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Ethanolic and aqueous extracts derived from Australian fungi inhibit cancer cell growth in vitro

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Abstract: Fifteen Australian macrofungi were investigated for cytotoxic activity. Ethanol, cold and hot water extracts of each species were screened for cytotoxic activity against normal mouse fibroblast cells (NIH/3T3), healthy human epithelial kidney cells (HEK-293), four cancer cell lines, gastric adenocarcinoma cells (AGS), two mammary gland adenocarcinoma cells (MDA-MB-231, MCF7) and colorectal adenocarcinoma cells (HT-29) with a validated MTT assay. Most extracts derived from *Omphalotus nidiformis*, *Cordyceps cranstounii* and *Cordyceps gunnii* demonstrated significant cytotoxic activity toward a variety of cancer cell lines. In contrast only some extracts from *Coprinus comatus*, *Cordyceps hawkesii*, *Hypholoma fasciculare*, *Lepista nuda*, *Leratiomyces ceres* and *Ophiocordyceps robertsii* displayed significant cytotoxic activity, which was usually selective for only one or two cancer cell lines tested. The least cytotoxic species evaluated in this study were *Agaricus bitorquis*, *Coprinopsis atrametaria*, *Psathyrella asperospora*, *Russula clelandii*, *Tricholoma* sp. AU2 and *Xerula mundroola.*

Key words: bioactivity, *Coprinus, Cordyceps*, cytotoxic, drug discovery, *Hypholoma, Lepista, Leratiomyces*, macrofungi, *Omphalotus, Ophiocordyceps*

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INTRODUCTION

The medicinal use of fungi has been documented in China, Russia, Japan and Korea, as well as in the United States and Canada (Wasser and Weis 1999). For example entire fungi and fungi extracts are used in traditional Chinese medicine for the treatment of cancer (Dong 2001, Huang 2007, Xu 2003, Zhang 2002, Zhu and Wei 2000), and these fungi preparations as well as isolated fungal fractions and compounds are used as adjuvants to surgery, radiotherapy or chemotherapy (Lindequist et al. 2005). Lentinan, derived from *Lentinula edodes*, schizophyllan from *Schizophyllum commune*, the Maitake D-fraction (β-glucan) from *Grifola frondosa* and krestin from *Trametes versicolor* (synonym *Coriolus versicolor*), are in clinical use for this purpose. Moreover mushroom consumption has been shown to prevent the development of cancer later in life with studies reporting an inverse correlation between mushroom intake and the risk of developing gastric (Kim et al. 2002) or breast cancer (Zhang et al. 2009).

This anti-cancer activity has been attributed partially to the polysaccharides present in basidiomycetes, which are high molecular weight carbohydrate polymers (ca. 500–2000 kDa) and arise as constituents of the fungal cell wall (Wasser 2002). These polysaccharides, as well as polysaccharide-protein complexes (glycoproteins), have been shown to exert their antitumor effects by influencing the immune system through the stimulation of macrophages, T lymphocytes and natural killer cells (Lindequist et al. 2005).

However other fungal high molecular weight compounds, such as flammulin, velutin and various lectins, have been shown to possess direct activity against cancer cells in vitro (Wang and Ng 2001, Wang et al. 2000). Recently a fungal polysaccharide derived from *Cordyceps jiangxiensis* was reported to exert a direct cytotoxic effect in vitro against the human gastric carcinoma cell line SGC-7901 (Xiao and Zhong 2008).

Moreover some low molecular weight fungal secondary metabolites also exhibit cytotoxicity against tumor cells. For example illudin, a tricyclic sesquiter-

pene from *Omphalotus olearius*, has served as the lead structure for the semisynthetic drug irofulven, which is currently in preclinical and clinical trials due to its effects against cancer-related kinases and apoptotic activity against a range of tumors (Baekelandt 2002). Cordycepin, isolated from several *Cordyceps* species, has shown an apoptotic effect on various cancer cell lines (Jin et al. 2008, Wu et al. 2007) while the cytotoxicity of *Lepista* species has been attributed to the clitoclines (Fortin et al. 2006).

