mean (SEM) of *n* number of animals. Maximum tissue contraction or relaxation response (E_{max}) and pEC_{50} were derived from nonlinear regression analysis of the obtained CRC. The pEC_{50} value is the negative logarithm of EC_{50} where EC_{50} is the concentration of drug which gives 50% of its maximum response. Statistical analyses were performed using one-way ANOVA, two-way ANOVA with Bonferroni correction and unpaired *t*-test. Results were considered statistically significant if p-value < 0.05.

Drugs

Carbamycholine chloride or carbachol (Nacalai Tesque, Japan), 5-hydroxytryptamine (Nacalai Tesque, Japan), (*S*)-(-)-propranolol hydrochloride (Santa Cruz, USA), tetraethylammonium chloride (Sigma–Aldrich, USA) and ipratropium bromide (Tocris, UK) were dissolved in purified water. Adenosine (Tocris, UK), salmeterol xinafoate (Tocris, UK) and nifedipine (Nacalai Tesque, Japan) were dissolved in DMSO (Sigma-Aldrich, USA).

Results

Composition of TM02 cultivar

The composition analysis results of *L. rhinocerotis* TM02 cultivar freeze-dried powder was summarized in Table 1. It was found that the powder material contains largely β-glucans, polysaccharides and glycoproteins. It also contains trace amount of adenosine and its derivatives including adenine, 3′-deoxyadenosine, ethyl- and hydroxyethyl-adenosine.

Bronchodilation effect of the CWE is time-dependent

In order to detect any relaxation induced by CWE, we used carbachol to pre-contract rat isolated airways. Fig. 1A shows that CWE produced marked relaxation in rat trachea and bronchus. In trachea, the tissue was significantly relaxed from 2.50 mg/ml to 3.75 mg/ml and in bronchus, tissue was significantly relaxed from 2.0 mg/ml to 2.5 mg/ml, when compared to vehicle control. The extract fully relaxed the trachea (E_{max}: 106.10 ± 4.69%; $p < 0.0001$) at 3.75 mg/ml and bronchus (E_{max}: 106.45 ± 8.51%; $p < 0.0001$) at 2.5 mg/ml. The relaxation effect of vehicle control in both tracheal and bronchial rings was negligible. Fig 1B shows a recording trace of the relaxation effect of CWE on the bronchus pre-contracted with carbachol.

To investigate impact of incubation time, tissues were exposed to the extract at varying time intervals of 5 min, 15 min, and 30 min between concentrations. The results demonstrated that at the same highest concentration used in trachea, which is CWE 2.75 mg/ml, magnitude of tissue relaxation increased with incubation duration (E_{max}: 11.92 ± 3.21%, 5 min; 61.31 ± 15.79%, 15 min; 118.19 ± 7.93%, 30 min) (Fig. 1C). Similar finding was observed in bronchus at CWE 2.0 mg/ml (E_{max}: 21.49 ± 11.66%, 5 min;

Table 1
Composition analysis of *L. rhinocerotis* TM02 cultivar freeze-dried powder.

Analyte	Content (mg/g)
Total polysaccharides	401.4
Beta-1,3/1,6-glucan	286.8
Glycoprotein	186.1
Alpha-glucan	29.91
3′-deoxyadenosine	0.873
Ethyl-adenosine	0.399
Adenosine	0.282
Hydroxyethyl-adenosine	0.268
Adenine	0.024

