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New approach to the characterization and quantification of *Antrodia cinnamomea* benzenoid components utilizing HPLC-PDA, qNMR and HPLC-tandem MS: Comparing the wild fruiting bodies and its artificial cultivated commercial products

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ABSTRACT

Antrodia cinnamomea (AC) is one of the Far East's treasured medicinal mushrooms and a major ingredient in different nutritional supplements targeting several serious ailments. Developing a sensitive simple quality control protocol for AC and its products is an unmet goal which we target in the current investigation through detecting its biomarkers. Among the AC components, benzenoid derivatives are considered therapeutically attractive due their anti-inflammatory and cytotoxic activities. We proposed a convenient method for concentrating the benzenoid-rich fraction (FNH), from AC wild fruiting bodies ethanolic extract (EEAC). Three benzenoids, 4,7-dimethoxy-5-methyl-1,3-benzodioxole (**1**), antrocamphin A (**2**) and 4,7-dimethoxy-5-methyl-6-(3-methylbut-3-en-1-ynyl)-1,3-benzodioxole (**3**) were purified and their structures were elucidated. Different wild AC samples and the separated benzenoids (**1**–**3**) exhibited anti-inflammatory activity through inhibiting superoxide anion generation and elastase release by human neutrophils in response to *N*-formyl-methionyl-leucyl-phenylalanine (FMLP)/cytochalasin B (CB). Compound **1** exhibited the most potent activity against superoxide anion generation (IC_{50} 5.08 μg/ml) while compounds **2** and **3** were less active (IC_{50} > 10 μg/ml) implying the advantageous use of **1**–**3** as biomarkers for AC.

HPLC-tandem MS analytical protocol was developed for the quantification of the three major benzenoids from different AC commercial products. The results showed that compound **1** is a unique characteristic biomarker for AC quality control. The concentrations of compounds **2** and **3** were higher in certain sources of AC fruiting bodies and were absent in mycelia, suggesting their usefulness as biomarkers for quality control of fruiting bodies products. The HPLC-tandem MS results were compared to the results obtained by quantitative NMR.

Abbreviations: AC, *Antrodia cinnamomea*; EEAC, ethanolic extract from *A. cinnamomea*; FNH, the *n*-hexane fraction from the EEAC (the benzenoid-rich fraction); HPLC-PDA, high performance liquid chromatography coupled to photodiode array detector; HPLC-tandem MS, high performance liquid chromatography coupled to triple quadrupole mass spectrometry; qNMR, quantitative nuclear magnetic resonance; WFB, wild fruiting bodies; SF, submerged fermentation; SSC, solid support culture; CWC, cutting wood culture; DC, dish culture; FM, combined formula of fruiting bodies and mycelia.

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1. Introduction

Herbal medicines and nutritional supplements are the chronicles of health care system for over 80% of the world population especially in Africa, Asia and Latin-America (WHO, 2002). The large market share of these products and the ambiguity of their quality control regulations raised serious concerns about natural product safety and efficacy (FDA, 2000; WHO, 2000). Folk medicine is a complex system dealing with multicomponent mixtures of different ingredients which vary depending on the climate, cultivation conditions, harvesting time, drying, storage conditions, and extraction procedures. These promiscuous factors along with the frequent reported cases of deliberate or coincidental adulterations imply the crucial need for uniform quality control protocols (Tistaert, Dejaegher, & Heyden, 2011).

Determination of specific chemical markers in natural products and nutritional supplements has emerged as a straight forward quality control procedure. The chemical nature of the selected markers should be accurately defined as well as their biological activity. The selection of chemical markers is usually affected by subjective assessments, empirical evidence and the commercial availability of reference standards. The prevailing practice focuses on analyzing constituents with the highest concentrations while minor compounds are usually ignored. This assumption dismisses the pharmacological and toxicological effects of minor components modifying the biological activity of herbal remedies and nutritional supplements. The development of more sophisticated spectroscopic techniques allowed the quality assessment of these supplements using chemical markers present in major as well as in minor concentrations.

Antrodia cinnamomea (AC), also known as *Antrodia camphorata* or *Taiwanofungus camphoratus*, is an endemic fungus, which parasitizes in the internal heartwood or dark humid wood surface of *Cinnamomum kanehirai* (Bull camphor tree) (Ao et al., 2009; Lu, Du, et al., 2009). It is used in Asian folk medicine to treat food, alcohol and drug intoxication, diarrhea, abdominal pain, hypertension, skin itching, and cancer (Ao et al., 2009; Shen et al., 2003). Recent investigations have evaluated AC biological activity suggesting its hepatoprotective, anticancer and anti-inflammatory effects (Du, Chang, et al., 2012; Geethangili & Tzeng, 2009; Huang et al., 2010; Lu, Hwang, et al., 2009; Shen et al., 2003).

