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Contributed Paper

# Blazeispirol A, a Chemotaxonomic Marker from Mycelia of the Medicinal Mushroom *Agaricus subrufescens*

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## Abstract

*Agaricus subrufescens* (almond mushroom) was first collected in America, but has been cultivated worldwide due to its medicinal properties. The potential health promoting benefits of *A. subrufescens* have been emphasized in several reports and include tumor growth reduction, antimicrobial, immunostimulatory and anti-allergy effects. A unique class of spiro-triterpenoids named blazeispirols was found in the cultured mycelia. Recently, it was found that blazeispirols are highly selective agonists of LXR alpha and extracts from the mycelia of *A. subrufescens* accordingly even showed cholesterol-lowering activities *in vivo* in an animal model. Preliminary results on the distribution of blazeispirols furthermore suggested that their occurrence is restricted to *A. subrufescens*. The objective of our study was to establish blazeispirol production in novel, parental and hybrid strains from various isolates of *A. subrufescens* originating from Brazil, France and Thailand. Eight strains of *A. subrufescens* were investigated by HPLC-MS after fermentation in ZM/2, YM 6.3 and SYM broth media. All strains produced blazeispirols in large quantities in ZM/2 medium, confirming that the major component of this complex, blazeispirol A, does not only constitute a novel pharmacological lead compound, but is also a phylogenetic and chemotaxonomic marker for *A. subrufescens* and even all hybrid strains retained production of the compound. The production of blazeispirol A by fermentation of *A. subrufescens*, however, is rather slow, hence, for a sustainable production of blazeispirols the fermentation process needs to be further optimised.

**Keywords:** *Agaricus subrufescens*, blazeispirol A, HPLC-MS, medicinal mushroom, mycelia

## 1. Introduction

Mushrooms are an important source of novel compounds and several species of fungi are used in functional foods or as medicines [1]. This is particularly true in China where mushrooms form a large component of Chinese Traditional Medicines, examples of medicinal mushrooms including species of the genera *Auricularia*, *Flammulina*, *Fomes*, *Fomitopsis*,

*Ganoderma*, *Grifola*, *Hericium*, *Inonotus*, *Lentinus*, *Piptoporus*, *Pleurotus*, *Schizophyllum* and *Tremella* have been used as alternative medicine in various Asian countries for a long time [2-6]. Mushrooms are even used in cosmetics to whiten skin and reducing skin aging [7].

The Almond mushroom (*A. subrufescens*) has been widely used for consumption and medicinal use [8]. The mushroom was first described in America and has been widely cultivated since the late 19$^{th}$ century [9]. Several decades later, the same species was discovered again in Sao Paolo, Brazil and called Piedade mushroom. In 1965, the mushroom (named *A. blazei* Murrill) was introduced to Asia from T. Furomoto who sent it to Japan for medicinal investigation. The potential health promoting benefits of *A. subrufescens* have been emphasized in several reports including tumor growth reduction, antimicrobial, immunostimulatory and anti-allergy effects [8, 10]. Those are attributed mainly to hydrophilic bioactive metabolites from the mushrooms (and to some extent, the cultured mycelia), such as the β-glucans [10] and other polysaccharides. The cultured mycelia, however, also yielded a unique class of spiro-triterpenoids named blazeispirols (A, B, C, D, E, F, X, Y and Z), which were discovered by Hirotani et al. [11-14]). At that time there were no prominent bioactivities reported from these compounds. Blazeispirols (A, D, E and X) were found to be highly selective antagonists of Liver X receptors (LXR) alpha. The compounds were extracted from the mycelia of *A. subrufescens*. Extracts with highly enriched blazeispirol contents also exhibited cholesterol-lowering activities *in vivo* in a murine animal model [15]. Furthermore, preliminary results on the distribution of blazeispirols suggest that their production is restricted to *A. subrufescens* [15]. Other bioactivities of different metabolites of *A. subrufescens* have been reported previously. For example, lectin, riboglucan, glucomannan, agaritine and blazien were shown to reduce tumor growth [16-21].