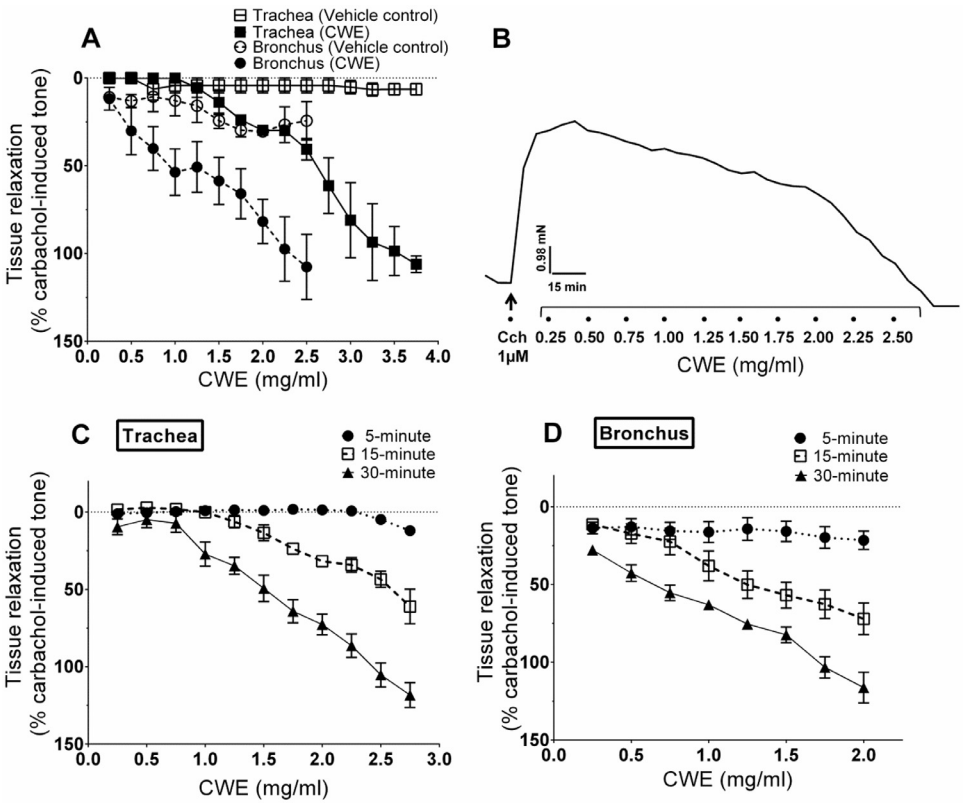

Fig 1. Effect of CWE cumulative CRC against carbachol-induced contractions of the rat isolated (A) trachea and bronchus. (B) Representative trace recording of the relaxation effect of CWE on rat isolated bronchus pre-contracted with carbachol. Effect of different time intervals between concentrations of CWE i.e. 5-, 15- and 30-min against carbachol-induced contractions of the rat isolated (C) trachea (D) bronchus. Tissue responses have been expressed as a percentage of carbachol-induced contraction and are shown as means ± SEM of 3–5 animals.

72.04 ± 10.05%, 15 min; 116.25 ± 9.78%, 30 min) (Fig. 1D). Overall, the findings suggest that the longer the tissues were exposed to the extract, a greater relaxation response was observed for each concentration tested.

Subsequent experiment tested the airway relaxation effect of positive controls. Results revealed that in the rat isolated airways pre-contracted by carbachol, ipratropium effectively relaxed the airways segment in trachea (E_{max}: 117.80 ± 6.22%; $p = 0.0004$, Fig. 2A) and in bronchus ($E_{max:}$ 113.40 ± 1.64%; $p = 0.0031$, Fig. 2B) compared to vehicle control. Salmeterol showed significant relaxation in bronchus (E_{max}: 104.40 ± 16.33%; $p = 0.0104$, Fig. 2B) but minimal effect in the trachea ($p > 0.05$, Fig. 2A) compared to vehicle control. The relaxation effect of vehicle control in both airway segments was negligible.

It was previously reported that adenosine can cause airway contraction indirectly via mast cell activation and postganglionic vagal neurons. However, the direct effect of adenosine on airway smooth muscle contractility remains inconclusive (Burnstock et al., 2012). Since adenosine and derivatives were detected in TM02 cultivar (see Table 1), we assessed the effect of adenosine in the basal tension and in carbachol-pre-contracted rat isolated trachea and bronchus. Our results revealed that adenosine did not affect the airway segments tested (data not shown).