To date the majority of chemical and pharmacological investigations have focused on American, European and Asian fungal species. Macrofungi occurring in Australia are unexplored to a large extent with considerable numbers of endemic species, many of which are not formally described, possessing unknown chemical, biological and pharmacological profiles (Hawksworth 2001, May and Simpson 2001). Ethnomycological research in Australia indicates only rare use of fungi as food or internal medicine by indigenous Australians (Mallet and Grgurinovic 1996) and hence the toxicity of native species is largely unknown. Knowledge of toxicity for the few species where this has been established is derived from poisoning cases due to confusion of native mushroom species with known edible northern hemisphere species (Pauli and Foot 2005).

Here we report on the screening of extracts from 15 fungi against a panel of four cancer and two healthy cell lines to evaluate their cytotoxic potential and possible value for anti-cancer drug development. Of the 15 species 11 are considered to occur naturally in Australia (native). Seven of these native species occur only in Australia; of the other native species two, *Ophiocordyceps robertsii* and *Cordyceps gunnii*, are found in New Zealand while *Hypholoma fasciculare* is cosmopolitan but appears to be native to Australia. *Leratiomyces ceres* is native to Australia and widespread as an exotic elsewhere.

MATERIALS AND METHODS

Fungi.—Fruit bodies were collected in May 2007 and 2008 at localities in the state of Victoria, Australia, and stored frozen (−18 C) before extraction. The species chosen were mostly from genera known to possess pharmacologically active compounds, and the particular species collected were those that were available in sufficient quantity for analysis. Each sample consisted of as many fruit bodies as needed to make up the 50 g (except for *Cordyceps cranstounii* and *Russula clelandii*) required for analysis; all parts of the fruit body were used, including the mummified host for the vegetable caterpillars *Cordyceps* and *Ophiocordyceps*. After picking fruit bodies were lightly cleaned of soil and immediately placed in snap-lock plastic bags and stored below 15 C until being placed in a freezer as soon as practicable after collecting.

Fungi were identified by one of the authors (TWM) from an examination of macro- and micromorphology in comparison to standard descriptions in the following floras, monographs and taxonomic treatments: Breitenbach and Kränzlin (1995) for *Agaricus bitorquis* and *Coprinopsis atrametaria* (as *Coprinus atramentarius*); Grgurinovic (1997) for *Coprinus comatus*, *Hypholoma fasciculare*, *Leratiomyces ceres* (as *Hypholoma aurantiacum*, syn. *Stropharia aurantiaca*), *Lepista nuda*, *Omphalotus nidiformis* (syn. *Pleurotus nidiformis*) and *Psathyrella asperospora* (as *Lacrymaria asperospora*); Miller and Hilton (1987) for *Russula clelandii*, interpreting that species broadly, including *R. lenkunya* as described by Grgurinovic (1997); Petersen (2008) for *Xerula mundroola*; and Willis (1959) for the three species of *Cordyceps* and *Ophiocordyceps robertsii* (as *Cordyceps robertsii*). There is no monographic treatment of Australian *Tricholoma*. Therefore the material is referred to as *Tricholoma* sp. AU2 because it differs from *T. eucalypticum* and *T. austrocolossum* (syn. *T. australe*) as described by Grgurinovic (1997) and also is not conspecific with *Tricholoma* sp. AU1 previously used in phytochemical studies (Ovenden et al. 2005). Names follow the draft Master Names List of Australian Fungi (RBG Melbourne, in prep) and most also are included in the Interactive Catalogue of Australian Fungi (May et al. 2010).

The status of species as native is based on usual occurrence in native vegetation or in association with native tree species, while exotic species are consistently found in gardens or other artificial habitats. The status of *Lepista nuda* is uncertain because it occurs in both native and exotic vegetation (Grey and Grey 2005). Voucher specimens (TABLE I) were deposited in the National Herbarium of Victoria (MEL).

