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In vitro growth-inhibitory effect of Cambodian essential oils against pneumonia causing bacteria in liquid and vapour phase and their toxicity to lung fibroblasts

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ABSTRACT

Essential oils hydrodistilled from seven Cambodian plant species (*Alpinia oxymitra*, *Boesenbergia rotunda*, *Cinnamomum cambodianum*, *Citrus lucida*, *Limnophila aromatica*, *Rhodamnia dumetorum*, and *Sindora siamensis*) were tested for their *in vitro* growth-inhibitory effect against pneumonia causing bacteria (*Haemophilus influenzae*, *Streptococcus pneumoniae*, *Staphylococcus aureus*) using the broth microdilution volatilisation method. Additionally, a modified thiazolyl blue tetrazolium bromide assay was performed for evaluation of their cytotoxic activity to human lung cells. All essential oils exposed some antibacterial efficacy; however, only *A. oxymitra* rhizome oil was active against all bacteria tested. *A. oxymitra* pericarp oil was found as the most effective antibacterial agent against *H. influenzae* in liquid and solid medium with the respective lowest minimum inhibitory concentrations of 64 and 32 μg/mL. Due to its high value for 80% inhibitory concentration of proliferation (>512 μg/mL), this essential oil may be considered as safe to human lung cell lines. Using dual-column/dual-detector system GC/MS analysis, β-pinene was identified as the main constituent of *A. oxymitra* leaves, pericarp and rhizome oils, while volatile oil from *A. oxymitra* seeds consisted predominantly of shyobunol. The major constituents of *B. rotunda*, *C. lucida*, *L. aromatica*, *R. dumetorum*, and *S. siamensis* oils were ocimene, decyl acetate, limonene, caryophyllene epoxide, and β-bourbonene, respectively. 1,8-cineole was the major compound of *C. cambodianum* bark and leaf essential oils. Based on these results, *A. oxymitra* pericarp oil can be considered as an effective antibacterial agent with application potential for the development of inhalation therapy against respiratory infections.

1. Introduction

Pneumonia belongs to the leading causes of morbidity and mortality, especially in low-income countries. The majority of severe episodes occurs in children under five years, the elderly and immuno-compromised individuals (Nguyen et al., 2017). In Cambodia, about 9100 children die from pneumonia every year (Ginsburg et al., 2014) and according to the World Health Organisation (WHO) (WHO, 2017) only 64.2% of children with pneumonia symptoms are taken to an appropriate healthcare provider. This acute respiratory infection of lung parenchyma is caused by bacterial pathogens such as *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Staphylococcus aureus* (Mandell, 2015). A timely antibiotic therapy can considerably reduce fatal cases of pneumonia (Sazawal and Black, 2003), nevertheless many low-income countries have limited access to health services and synthetic drugs as well, whereas less than 40% of children are treated with antibiotics in Cambodia (WHO, 2017).

The plant essential oils are of great potential for the development of novel antimicrobial preparations. They have been widely used for their diverse biological effects since the Middle Ages (Bakkali et al., 2008). Since the presence of volatile compounds is characteristic for some plant taxa, chemotaxonomic research is a frequent approach to their exploration. Due to the volatility of essential oils, they are suitable for inhalation therapy, which is an effective way for the healing of respiratory ailments such as pneumonia. Their vapours can act directly on the site of infection in the respiratory system and simultaneously restrict systemic exposure, degradation of active components in the gastrointestinal tract

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and associated toxicity (Kuzmov and Minko, 2015). In addition, essential oils contain a broad spectrum of chemically diverse substances with antimicrobial effect: thus it is more difficult for bacterial pathogens to develop resistance to these multi component mixtures than to single-ingredient conventional antibiotics (Yang et al., 2015). During the last few years, several inhalation devices and suitable delivery systems for essential oils in the treatment of respiratory infections (*e.g.* pocket inhaler, aromatherapy patch, decongestant on a foraminous carrier, and encapsulated essential oils) have been developed and patented (Horvath and Acs, 2015).

In Cambodia, after several decades of human destruction and the collapse of all social welfare systems during the Pol Pot regime, medicinal plants are considered as a very important factor for health security, and traditional Khmer herbal medicine remains the oldest and the most accessible source of primary health care (Bith-Melander and Efird, 2008). Cambodia also possesses rich natural resources and unique original ecosystems *e.g.* the Cardamom Mountains, which contain a number of endemic plant taxa belonging to essential oil-bearing families such as Zingiberaceae, Lauraceae and Myrtaceae (Chassagne et al., 2016). Nevertheless, scientific validation and identification of many Cambodian medicinal plants, as well as assessment of their anti-infective properties, active substances content and safety, are desirable. Recently, several *in vitro* studies have investigated biological activity and revealed some antibacterial potential of essential oils derived from different parts of Cambodian plant species that are easily available in traditional markets and in wild nature (Norajit et al., 2007; Phanthong et al., 2013). However, no experiments determining their antibacterial potential in vapour phase against pathogens causing pneumonia had been carried out until now.

In this article, we report a detailed examination of *in vitro* growth-inhibitory effect of essential oils from seven Cambodian medicinal and edible plant species against pneumonia causing bacteria in liquid and vapour phase by using a new broth microdilution volatilisation method recently developed by our team (Houdkova et al., 2017). This is the first practical application of this novel method in the field of essential oils. Additionally, the cytotoxicity and chemical composition of tested essential oils were analysed with the aim of assessing the relationship between their antimicrobial potential, chemistry and safety for treatment of pneumonia.