CWE attenuates carbachol and 5-hydroxytryptamine (5-HT)-induced contractions

As the rat isolated airways in our study were not responsive to other contractile agent i.e. histamine, we have therefore used muscarinic agonist (carbachol) and 5-HT to assess the incubation effect of CWE. Pre-incubation of tissues with CWE 3.5 mg/ml has significantly reduced the E_{max} of carbachol-induced CRC in rat trachea ($p = 0.0012$) and bronchus ($p = 0.001$) (Fig. 3A and 3B). Meanwhile, adenosine did not have any significant effect on the E_{max} and pEC_{50} values of carbachol CRC compared to vehicle control group in both trachea and bronchus

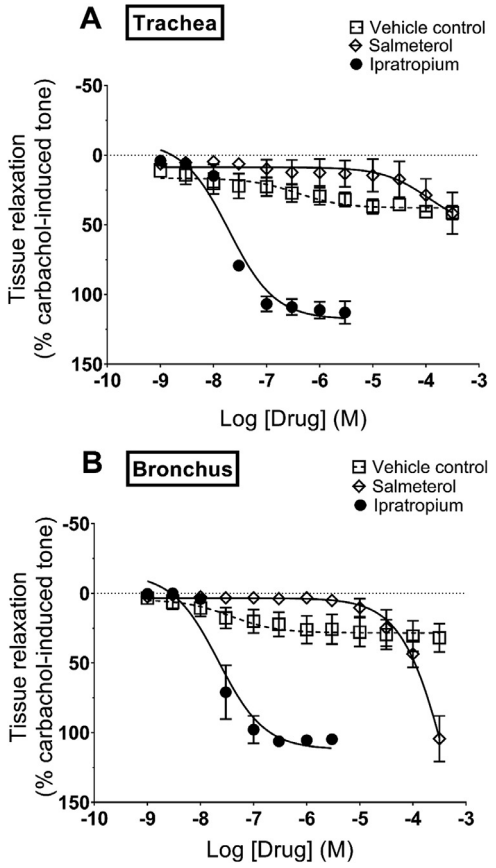

Fig 2. Effect of ipratropium and salmeterol cumulative CRC against carbachol-induced contractions of the rat isolated (A) trachea and (B) bronchus. Tissue responses have been expressed as the percentage of carbachol-induced tone and are shown as means ± SEM of 3 animals.