We collected, identified, and isolated *A. subrufescens* strain CA 918 from grassland at Mae Fah Luang University. We then bred our Thai strain with a French strain and a Brazilian strain from INRA, France. The objective of this study was to examine the production of blazeispirols of these strains.

## 2. MATERIALS AND METHODS

### 2.1 Mushroom Strains

Three parental strains were obtained from Brazil, France and Thailand. The strain from Brazil, WC837 (= CA454 in the Collection of the Germplasm of the Agarics at Bordeaux, CGAB at INRA, France) was provided by D. J. Royse and Vija L. Wilkinson. WC837 is similar to ATCC 76739 which was used by Mizuno et al. [20] and originally provided by T. Furumoto who is known to have collected *A. subrufescens* in Brazil (São Paulo State). The French strain, CA487, was isolated as a tissue culture by J. C. Blanchard. The strain was identified by J. Guinberteau. The Thai strain, CA918, was isolated as a tissue culture by S. C. Karunarathna, P. Callac and S. Rapior on the campus Mae Fah Luang University, Chiang Rai, Thailand. Two Thai-Brazilian strains (CA918-075 X CA454-4 and CA918-076 X CA454-4) and Three Thai-French strains (CA918-075 X CA487-35, CA918-075 X CA487-100 and CA918-076 X CA487-35) were obtained via breeding the parental strains.

Strains including hybrids were deposited in culture collections as follows: The Brazilian strain, WC837 (= CA454), The French strain, CA487 and Brazilian-French hybrids in the Collection of the Germplasm of the Agarics at Bordeaux (CGAB at INRA, France); The Thai strain, CA918 (= MFLUCC 11-0653), French-Brazilian and Thai-Brazilian hybrids in CGAB (INRA, France) and in Mae Fah Luang University Culture Collection, Thailand.

**2.2 Small Scale Fermentation**

Mycelium plugs of eight strains of *A. subrufescens* from the actively growing edge of colonies on YM 6.3 agar medium (Glucose 0.4%, malt extract 1%, yeast extract 0.4%, Agar 20% and tap water: adjusted to 6.3 pH) were inoculated to 200 mL of ZM/2 broth medium (Molasses 0.5%, oatmeal 0.5%, glucose 0.15%, sucrose 0.4%, mannitol 0.4%, edamine S 0.05%, $Na_2SO_4$ 0.05%, $CaCO_3$ 0.15%, pH 7.2, prepared with tap water), YM 6.3 broth medium (Glucose 0.4%, malt extract 1%, yeast extract 0.4%, tap water: adjusted to 6.3 pH) and SYM broth medium (Sucrose 1%, yeast extract 0.5%, malt extract 3%, distilled water) were prepared in 500 mL Erlenmeyer flasks. All cultures were incubated at 25 °C on a rotary shaker at 140 rpm in darkness for 60 days prior to preparation of crude extracts for HPLC- DAD/ MS analysis.

**2.3 Mycelium Extraction**

Mycelial cultures were harvested after 60 days fermentation. The procedure followed the method of Grothe et al. [15]. After harvesting, the wet mycelium was separated from the fluid by centrifugation and filtration. The culture fluid was discarded for lack of bioactivity. The wet mycelium was extracted with acetone for 30 min twice in an ultrasonic bath (25°C). After filtration, the acetone was evaporated *in vacuo* at 40°C. The remaining aqueous residue was diluted with water and extracted twice with the same volume of ethyl acetate (EA). After drying over anhydrous sodium sulfate, the organic solvent was removed in vacuo at 40°C to yield the crude extract. The crude mycelial extract was dissolved in methanol (concentration adjusted to 1 mg/ml) and used for HPLC-DAD/ MS analysis.

**2.4 HPLC Screening**

An aliquot of the crude extract (100µl) was analyzed by HPLC-MS on a reverse phase HPLC column (VP Nucleodur C 18, 21 × 250 mm, 7 µm) solvents: (A) $H_2O$+0.5% formic acid, (B) Acetonitrile using the linear gradient system from 20% ACN to 100% ACN for 30 minutes. The flow rate was 20 ml/min at room temperature [22].

Peaks in the crude extract were compared with the standard of blazeispirol A, also relying on the mass spectrum in the positive ESI mode, as well as its characteristic UV/Vis chromophore and retention time ($R_t$).