Extraction and sample preparation.—Frozen fungal fruit bodies (50 g except for *Cordyceps cranstounii*, 38 gm and *Russula clelandii*, 37g) were chopped and promptly pulverized in ethanol with a homogenizer (IKA Works Inc.: T18) and extracted successively with 100% ethanol (2 × 500 mL, 20–25 C), cold water (1 × 1000 mL, 20–25 C) and hot water (1 × 1000 mL, 60–80 C) for 6 h, with vigorous stirring. Fungal material was air dried overnight to remove ethanol residues. Extracts were filtered (2× Whatman No. 2, 9 cm diam) and dried with a rotary evaporator (Buchi) or freeze dryer (Gilchrist) and stored frozen (−18 C) until required. The crude extracts were resolubilize in sterile aqueous DMSO (1% final concentration, for ethanol extracts) or MilliQ water (water extracts) and tested for cytotoxic activity at four concentrations (5.0, 0.5, 0.05 and 0.005 mg/mL).

Cell culturing and cytotoxicity assay.—All cell lines were purchased from ATCC (Manassas, Virginia 20108). Lines were cultured in Advanced Dulbecco's modified Eagle's medium (DMEM, Gibco: 12491) supplemented with inactivated newborn calf serum (NBCS, 10%, Gibco: 26010-074) and L-glutamine (200 mM, Gibco: 25030) at 37 C in 5% CO_2 (Heraeus BBIS CO_2 incubator). In vitro cytotoxicity was determined against normal mouse fibroblast cells (NIH/3T3: CRL-1658) and five human cell lines, gastric adenocarcinoma cells (AGS: CRL-1739), two mammary gland

TABLE I. Fungi species collected for this investigation

Species	Location (all Australia, Victoria)	Latitude/longitude	Voucher number	Family	Native/exotic	Distribution
Agaricus bitorquis	Melbourne, Royal Botanic Gardens	37°50′S, 144°59′E	MEL 2116997	*Agaricaceae*	Exotic	Cosmopolitan
Coprinopsis atramentaria	Melbourne, Royal Botanic Gardens	37°49′S, 144°58′E	MEL 2063519	*Psathyrellaceae*	Exotic	Cosmopolitan
Coprinus comatus	Melbourne, Royal Botanic Gardens	37°49′S, 144°58′E	MEL 2025978	*Agaricaceae*	Exotic	Cosmopolitan
Cordyceps cranstounii	Healesville district, Black Spur, Dom Dom Saddle	37°36′S, 145°38′E	MEL 2323333	*Clavicipitaceae*	Native	Australia
Cordyceps gunnii	Healesville district, Black Spur, Dom Dom Saddle	37°36′S, 145°38′E	MEL 2192235	*Clavicipitaceae*	Native	Australia and New Zealand
Cordyceps hawkesii	Healesville district, Black Spur, Dom Dom Saddle	37°36′S, 145°38′E	T.W. May RBG24 (MEL)	*Clavicipitaceae*	Native	Australia
Hypholoma fasciculare	Healesville district, Black Spur, Dom Dom Saddle	37°36′S, 145°38′E	MEL 2192215	*Strophariaceae*	Native	Cosmopolitan
Lepista nuda	Healesville district, Black Spur, Dom Dom Saddle	37°35′S, 145°38′E	MEL 2192242	*Tricholomataceae*	Native (?)	Cosmopolitan
Leratiomyces ceres	Melbourne, Royal Botanic Gardens	37°50′S, 144°59′E	MEL 2192245	*Strophariaceae*	Native	Only Australia (widely introduced elsewhere)
Omphalotus nidiformis	Melbourne, Domain, near Royal Botanic Gardens	37°49′S, 144°59′E	MEL 2192244	*Omphalotaceae*	Native	Australia
Ophiocordyceps robertsii	Healesville district, Black Spur, Dom Dom Saddle	37°36′S, 145°39′E	MEL 2192231	*Clavicipitaceae*	Native	Australia and New Zealand
Psathyrella asperospora	Melbourne, Royal Botanic Gardens	37°49′S, 144°58′E	MEL 2061945	*Psathyrellaceae*	Native	Australia
Russula clelandii	Warrandyte State Park, Jumping Creek Reserve	37°44′S, 145°14′E	MEL 2192226	*Russulaceae*	Native	Australia
Tricholoma sp. AU2	Warrandyte State Park, Jumping Creek Reserve	37°44′S, 145°14′E	MEL 2044320	*Tricholomataceae*	Native	Australia
Xerula mundroola	Healesville district, Black Spur, Dom Dom Saddle	37°36′S, 145°38′E	MEL 2192237	*Physalacriaceae*	Native	Australia