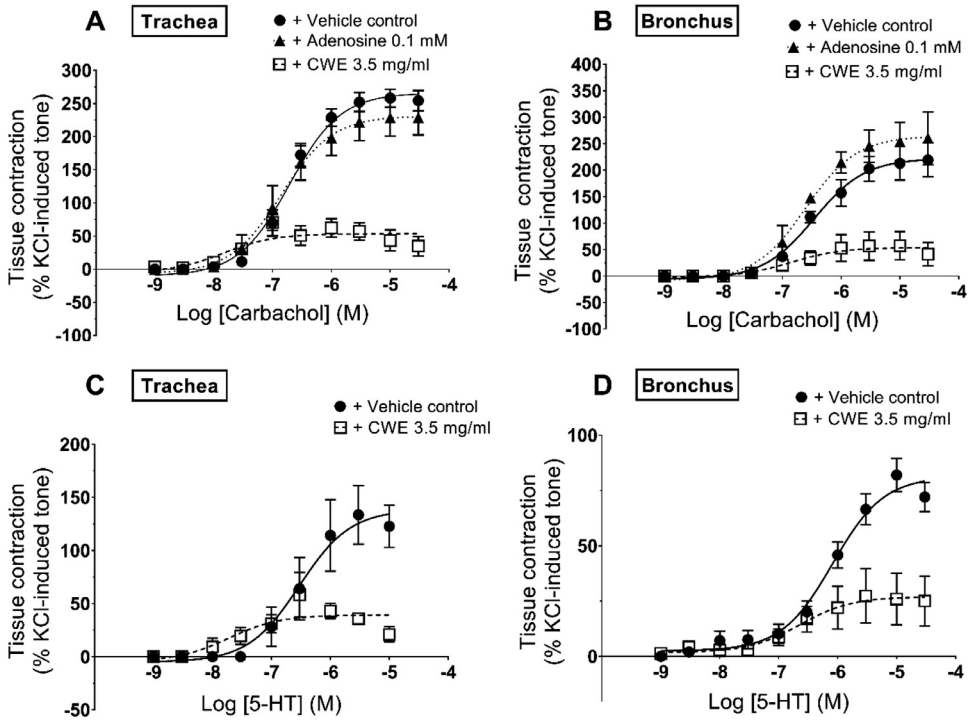

Fig 3. Effect of CWE and adenosine pre-incubation on carbachol CRC in rat isolated (A) trachea and (B) bronchus. Effect of CWE pre-incubation on 5-hydroxytrptamine CRC in rat isolated (C) trachea and (D) bronchus. Tissues were pre-incubated with adenosine, CWE or vehicle control 30 min before carbachol or 5-HT CRC. Tissue contractions are expressed as a percentage of 60 mM KCl-induced contraction and shown as mean values ± SEM of 3–9 animals.

Table 2
The maximum response (E_{max}) and pEC_{50} of carbachol and 5-hydroxytrptamine (5HT) derived from the respective concentration response curve in the presence of different treatment. Unpaired *t*-test where comparison of the mean was made between vehicle control and the treatment group; $^*p < 0.05$; $p < 0.01$. The data represented the means ± SEM of n number of animals.

		Trachea			Bronchus		
Compounds		n	E_{max} (%)	pEC_{50}	n	E_{max} (%)	pEC_{50}
Carbachol	Control (water)	4	237.50 ± 17.99	6.88 ± 0.10	4	286.80 ± 28.43	6.42 ± 0.11
	+ CWE (3.5 mg/ml)	3	53.46 ± 13.21**	7.82 ± 0.66**	4	54.97 ± 26.04**	6.98 ± 0.22*
Carbachol	Control (DMSO 0.1%)	3	260.50 ± 15.90	6.71 ± 0.09	3	220.50 ± 31.39	6.42 ± 0.08
	+ Adenosine 0.1 mM	3	229.50 ± 25.43	6.80 ± 0.06	3	260.8 ± 44.11	6.62 ± 0.09
5HT	Control (water)	3	139.70 ± 13.20	6.45 ± 0.11	9	83.37 ± 7.36	6.19 ± 0.16
	+ CWE (3.5 mg/ml)	3	53.46 ± 7.60**	7.31 ± 0.39*	9	27.41 ± 12.59*	7.01 ± 0.92

($p > 0.05$, Fig. 3A and 3B). Similarly, the maximum response of 5-HT CRC was significantly reduced by CWE 3.5 mg/ml pre-incubation in trachea ($p = 0.0048$) and in bronchus ($p = 0.0014$) (Fig. 3C and 3D). CWE has slightly shifted the 5-HT curve to the left in trachea ($p = 0.0487$), but not in bronchus. The pEC_{50} and E_{max} values were recorded in Table 2. We also observed that the E_{max} of 5-HT CRC in trachea was significantly higher than that in bronchus ($p = 0.0035$).

Bronchodilation effect of CWE in airway segments is linked to regulation of calcium entry

Additionally, we investigated the roles of beta-adrenoceptor and potassium channel in the bronchorelaxation effect induced by CWE. As shown in Fig. 4, incubation of tissues with propranolol or TEA did not significantly reduce the relaxation response induced by CWE in both tracheal and bronchial rings ($p > 0.05$).