**2.5 Isolation of Blazeispirol A**

Blazeispirol A was isolated from the mycelium of *Agaricus subrufescens* (Thai-Brazilian hybrid strain). For this purpose, 50g of powdered mycelium was extracted twice with acetone for 30 min in an ultrasonic bath (25 °C) and the organic solvent was evaporated in vacuo at 40°C. The remaining crude extract was subjected to flash chromatography on silica gel and two fractions were obtained subsequently using a linear gradient of n-heptane/ethyl acetate (0-40% ethyl acetate): A (145 mg; containing blazeispirol A) and B (44 mg). The final purification was performed by preparative HPLC on normal phase material (column: VP 250/21 Nucleosil 100-7, Macherey-Nagel; solvent A: n-heptane, solvent B: methyl-tertbutylether, gradient system: 0% B increasing to 20% B in 30 min, increasing to 100% B in 5min; flowrate = 10 mL·min-1). Blazeispirol A was collected according to UV absorption at 210 nm and 280 nm and characterized by HPLC-DAD/MS (Agilent 1260 Infinity Systems coupled to an ion trap MS amaZon speed™ and TOF-MS MaXis, Bruker; C18 Acquity UPLC BEH column (2.1 × 50 mm, 1.7 µm), Waters; solvent A: $H_2O$ + 0.1% formic acid, solvent B: AcCN + 0.1% formic acid, gradient system: 5% B for 0.5 min increasing to 100% B in 19.5 min, maintaining 100% B for 5 min, flowrate = 0.6 mL min−1) and NMR-spectroscopy (Bruker Avance III 500 spectrometer, recorded in $CDCl_3$ at 500 and 100MHz, respectively);

the analytical data were consistent with the literature [14].

## 3. RESULTS

### 3.1 HPLC Analysis

HPLC- DAD/ MS analysis of all extracts showed that the major peak was eluted at 16.4 min (see Figure 1). These compounds exhibited virtually the same characteristic UV/Vis chromophores and appeared at the same retention time as the standard (Figure 2, 4). By comparison with known compounds in the library, these compounds were likely to constitute blazeispirol A. Further confirmation was established by MS analysis; the compound had a molecular mass [M+H-$H_2O$] of 381.3 Da, which matched with that reported for blazeispirol A [15] (Figure 3, 4; see crude extracts of *A. subrufescens* which produces blazeispirol A in Table 1).

Four out of eight strains which were fermented in SYM broth medium contained blazeispirol A. While, in six out of eight strains which were fermented in YM broth medium we detected blazeispirol A, whereas all strains which were fermented in ZM/2 medium produced blazeispirol A (Table1).

Extracts from the eight strains exhibited UV absorption maxima at 225 nm (Figure 2) suggesting that the compounds are structurally related.

## 4. DISCUSSION

Medicinal mushrooms have been utilised for over 300 years throughout the world [6, 23]). Mushrooms produce several bioactive compounds; such as polysaccharides of the β-glucan type [2, 24] and riboglucan [16]. These compounds which have been found in *Agaricus subrufescens* exhibited anticancer properties and immunostimulatory properties and found in *Agaricus subrufescens* [2]. Extracts of *A. campestris* were described to have anti-hypertriglyceridemic, insulin releasing, and insulin like activities, whereas those of *A. sylvaticus* contain phenolic compounds, polyketides and terpenes resulting in reduced cholesterol and triglyceride levels in diabetic treatments [3]. In addition, *A. bisporus* produces acidic polysaccharides, dietary fiber and anti-oxidants which have anti- inflammatory, anti-diabetic, hypoglycemic and hypocholesterolemic effects [2, 3]. Another group of medicinal compounds are triterpenoids, from Ganoderma, which contribute to the inhibition of histamine [25-27]. However, these triterpenoids are commonly found in numerous mushrooms. The spiro triterpenoids of the blazeispirols type are specifically produced in mycelial cultures of the almond mushroom, *Agaricus subrufescens*.