TABLE II. Yields of ethanol, cold water and hot water extracts for fungi species assayed in this study

Species	Mass of fungi extracted (g)	Mass of extract (g) (percent yield w/w)		
		Ethanol (EtOH)	Cold water (CW)	Hot water (HW)
Agaricus bitorquis	50	1.84 (3.69)	1.33 (2.66)	0.27 (0.53)
Coprinopsis atramentaria	50	0.88 (1.76)	1.81 (3.62)	0.16 (0.32)
Coprinus comatus	50	1.11 (2.18)	1.26 (2.47)	0.17 (0.33)
Cordyceps cranstounii[a]	38	2.18 (5.74)	0.98 (2.58)	0.27 (0.71)
Cordycpes gunnii	50	1.26 (2.52)	7.70 (15.4)	0.40 (0.80)
Cordyceps hawkesii	50	2.65 (5.30)	1.97 (3.94)	0.33 (0.66)
Hypholoma fasciculare	50	0.95 (1.90)	0.83 (1.66)	0.22 (0.45)
Lepista nuda	50	1.75 (3.43)	2.89 (5.67)	0.27 (0.53)
Leratiomyces ceres	50	0.96 (1.93)	0.99 (1.98)	0.56 (1.11)
Omphalotus nidiformis	50	4.95 (9.90)	1.77 (3.54)	2.60 (5.20)
Ophiocordyceps robertsii	50	2.76 (5.52)	2.10 (4.20)	0.60 (1.20)
Psathyrella asperospora	50	1.11 (2.13)	1.66 (3.19)	0.23 (0.44)
Russula clelandii [a]	37	0.76 (2.05)	0.96 (2.59)	0.15 (0.41)
Tricholoma sp. AU2	50	0.96 (1.93)	0.34 (0.68)	0.08 (0.16)
Xerula mundroola	50	1.24 (2.48)	0.53 (1.06)	0.40 (0.80)

[a] For these species less than 50 g was available.

adenocarcinoma cells (MDA-MB-231: HTB-26, human estrogen-receptor negative (ER−); MCF7: HTB-22, human estrogen-receptor positive (ER+), healthy human epithelial kidney cells (HEK-293: CRL-1573) and colorectal adenocarcinoma cells (HT-29: HTB-38) with the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay developed by Mosmann (1983) with minor modifications as described by Popiolkiewicz et al. (2005) and as noted in the text.

Cells and medium (150 μL) were seeded into 96 well plates (Nunc: 167008; MCF7 and AGS: 1.50×10^4; NIH/3T3: 0.90×10^4; HT-29: 3.00×10^4; MDA-MB-231: 1.75×10^4; HEK-293: 2.00×10^4 cells/well). After 24 h incubation and attachment the cells were treated with different concentrations of mushroom extract (20 μL) and PBS (30 μL) for 48 h. After washing and 2 h incubation with MTT solution (0.5 mg/mL for cancer cell lines and 1 mg/mL for healthy cell lines) cells were lysed with DMSO. The absorbance was measured after 45 min with a microplate reader (Wallac 1420 Multilabel counter, PerkinElmer) at a wavelength of 560 nm. MilliQ-water and 1.0% dimethylsulfoxide (DMSO) served as the negative control for water and ethanol extracts respectively, while 25% DMSO and cycloheximide served as the positive control. The MTT assay was validated with concentrations of DMSO (0.5–25%). The results were generated from two independent experiments with each experiment performed in triplicate.