It is known that a higher level of intracellular Ca^{2+} leads to smooth muscle contraction. The source of Ca^{2+} may be from the sarcoplasmic reticulum store or through opened membrane Ca^{2+} channels. To assess the involvement of extracellular Ca^{2+} in tissue contraction, we removed extracellular Ca^{2+} source during pre-incubation with CWE or nifedipine and subsequently contracted the tissues with re-introduction of Ca^{2+}. Pre-incubation of isolated trachea with CWE (E_{max}: 16.21 ± 7.93%, $p < 0.0001$) and nifedipine ($E_{max:}$ 40.87 ± 10.96%, $p < 0.001$) showed a significantly supressed Ca^{2+}-induced contraction compared to control (101.88 ± 9.10%) (Fig. 5A). A similar outcome was observed in bronchus, in which CWE (E_{max}: 92.01 ± 14.13%, $p < 0.01$) and nifedipine (E_{max}: 31.31 ± 8.76%, $p < 0.0001$) also significantly reduced the maximum response of Ca^{2+}-induced contraction compared to control (144.51 ± 8.23%) (Fig. 5B). The entry of Ca^{2+} in response to KCl is shown to be mainly mediated via L-type calcium channel as nifedipine substantially suppressed the Ca^{2+}-induced contraction in both K^+-depolarised tracheal and bronchial rings.

Discussion

Medicinal fungi such as *Cordyceps sinensis* and *Ganoderma lucidum* are reported to possess anti-asthmatic properties, as bronchodilators and immunomodulators, but the active chemical constituents have not been fully identified (Liu et al., 2003; Yue et al., 2008). Many Basidiomycetes mushrooms contain biologically active polysaccharides, mainly β-glucans, with immunomodulating activities (Wasser, 2003). Previous phytochemical analysis revealed that CWE contains varying composition of carbohydrates and proteins due to the differences in extraction methods and cultivar (Lau et al., 2015). Our composition analysis results are agreeable with previous study that carbohydrates are the major constituent in TM02 cultivar (Yap et al., 2013), with β-glucan being the major polysaccharide. We have also identified a series

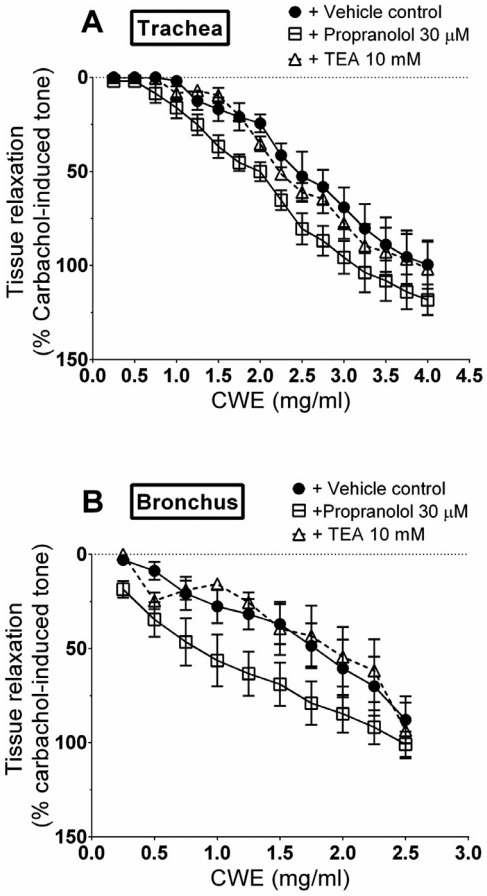

Fig 4. Role of beta-adrenoceptor and potassium channel in CWE-induced airway relaxation in rat isolated (A) trachea and (B) bronchus. Tissues were pre-incubated with propranolol, TEA, or vehicle control 30 min before contraction with carbachol, followed by CWE CRC. Tissue responses have been expressed as a percentage of carbachol-induced contraction and are shown as means ± SEM of 3–6 animals.