*Agaricus subrufescens* is an edible mushroom with considerable medicinal properties [8, 10]. There have been many studies on the medicinal properties of *A. subrufescens*; the taxon has one of the highest concentrations of β-glucans [28]. β-glucans are polysaccharide shown to have anti-cancer properties [29, 30]. Agaritine is a low molecular weight compound which inhibits leukemic cells and is found in *A. subrufescens* [31, 32]. Extracts of the fruiting body have also been shown to be protective against bacterial infection in mice [33], to be antidiabetic [3] and have antiviral activity [34, 35], but blazeispirols have never been found in the basidiomes of *A. subrufescens*.

Blazeispirols were discovered by Hirotani [11-14] and recently several new minor analogs were isolated by Grothe et al [15]. Blazeispirols were found in *A. subrufescens* as highly selective agonists to LXR receptor alpha. The crude extract from eight strains of *A. subrufescens* including Brazilian, French and Thai and their hybrids contained blazeispirols. Blazeispirol A is produced by mycelium fermentation [15], but the compound has never been found in fruiting bodies of *A. subrufescens*. In this study, blazeispirol A, occurred in mycelium extraction of all tested strains of *A. subrufescens*. Culture media components effected the production of

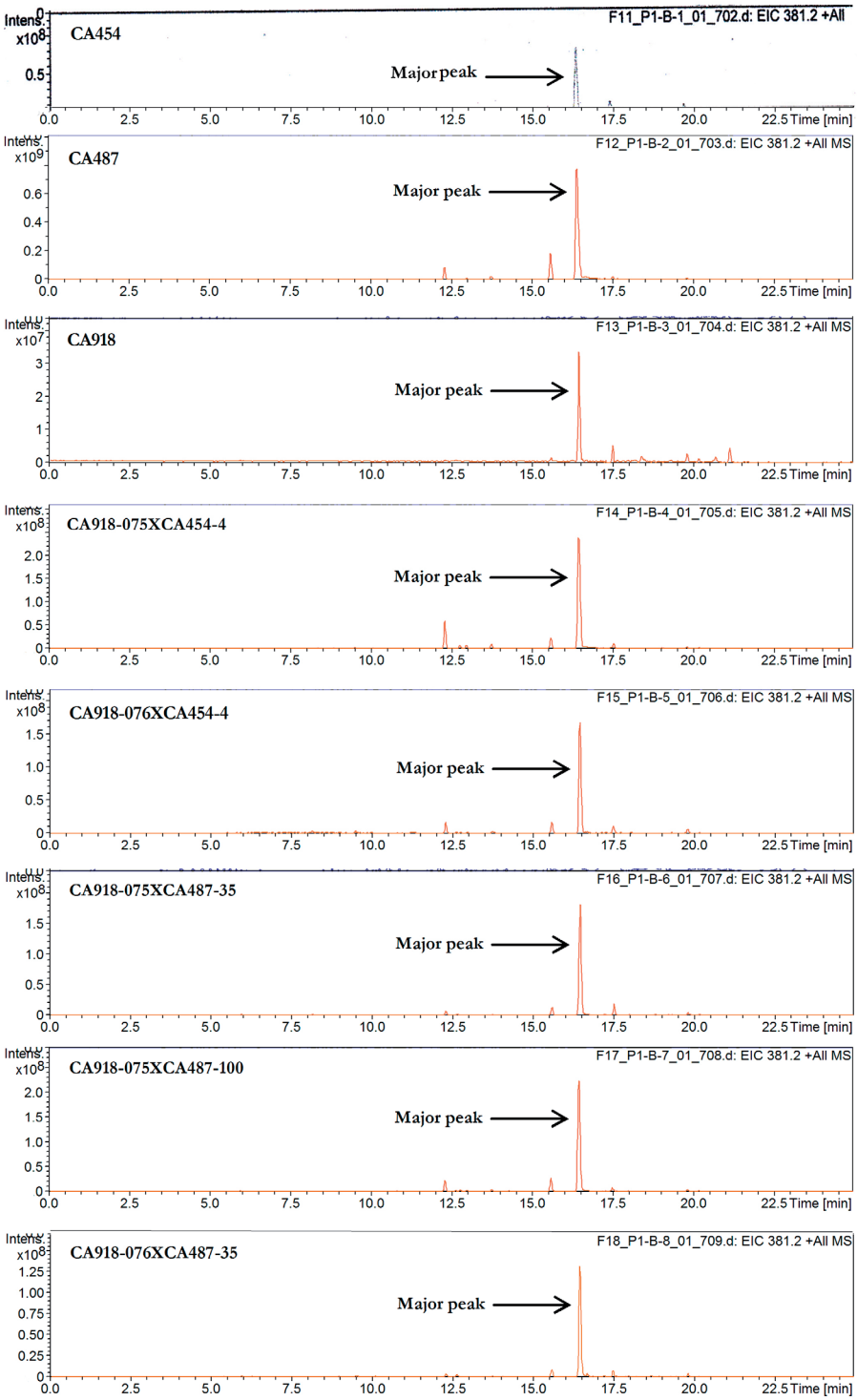


**Figure 1.** Major peak eluted at 16.4 min in the eight samples of parental and hybrid strains of *A. subrufescens*.

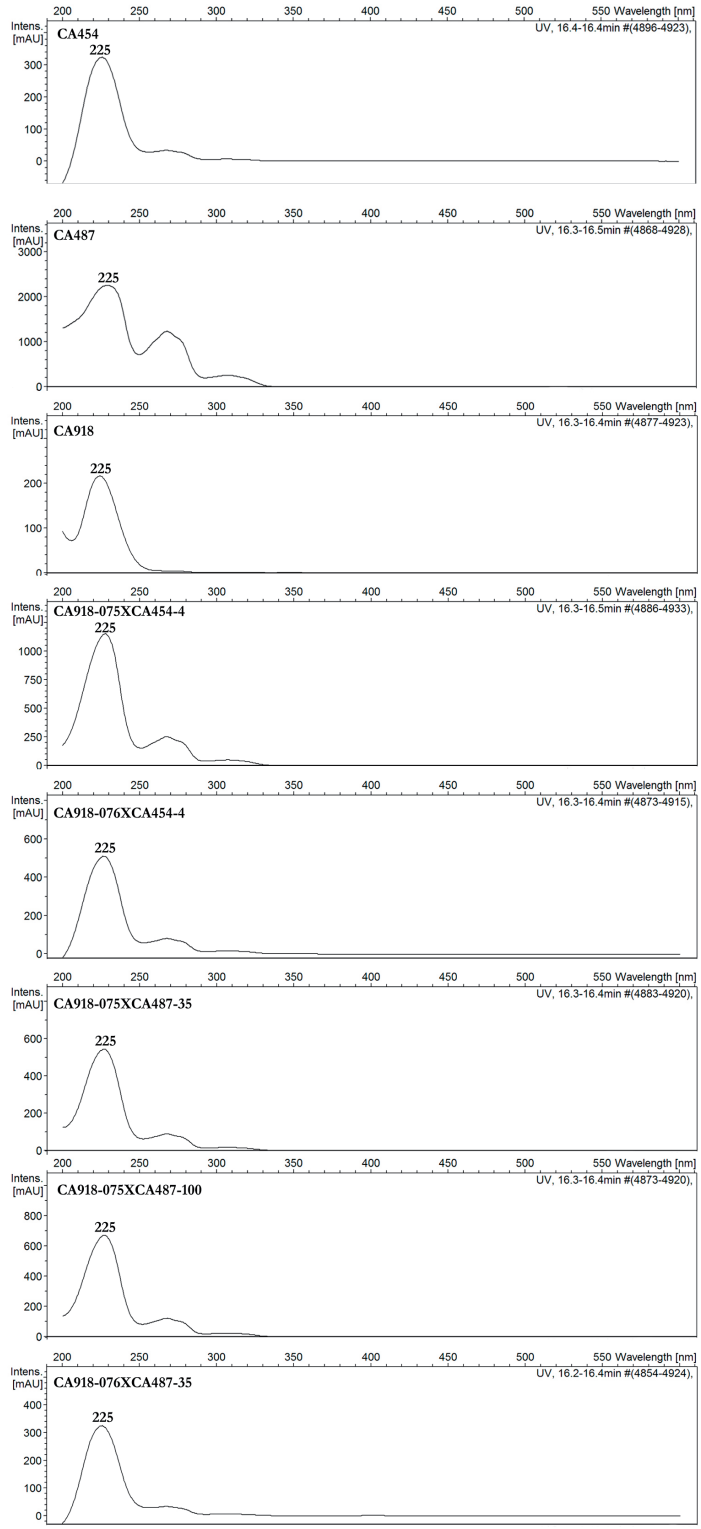


**Figure 2.** HPLC-UV/ Vis spectra of blazeispirol A in extracts of parental and hybrid strains of *A. subrufescens.*

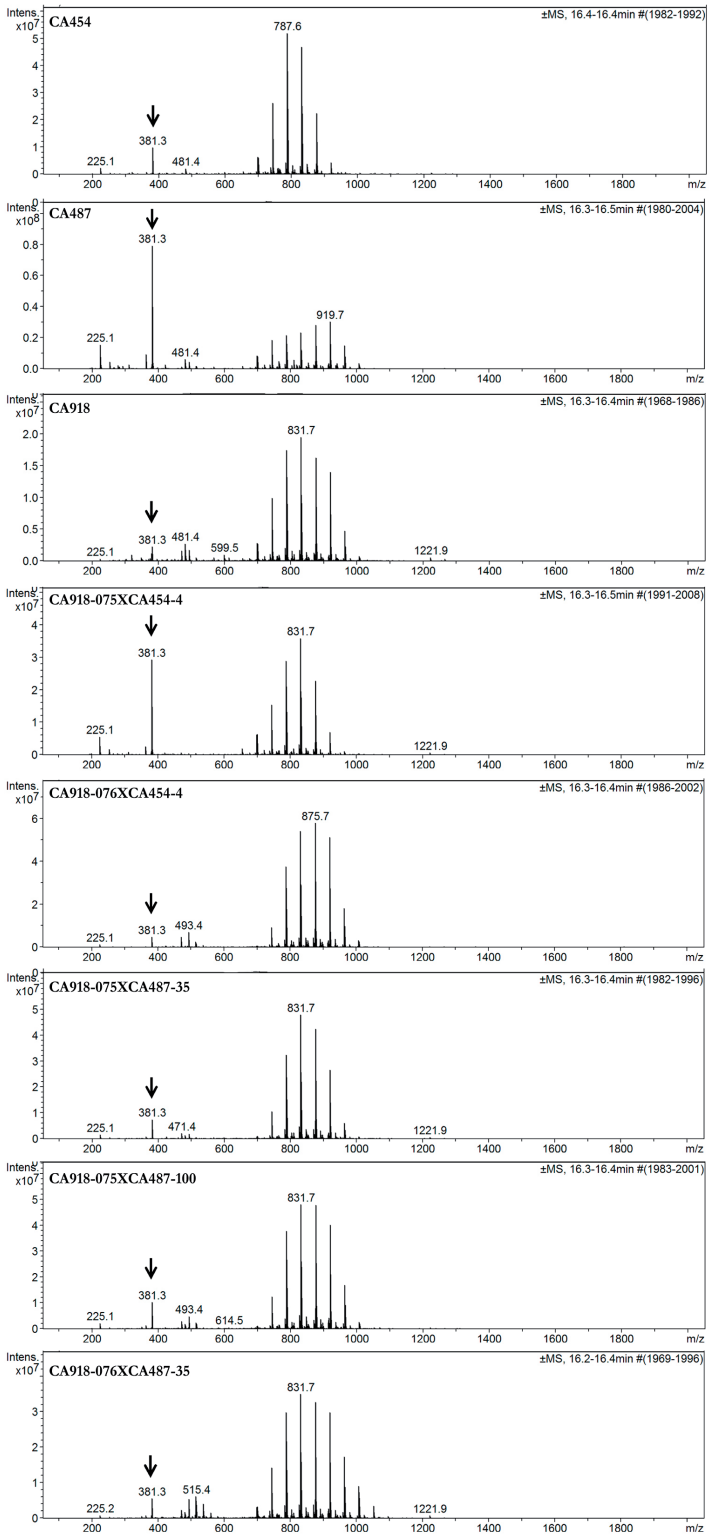


**Figure 3.** HPLC-MS analysis, showed molecular masses [M+H-$H_2O$] of 381.3 Da, found in extracts of parental and hybrid strains of *A. subrufescens* (see arrows).