Statistical analysis.—Probit regression analysis was carried out for dose response data with LPD line software available online (http://www.ehabsoft.com/ldpline/, Bakr 2009) and was used to calculate IC_{50} values.

RESULTS

Fifteen Australian macrofungi, representing 13 genera, were successively extracted three times generating ethanolic, cold and hot water extracts. The yields obtained for each fungal extract are provided (TABLE II).

In vitro cytotoxicity of mushroom extracts.—Extracts were screened for cytotoxic activity against healthy mouse and human kidney, as well as four human cancer cell lines (gastric, colon and breast). The 50% inhibitory concentration (IC_{50}) values of the ethanol and aqueous extracts of the fungi are summarized (TABLE III). In this study crude extracts with IC_{50} values below 1.0 mg/mL were considered significantly active and of those extracts exhibiting IC_{50} values below 0.1 mg/mL were deemed potently active. In the case of crude extracts that are composed of a complex mixture of metabolites, IC_{50} values of 0.1–1 mg/mL, while somewhat high, might be indicative of cytotoxic compounds that are present at low concentrations. It is anticipated that enriching these compounds through bioassay-guided fractionation will result in significant increases in cytotoxic activity of several orders of magnitude. Approximately half of the 45 extracts tested exhibited significant cytotoxicity against at least one cell line, with no particular solvent extract (ethanol, cold or hot water) being predominantly active.

Of the panel of cell lines selected for this study our results (TABLE III) show that the normal mouse fibroblast NIH/3T3 cell line was most susceptible while the healthy human cell line HEK-293 was least susceptible to the fungi extracts. Extracts of only three fungi, *Cordyceps cranstounii*, *Cordyceps hawkesii* and *Lepista nuda*, exerted any cytotoxic activity against the HEK-293 cell line.

TABLE III. Concentration at which cell growth is inhibited to 50% (IC_{50}) by the three most active fungi extracts

Species	Extract	Cytotoxic activity (IC_{50}) (mg/ mL)					
		NIH/3T3	HEK-293	HT-29	MDA-MB-231	MCF7	AGS
Cordyceps cranstounii	EtOH	0.65	1.72	2.79	1.65	> 5.00	3.27
	CW	0.14	NA	0.41	0.53	NDD	0.41
	HW	0.11	NA	0.12	0.18	1.88	0.26
Cordyceps gunnii	EtOH	0.99	> 5.00	1.51	0.42	3.83	1.02
	CW	0.37	NA	3.35	0.72	> 5.00	1.22
	HW	0.37	NA	0.72	0.35	> 5.00	0.47
Omphalotus nidiformis	EtOH	0.63	NA	0.06	0.04	1.11	0.06
	CW	0.37	NA	0.71	0.51	NA	0.23
	HW	0.37	> 5.00	0.49	0.84	> 5.00	1.04
Cycloheximide†	_	0.0003		0.0036	0.0004	0.0613	0.001

EtOH = ethanol; CW = cold water; HW = hot water; NDD = non dose dependent cytotoxic activity observed; NA = no cytotoxic activity observed at any of the concentrations evaluated; > 5.00 = extract exhibited low cytotoxic activity at one or more concentrations evaluated; † cytotoxic positive control.

Selective cancer cell cytotoxic activity.—Among the 45 evaluated the cold water extract from *Xerula mundroola* (IC_{50} 0.02 mg/mL), all of *Leratiomyces ceres* (IC_{50} 0.15–0.83 mg/mL) and the ethanolic extract of *Hypholoma fasciculare* (IC_{50} 0.18 mg/mL) exhibited selective and significant cytotoxicity toward colon cancer (HT-29) cells but displayed low or no cytotoxicity toward all other cancer cell lines screened.