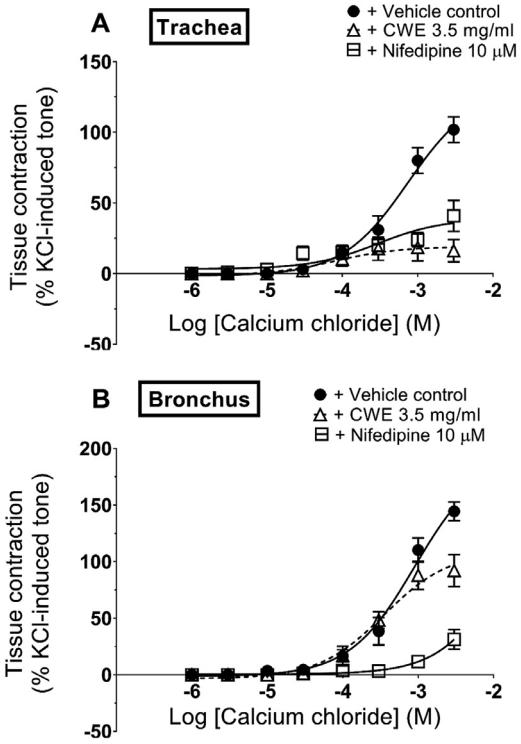

Fig 5. Effect of CWE on calcium-induced contractions in rat isolated (A) trachea and (B) bronchus. Tissues were pre-incubated with CWE, nifedipine or vehicle control in calcium-free environment for 30 min, followed by addition of calcium chloride to reintroduce calcium ion. Tissue responses have been expressed as a percentage of KCl-induced contraction and are shown as means ± SEM of 5–8 animals. One-way ANOVA comparison of the mean (E_{max}) between control, treatment groups (A): $p < 0.001$ and (B) $p < 0.01$.

of adenosine derivatives in TM02 cultivar.

Our results demonstrated that CWE displayed bronchodilator properties in rat airways, in support of its traditional claims for relieving asthma and chronic cough. This study showed that a longer incubation period of CWE enhanced the bronchorelaxation response of the airway rings. Previous papers have proposed that the bioactive components in CWE could be polysaccharides (Yap et al., 2013), polysaccharide-protein complexes or proteins (Lee et al., 2012, 2014). It is speculated that the carbohydrates form complexes with these proteins to become large molecular weight bioactive molecules, and the resultant polysaccharide-protein complexes are less likely to penetrate tissues directly (El Enshasy and Hatti-Kaul, 2013; Schmidt et al., 2009). Therefore, we propose that the observed effects were modulated by cell surface receptors (Brown and Gordon, 2003; El Enshasy and Hatti-Kaul, 2013), whereby the longer response time observed may be linked to time required for these large molecules to adsorb and bind to these surface proteins/receptors. In an earlier study, a polysaccharide isolated from marine filamentous fungus *Phoma herbarum* exhibited a time-dependent binding to macrophages, possibly via the Toll-like receptors or complement receptor CR3 which led to significant activation of P38 kinase (Chen et al., 2009). The observation that the potency of CWE was enhanced with prolonged incubation is somewhat resembling the action of the polysaccharide isolated from *Phoma herbarum*. Hence, it is plausible that the large polysaccharides, proteins or polysaccharide-protein complex may partly contribute to the time-dependent properties of the mushroom's airway relaxant effect. These results form the basis for further studies looking into the bronchodilation effect of different molecular weights of CWE through fractionation.

Adenosine has been reported to exhibit differing constrictor and relaxant responses on airways, mainly due to the expression of various adenosine receptor subtypes in different species. Previous studies reported that adenosine contracted the airways via A_1 and A_{2B} receptors in rat and mice and via A_3 receptor in rat, guinea pig, and mice (Spicuzza et al., 2006). On the other hand, adenosine and analogues caused relaxation in carbachol-precontracted guinea pig airway smooth muscle (Burnstock et al., 2012). A previous study using Brown Norway rat showed that only sensitized and ovalbumin-challenged rats airways responded to adenosine and adenosine receptor agonists (Hannon et al., 2002). Similar to our findings, Hannon et al. (2002) did not observe any response to adenosine in non-sensitized rats (control). This reconciles with an observation reported in human where adenosine-induced contraction is more pronounced in asthmatics patients when compared with healthy subjects (Spicuzza et al., 2006). Expression of A_1 receptor on bronchial tissue was also shown to be higher in ovalbumin-challenged Wistar rats compared to control rats (Alfieri et al., 2012). We did not observe any response to adenosine in the airways segments as these tissues were obtained from untreated and non-ovalbumin sensitized rats. Therefore, we have excluded the possibility of adenosine being the main bioactive component in *L. rhinocerotis*. Nevertheless, as crude extract contains various phytochemical constituents, we cannot rule out the possibility of synergistic effect from multiple components causing the bronchorelaxation effect observed.