2. Materials and methods

2.1. Plant material

Based on chemotaxonomic criteria, seven local plant species (*Alpinia oxymitra* K. Schum., *Boesenbergia rotunda* (L.) Mansf., *Cinnamomum cambodianum* Lecomte, *Citrus lucida* (Scheff.) Mabb., *Limnophila aromatica* (Lam.) Merr., *Rhodamnia dumetorum* (DC.) Merr. & L.M. Perry, *Sindora siamensis* Miq.) were selected as phytochemically less explored representatives of taxa containing essential oils. The plant material was collected between July and September 2016. *A. oxymitra*, *C. cambodianum*, *R. dumetorum*, and *S. siamensis* were collected from various districts of Cambodia (Cardamom Mountains, Elephant Mountains, Chant Saen Commune in Oudong District) from wild populations of at least three independent plants. *B. rotunda*, *C. lucida*, and *L. aromatica* were purchased in local markets (Psar Thmei, Chbar Ampov, and Cham Kar Dong in Phnom Penh). Identification of species was performed in the field by ethnobotany expert Prof Ladislav Kokoska, currently head of the Laboratory of Ethnobotany and Ethnopharmacology of the Faculty of Tropical AgriSciences, Czech University of Life Sciences Prague. Voucher specimens were deposited in the herbarium of the Department of Botany and Plant Physiology of the Faculty of Agrobiology, Food and Natural Resources of the Czech University of Life Sciences Prague (Czech Republic). A detailed description of collected plant samples is summarised in Table 1.

2.2. Preparation of essential oils

Essential oils were obtained by hydrodistillation of dried plant material (except *C. lucida* fruit peel which was obtained by the grating of fresh fruits using a stainless steel grater) in 1 L of distilled water for 3 h using a Clevenger-type apparatus (Merci, Brno, CZ) according to the procedures described in the European pharmacopoeia (2013). The essential oils were stored in sealed glass vials at 4 °C. The data on yields (*v/w*, based on the dry plant weight) of obtained essential oils are shown in Table 1.

2.3. Bacterial strains and culture media

The following standard strains of the American Type Culture Collection (ATCC) were used: *Haemophilus influenzae* ATCC 49247, *Staphylococcus aureus* ATCC 29213, and *Streptococcus pneumoniae* ATCC 49619. The cultivation and assay media (broth/agar) were Mueller-Hinton (MH) complemented by yeast extract and Haemophilus Tested Medium (*H. influenzae*), MH (*S. aureus*), and Brain Heart Infusion (*S. pneumoniae*). The pH of broths was equilibrated to a final value of 7.6 using Trizma base (Sigma-Aldrich, Prague, CZ). All microbial strains and cultivation media were purchased from Oxoid (Basingstoke, UK).

Stock cultures of bacterial strains were cultivated in appropriate medium at 37 °C for 24 h prior to the testing and then the turbidity of the bacterial suspension was adjusted to 0.50 McFarland standard using Densi-La-Meter II (Lachema, Brno, CZ) to get the final concentration of 10^7 CFU/mL. The susceptibilities of *H. influenzae*, *S. aureus*, and *S. pneumoniae* to ampicillin (84.5%, CAS 69-52-3), oxacillin (86.3%, CAS 7240-38-2) and amoxicillin (90%, CAS 26787-78-0), respectively, purchased from Sigma-Aldrich (Prague, CZ), were checked as positive antibiotic controls (CLSI, 2015).

2.4. Antimicrobial assay

The antibacterial potential of plant essential oils in liquid and vapour phase was determined using a broth microdilution volatilisation

Table 1
Plant species selected for antibacterial and cytotoxicity testing.

Scientific name	Family	Collection number	Area of collection	Part used	Weight of sample (g)	Essential oil yield % (*v/w*)	Essential oil colour
Alpinia oxymitra K. Schum.	Zingiberaceae	02463KBFR6	Mt Aoral	Leaves	49.00	0.35	Colourless
				Pericarp	12.30	0.32	Pale yellow
				Rhizomes	125.40	0.04	Colourless
				Seeds	21.90	4.65	Colourless
Boesenbergia rotunda (L.) Mansf.	Zingiberaceae	–	Chbar Ampov	Rhizomes	109.90	0.34	Colourless
Cinnamomum cambodianum Lecomte	Lauraceae	02455KBFR7	Mt Aoral	Bark	44.50	0.45	Pale yellow
				Leaves	41.70	0.38	Pale yellow
Citrus lucida (Scheff.) Mabb.	Rutaceae	02476KBFRA	Cham Kar Dong	Fruit peel	74.50	0.43	Pale yellow
Limnophila aromatica (Lam.) Merr.	Plantaginaceae	02469KBFRC	Psar Thmei	Aerial part	13.80	1.20	Slightly yellow
Rhodamnia dumetorum (DC.) Merr. & L.M.Perry	Myrtaceae	02458KBFRA	Oudong	Leaves	164.80	0.18	Bright yellow
Sindora siamensis Miq.	Leguminosae	02481KBFR6	Angkor Chey	Fruit husk	44.00	0.45	Pale yellow