High non-selective cytotoxic activity.—The IC_{50} values of all *Omphalotus nidiformis* extracts were low for most cell lines, 0.04–1.11 mg/mL, with the ethanolic extract displaying potent cytotoxicity against gastric (AGS), colon (HT-29) and estrogen-independent breast cancer (MDA-MB-231) cell lines. The cold and hot water extracts from *O. nidiformis* also were significantly active against those cell lines.

A similar pattern of significant cytotoxic activity against gastric (AGS), colon (HT-29) and estrogen independent breast cancer (MDA-MB-231) was observed for the cold and hot water extracts from *C. cranstounii* and the hot water extracts of *Cordyceps gunnii*.

Low or no cytotoxic activity.—*Agaricus bitorquis, Coprinopsis atrametaria, Psathyrella asperospora, Russula clelandii, Tricholoma* sp. AU2 and *Xerula mundroola* were the least cytotoxic species evaluated in this study; generally low or no cytotoxic effects were observed for the various extracts from these species. Of the extracts that exhibited low or no toxicity and/or proliferative effects (results not shown) the majority corresponded to cold or hot water extracts.

DISCUSSION

Ethanol, cold and hot water extracts of predominantly native Australian macrofungi were investigated for cytotoxic activity. Successive extractions provided a rough separation of compounds on the basis of their solubility. Ethanol extracts are likely to contain both polar and nonpolar metabolites, including terpenoids (Lindequist et al. 2005), sterols, fatty acids, polypeptides and amino acids (von Usedom 2003). Conversely water extracts are likely to contain water-soluble compounds including carbohydrates such as polysaccharides and small amounts of proteins and minerals (von Usedom 2003). Moreover many of the traditional preparations of medicinal mushrooms employ either hot or cold aqueous preparations and it has been suggested that a hot water extract from *Inonotus obliquus* might suppress cellular proliferation in human stomach cancer (Watanabe 2004). Therefore hot water extracts also were evaluated in this study. Gastric, colorectal and two breast cancer cell lines were selected for the test panel because they represent some of the most prevalent types of cancer (World Health Organization 2008). Of interest all species showed low or no cytotoxic activity against human healthy cells and some had significant (*Omphalotus nidiformis*, *Cordyceps. cranstounii* and *Cordyceps gunnii*) and selective (*Xerula mundroola*, *Leratiomyces ceres*, *Hypholoma. fasciculare*) activity against cancer cells.

In our study *O. nidiformis* extracts overall were the most cytotoxic extracts. Species of *Omphalotus* contain several types of illudins, a class of oxygenated sesquiterpenes that possess antitumor, cytotoxic and antibiotic activity (Kelner et al. 1990). The toxicity of *O. nidiformis*, colloquially known as ghost fungus due to luminescence, is presumably due to the illudins, as is the case for other species in the genus (Benjamin 1995). In common with northern hemisphere species of the genus *O. nidiformis* contains illudins such as M and S (Kirchmair et al. 1999) but also three novel

illudins (F, G and H) (Burgess et al. 1999). *O. nidiformis* is widespread in temperate Australia (Grey and Grey 2005), and there have been a number of human poisonings in Australia due to *O. nidiformis* being confused with edible *Pleurotus* species (Hender et al. 2000). Given that illudins are extractable with non-polar solvents and the highest cytotoxic activity was detected in ethanolic extracts, we presume that the illudins are responsible, or at least contribute, to the activity. The mushroom can be grown in pure culture (Burgess et al. 1999) and it therefore will be practicable to carry out further studies to establish whether the compounds responsible for the activity are indeed illudins and whether they are those shared with other species or are possible unique to *O. nidiformis*.