Bronchodilators such as β_2-adrenoceptor agonist (salmeterol) and muscarinic antagonist (ipratropium) are often used as reliever in asthma and COPD. Our results clearly showed that the efficacy of CWE is comparable to ipratropium, and better than salmeterol in relaxing airway contraction. Both CWE and ipratropium successfully relaxed

tracheal and bronchial rings for more than 100%. In this study, salmeterol exhibited less pronounced airway relaxation effect compared to ipratropium. A previous study showed that the density of β_2-adrenoceptor increases from the upper to lower airways by using radioligand binding assays in guinea pig (Carswell and Nahorski, 1983). Higher intensity of β_2-adrenoceptor mRNA was also identified in smaller airway smooth muscle of human and rat (Hamid et al., 1991). Hence, the possibility of low β_2-adrenoceptor abundance in upper airway may explain the lack of airway relaxant effect of salmeterol in trachea.

In our study, it was noted that CWE possessed antagonistic effects on airway contraction induced by carbachol as well as 5-HT, from the marked reduction in the maximum responses. The antagonistic effects appear to be non-competitive as increasing concentrations of both agonists did not reverse the inhibition. The similar inhibitory effect of CWE on both carbachol and 5-HT infers that the relaxation of the airway smooth muscles is mediated via regulation of downstream calcium signalling pathway of the $G_{\alpha q}$-protein-coupled receptor. Additionally, the results from mechanistic studies using propranolol and TEA have ruled out that CWE stimulates the β_2-adrenoceptor or the membrane K^+ channel to elicit bronchorelaxation. We therefore proposed that one of the plausible mechanisms of action of CWE is blocking Ca^{2+} influx into the airway smooth muscle.

Subsequent calcium-free experiment demonstrated that pre-treatment with CWE significantly suppressed calcium-induced contractions in trachea and bronchus. We confirmed the importance of Ca^{2+} influx in modulating the airways patency by using nifedipine, a L-type calcium channel blocker. Nifedipine exhibited the same degree of pronounced inhibition in trachea and bronchus thus implies that the expression of the L-type calcium channel is similar in these airway branches. Our claim that CWE affects extracellular Ca^{2+} influx into cells is further substantiated by another set of evidence from calcium imaging assays. Incubation of CWE significantly suppressed Ca^{2+} flux into rat dorsal root ganglion cells and this effect is mirrored by nifedipine (see Supplementary Material).

Airway smooth muscle contraction takes place almost immediately after exposure to allergen or irritation during acute asthma attack. The late-phase asthmatic reaction, which may occur h following the initial allergen provocation, is associated with the recruitment and activation of various proinflammatory mediators, contributing to airway hyperresponsiveness. The time-dependent and antagonistic properties of the CWE on airway segments contracted by muscarinic and 5-HT receptors activation suggests that instead of providing immediate bronchodilatory effect for acute asthma attack, *L. rhinocerotis* may have potential benefit for preventing and relieving airway obstruction which takes place in the late-phase asthmatic reaction and COPD. This airway relaxant effect of CWE corroborates with a recently published paper on the in vivo inhibitory effects of *L. rhinocerotis* on ovalbumin-induced airway inflammation (Johnathan et al., 2016).

Conclusion

To the best of our knowledge, our group is the first to report on the bronchodilator effects of *L. rhinocerotis* on respiratory tract supporting its traditional uses for asthma and cough by relieving the symptom of breathing difficulties. Our findings also showed that the effect of *L. rhinocerotis* is concentration- and incubation time-dependent, with efficacy comparable and better than bronchodilators, ipratropium and salmeterol. The airway relaxation effect of *L. rhinocerotis* is not linked to activation of β_2-adrenoceptor or potassium channels but likely to inhibit the influx of extracellular Ca^{2+}.

Conflict of interest

Ligno Biotech Sdn Bhd supplied the sclerotial powder of Lignosus rhinocerotis. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2018.03.025.

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