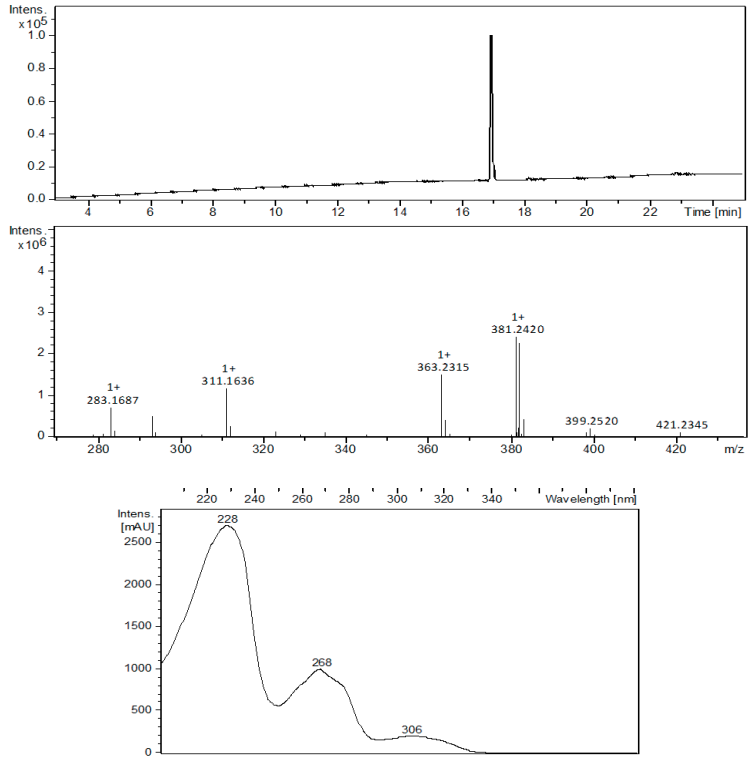


**Figure 4.** HPLC chromatogram, HR-MS spectrum and UV/Vis spectrum of the isolated blazeispirol A.

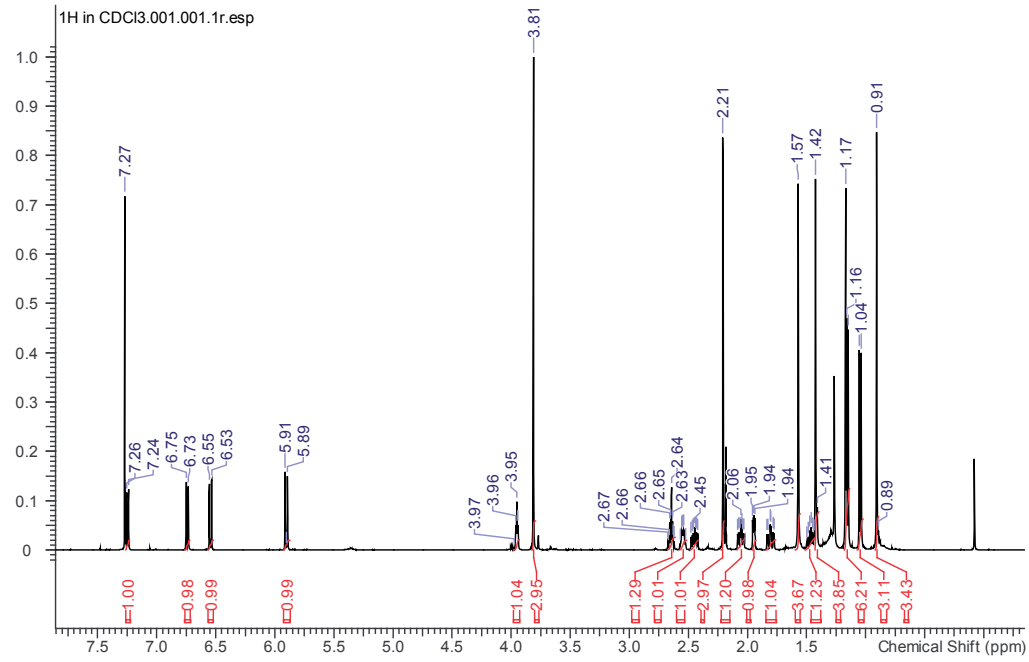


**Figure 5.** [1]H-NMR spectrum of the isolated blazeispirol A.

**Table 1.** Effect of culture media to blazeispirol A production from *Agaricus subrufescens* and crude extracts (mg) of blazeispirol A produced in ZM/2 media.

| Original code | Culture Media | | | Crude extracts (mg) of ZM/2 medium |
|---|---|---|---|---|
| | ZM/2 | SYM | YM | |
| CA454 | + | + | + | 7.2 mg |
| CA487 | + | + | + | 35.6 mg |
| CA918 | + | - | - | 12.3 mg |
| CA918-075 × CA454-004 | + | - | + | 11.6 mg |
| CA918-076 × CA454-004 | + | - | + | 11 mg |
| CA918-075 × CA487-035 | + | + | + | 13.9 mg |
| CA918-075 × CA487-100 | + | - | - | 13.5 mg |
| CA918-076 × CA487-035 | + | + | + | 17.5 mg |

**Note.** + blazeispirol A production was detected in this medium,
- no blazeispirol A production was deteteed in this medium.

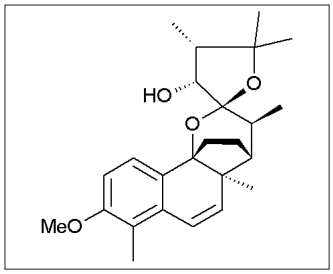


**Figure 6.** Chemical structure of blazeispirol A [15].

blazeispirol A. All strains fermented in ZM/2 medium produced blazeispirol A as suggested in Grothe et al. [15], who reported that ZM/2 medium is an optimal medium for blazeispirols production.

Temperature is one of the factors regulating secondary metabolite production. The temperature range that is suitable for secondary metabolite production is usually much lower or higher than the optimum growth temperature [36]. In this study, the growth and blazeispirol A production were only established at 25°C. Blazeispirol A production could be further optimized by changing temperature and pH.