Illudins also have been reported from *Coprinus comatus* (Gonzalez del Val et al. 2003). Not surprising therefore studies have shown anti-proliferative effects of ethanol and ethyl acetate extracts against prostate cancer cells (LNCaP) (Zaidman et al. 2008) and of ethyl acetate extracts against ovarian cancer cells (ES-2) (Rouhana-Toubi et al. 2009). A cold water extract also showed activity against estrogen-dependent and independent breast cancer cells (Gu and Leonard 2006). In contrast we observed activity for the ethanolic instead of the water extract of *C. comatus*, which might indicate that the cytotoxic compounds were solubilized in our initial ethanol extraction and could be attributed to the illudins.

Given that illudins are known from *Coprinopsis atramentaria* (as *Coprinus atramentarius*) (Lee et al. 1996), it was surprising that none of the extracts from the Australian specimens of this species exhibited significant cytotoxicity. *Coprinus* and *Coprinopsis* species examined in this study are thought to have been introduced to Australia. *C. atramentaria* has a wide distribution globally (May et al. 2010), and perhaps different growing conditions throughout its range contribute to different compositions of constituents as might different genotypes.

Extracts from Australian *C. cranstounii* and *C. gunnii* exhibited significant cytotoxic effects against a variety of cancer cell lines. In contrast the remaining two species of *Cordyceps* sensu lato (*C. hawkesii* and *Ophiocordyceps roberstii*) showed significantly less cytotoxic potential. Cytotoxic activity has not been reported for any of the four native *Cordyceps* and *Ophiocordyceps* species examined in this study, however species of *Cordyceps* sensu lato are used in traditional Chinese medicine and consequently the chemistry and pharmacology of the medicinal *Cordyceps militaris* and *Ophiocordyceps sinensis* (syn. *Cordyceps sinensis*) have been examined extensively, with more than 60 cytotoxic activity reports of species from *Cordyceps* sensu lato.

Genus *Cordyceps* in the broad sense (sensu lato) contains entomopathogenic fungi that parasitize a range of arthropods and fungi, including caterpillar larvae. A number of segregate genera from *Cordyceps* sensu lato have been recognized (Sung et al. 2007), although not all species have been placed in this new classification yet.

Liu et al. (2002) considered *C. hawkesii* to be a synonym of *C. gunnii*, based on similarities of ITS sequences among Chinese material under these names. However reports of *C. gunnii*, *C. hawkesii* and *O. robertsii* (as *C. robertsii*) from China (Liu et al. 2002) appear to be misidentifications because the provided ITS sequences of Chinese collections have low BLAST matches against those of Australian collections. For example AJ309344 (*C. gunnii*, Australia) has only 86% match against AJ309340 (*C. gunnii*, China), whereas Chinese isolates (variously identified as *C. gunnii*, *C. hawkesii* or *Paecilomyces anamorphs* of the two) have matches of 94% or higher. Until sequence data are available for a range of Australian collections we prefer to recognize *C. hawkesii* as distinct from *C. gunnii* based on morphological characters, in particular the well demarcated fertile portion of the club (Grey and Grey 2005, Willis 1959).

This study is the first cytotoxic evaluation of the Australian native mushrooms *C. cranstounii*, *C. gunnii*, *C. hawkesii*, *O. robertsii*, *Psathyrella asperospora*, *L. ceres*, *Tricholoma* sp. AU2, *X. mundroola* and *Russula clelandii* and has confirmed the in vitro cytotoxic effects for *O. nidiformis* against various cancer cell lines. We furthermore showed that extracts from the cosmopolitan species *C. atramentaria* collected in Australia do not exert significant cytotoxic effects against the cell lines tested. We also identified a marked difference between the cytotoxic profile of Australian material of *C. hawkesii* and *C. gunnii* that supports their treatment as distinct species.

Our study provides a first comprehensive evaluation of the cytotoxicity of various Australian fungi species and forms an important basis for the isolation and structural elucidation of cytotoxic compounds from these species in the future.

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