Wild AC fruiting bodies grow at an extremely slow rate as they need one year to grow to the size of one Euro resulting in expensive marketed products (1 kg may reach US$ 15,000–28,000) (Du, Chang, et al., 2012). Therefore, different artificial cultivation technologies have been developed increasing its mass production. This has led to the introduction of several AC dietary supplements for cancer prevention and hepatoprotection. AC products are currently available in Taiwan, Japan, Singapore, Korea, Malaysia, China and even in some European countries such as England and Germany.

Despite the wide use of this treasured mushroom, its production and marketing face a critical challenge. Each provider claims that their marketed products are identical in terms of activity and content to the wild AC products. Fungal metabolites are highly affected by media conditions and culture techniques resulting in products with inconsistent biological activity (Ao et al., 2009). The chemical profile of AC major constituents in the cultivated and wild products may match; however this does not indicate similar biological activity because the activity depends on the synergistic and antagonistic effect of the entire spectrum of active ingredients. Moreover, marketed products are prepared either from mycelia or fruiting bodies or both, with variable concentrations of active constituents in each tissue, precluding the accurate estimation of the product biological activity. Therefore, developing an efficient quality control protocol to determine the concentration of active ingredients present in minor quantities will provide an accurate picture of the expected quality and biological activity of AC products. Although some studies discussed the characterization and quantification of particular AC components, they either focused on the fruiting bodies or mycelia (Lin et al., 2011; Zhao & Leung, 2010). To the best of our knowledge, no scientific report has compared the concentrations of active ingredients in the fruiting bodies and mycelia.

Our group has studied AC and its active constituents for several years showing that AC polysaccharides promoted functional maturation of dendritic cells in the expression of phenotypic characteristics, IL-12 production and chemotaxic activity (Lu, Du, et al., 2009). We also established the chemical profile of AC triterpenoids using NMR and HPLC-PDA/MS and successfully separated for the first time a mixture of ergostane triterpenes stereo-isomeric pairs with a chiral center at C-25 (Du, Wu, et al., 2012; Wu, Lu, Chang, Du, & Wu, 2010). Moreover, we proposed the first total synthesis of one of the AC benzenoids (antrocamphin A) and its analogs (Lee et al., 2011).

Benzenoids represent interesting chemical markers for AC products because they are present in minor quantities, can be quantified spectroscopically, and possess a wide spectrum of biological activities such as hepatoprotection and cytotoxicity against several cancer lines (Ao et al., 2009; Geethangili & Tzeng, 2009). Despite these merits, their utilization as chemical markers has been plagued by the lack of reliable experimental procedures to determine their concentration in mushroom samples. In the present study, we utilized special fractionation procedures to concentrate the beznenoid-rich fraction (FNH) from AC ethanolic extract (EEAC). The chemical profile of the FNH active components was illustrated through the isolation, purification, and structural elucidation of the three major AC benzenoids (**1**–**3**) using NMR and HPLC-PDA. Benzenoid quantification was performed by comparing the quantitative NMR (qNMR) results with those of the liquid chromatography coupled to triple quadrupole mass spectrometry results. The accuracy of the used spectroscopic techniques was evaluated. Additionally, the anti-inflammatory activity of FNH and the isolated benzenoids (**1**–**3**) were evaluated against superoxide anion generation and elastase release by human neutrophils in response to *N*-formyl-methionyl-leucyl-phenylalanine (FMLP)/cytochalasin B (CB). The results of the current investigation provide an efficient quality control platform which can be utilized for all AC products and other benzenoid containing remedies.

2. Materials and methods

2.1. Standard compounds and AC materials

Standard compounds of (1-3) used for comparative analysis experiments were isolated from the *n*-hexane fraction (FNH) of EEAC. The Internal standard used for tandem MS (1,2,5-trimethoxy-4-(3-methylbut-3-en-1-ynyl)benzene) was synthesized in our laboratory (Lee et al., 2011) (Fig. 1). The purity of these benzenoids was >97%, which was assessed with a three-point peak purity method and determined using Shimadzu “Class VP” software. The tested AC materials in the present study obtained from five available sources and a combined of fruiting bodies and mycelia formula (Table 3). In addition to the wild fruiting bodies (WFB), other four AC products were collected from different Taiwanese biotechnology companies and these sources are: submerged fermentation (SF), solid support culture (SSC), cutting wood culture (CWC), dish culture (DC). Among these sources, batches of SF 1–9, SSC 1–3, DC 1 are composed of mycelia however CWC 1–6 and DC 2–3 are composed of fruiting bodies. FM is a combined fruiting bodies and mycelia formula.

2.2. EEAC fractionation procedures

Over the past few years, several research groups have developed different extraction and fractionation procedures for the AC fruiting bodies and mycelia. Despite these efforts, the developed protocols were inefficient to concentrate biologically active components in specific fractions (Ao et al., 2009; Du, Wu, et al., 2012; Shen et al., 2003). Developing an efficient fractionation protocol for concentrating