Blazeispirol A was obtained from mycelial extracts incubated at 25°C on a rotary shaker, in ZM/2 broth media, in darkness. The highest yield of blazeispirol A was found in the parental French strain, followed by the Thai-Brazilian (CA918-075 x CA 454-4) and Thai-French hybrid (CA918-075 x CA487-100). All hybrid strains exhibited better yields of blazeispirol A than the parental Thai and Brazilian (see HPLC profiles in Figure 1). However, these results are preliminary and need further verification, above all during scale-up and under monitoring of the production by HPLC during the course of the fermentation of these strains. Previous results on the distribution of blazeispirols suggested that their production is restricted to *A. subrufescens,* and allied species, while other species in *Agaricus* (e.g. *A. arvensis*, *A. augustus*, *A. bisporus*, *A. bitorquis*, *A. campestris* var. *campestris*, *A. macrosporus*, *A. semotus*, *A. silvicola*, *A. trisulphuratus* and *A. xanthoderma* did not produce blazeispirols under various fermentation conditions [15]. Hence blazeispirols could be specific to *A. subrufescens*.

## 5. CONCLUSIONS

In this study, three strains of *A. subrufescens* and their hybrids (five strains) were tested for blazeispirol A production in fermentation broth. All strains produced "blazeispirol A". The highest yield of crude extract was obtained from the parental French strain, followed by the Thai-Brazilian and Thai-French hybrid strains. All hybrid strains yielded higher quantities of blazeispirol A than the parental Thai and Brazilian strains. The fermentation of blazeispirol A, still however, remains to be optimised for mass production. It would be desirable to breed a strain that produce blazeispirol A in the fruiting bodies because these could be produced in large quantities and the mushroom themselves can be eaten as they have potential nutriceutical properties, including cholesterol-lowering and other beneficial effects.

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