International Journal of Pharmacy and Pharmacology ISSN: 2326-7267 Vol. 9 (12), pp. 001-005, December, 2020. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Antimicrobial properties of protein extracts from wild mushroom fungi and native plant species against hospital pathogens

Michael Hearst[1,2], David Nelson[3], Graham McCollum[3], Linda M. Ballard[4], B. Cherie Millar[3], Sara Moore[5], Stephen McClean[5], John E. Moore[3,5] and Juluri R. Rao[1,5]*

[1]Applied Plant Science Division, Agri-Food and Biosciences Institute, Newforge Lane, Belfast, Northern Ireland, BT9 5PX.
[2]Grosvenor Grammar School, Cameronian Drive, Belfast, Northern Ireland, BT5 6AX.
[3]Northern Ireland Public Health Laboratory, Department of Bacteriology, Belfast City Hospital, Lisburn Road, Belfast, Northern Ireland, BT9 7AD.
[4]Ulster Folk and Transport Museum, Cultra, Holywood, Co. Down, Northern Ireland, BT18 0EU.
[5]School of Biomedical Sciences, Centre for Molecular Biosciences, University of Ulster, Cromore Road, Coleraine, Co. Londonderry, Northern Ireland, BT52 1SA.

Accepted 08 November, 2020

Protein extracts of either native or exotic rare mushroom fungi and plants that are normally known for novel therapeutics including immune modulation were investigated for their potential antimicrobial effects. Data obtained using the Kirby-Bauer's disc-diffusion assay methods showed that a number of locally sourced wild mushroom fungi (e.g. *Ganoderma resinaceum*, *Russula fragilis* and *Inocybe grammata*) had proteins with inherent antimicrobial properties against a number of typical hospital pathogens. The wild type fungus *Mycena pura* exhibited strong antagonism against *Escherichia coli*, an organism often commonly associated with nosocomial infections both locally and worldwide. Polyacrylamide gel electrophoresis (PAGE) of protein extracts revealed unique protein banding patterns for the exotic fungal species and possessed significant inhibitory effects against a range of nosocomial pathogens including MRSA, *Salmonella*, *Candida* and *Aspergillus* species. This small-scale study revealed the occurrence of wild fungal peptides of potential therapeutic significance and antimicrobial potential for exploitation in complementary therapies in clinical and veterinary medicine.

Key words: Exotic fungi and medicinal plants, antibacterial activity.

INTRODUCTION

Medicinal fungi such as Shiitake, *Lentinula edodes,* have a well-documented history of use in traditional oriental therapies. Not only is this an ancient practice, but still today, medical practice in Japan, China, Korea, and other Asian countries continues to rely on fungal-derived antibiotics. In search of novel therapeutic alternatives, many fungal and plant based studies have found compounds with various clinical properties, ranging from anti-carcinogenic, anti-inflammatory, immunosuppressive and, last but not the least we recently report-ted new compounds (Rao et al., 2009) and the antimicrobial potentials in locally sourced Shiitake mushrooms (Hearst, 2009).

According to the World Heath Organisation, (http://www.who.int/mediacentre/factsheets/fs194/en/) "the bacterial infections which contribute most to human disease are also those in which emerging and microbial resistance is most evident: diarrhoeal diseases, respiratory tract infections, meningitis, sexually trans-mitted infections, and hospital-acquired (nosocomial) infections." The following are some common examples;

*Corresponding author. E-mail: jr.rao@afbini.gov.uk, j.rao@ ul ster. ac.uk. Tel: +44 (28) 9025 5258. Fax: +44 (28) 9025 5007.

penicillin-resistant *Streptococcus pneumoniae*, vancomycin-resistant *Enterococci*, methicillin-resistant *Staphylococcus aureus*, and multi-resistant *Salmonellae* and *Mycobacterium tuberculosis*. With the recent emergence of the resistant *E. coli* linked NDM-1 'superbug' there is an urgent need to combat pathogens. Antimicrobial resistance in both medicine and agriculture is now a glaring reality. It represents a significant challenge of global dimensions to human and veterinary medicine with the prospect of therapeutic failure for life-saving treatments (e.g. "The Copenhagen Recommendations: Report from the Invitational EU Conference on The Microbial Threat" http://www.im.dk/publikationer/micro98/index.htm).

A new science Venomics (e.g. http://www.science.ulster.ac.uk/pbrg/) focuses on novel bioactive peptides and small molecules from a variety of unusual biological sources such as snake and scorpion venoms, amphibian defensive skin secretions. The proteomics of the molecules of interest include those displaying anti-microbial, anti-cancer, vasoactive, insecticidal or anthelmintic properties and their therapeutic evaluations against human tumors and their cell lines. In terms of antimicrobials, several of the amphibian peptides (Thompson et al., 2007) were purified to homogeneity and they displayed potent antimicrobial activity with minimum inhibitory concentrations starting at 0.4 µM when tested against a range of Gram-positive and Gram-negative bacteria including *Escherichia coli*, *Staphylococcus aureus* and *Micrococcus luteus*. Together with such exciting published data emerging from unusual fauna, and despite curative effects to illnesses, there has been a shortfall of scientifically accredited data or reports examining the concomitant antimicrobial combative potencies manifested in rare fungal genera such as local or exotic wild mushrooms and native herbal plants. With the above in mind, a preliminary scouting study was undertaken to uncover potential antimicrobial constituent proteins in both native or exotic rare mushroom fungi and plants for combating several bacterial pathogens including the hospital superbugs. These included *Anenome nemorosa*, *Filipendula ulmaria* and *Sambucus nigra*, together with the fungi *Ganoderma resinaceum*, *Mycena pura*, *Inocybe grammata* and *Russula fragilis*.

MATERIALS AND METHODS

Mushroom fungi and plant tissues were freeze dried for approximately 72 h (Edwards Super Modulyo Freeze Drier). A plant protein (P-PER®) extraction kit, catalogue no. 89803 (Thermo Scientific https://www.thermoscientific.com/wps/portal/ts/products/) was used to extract proteins, as per the Manufacturer's protocol. 20 mg of dried tissue was placed into the polypropylene bag provided, which contained an inner layer mesh screen. The prepared extraction solution was added to the bag and a pestle was used to mechanically break up the tissue, ensuring thorough maceration. Once a homogeneous mixture was achieved, the liquid was pipetted into a sterile Eppendorf tube, centrifuged (@3,500 rpm, 5 min), and a pipette tip was inserted through the upper organic layer into the aqueous layer to remove the protein solution which was transferred into a fresh sterile Eppendorf tube, for storage until assay. Kirby-Bauer disc assays were performed as described previously (Hearst et al., 2009) in which the test discs were impregnated with protein extracts (suppled @ 1.0 mg protein ml^{-1}) and placed over lawns of freshly grown cultures of representative microorganisms. Discs carrying the antibiotic Ciprofloxacin (Oxoid), served as a positive control together with those impregnated with protein extraction kit reagents as background effect or negative controls.

Identification of local wild mushrooms

Formal visual appearance determinations of wild fungi collected were made by consulting a manual (Phillips and Shearer, 1981). The identities of the typical wild mushroom fungi were further confirmed by means of Polymerase Chain reaction (PCR) assays. PCR were carried out using fungal 18S rDNA universal ITS 1 and ITS 4 primers (ITS1: TCC GTA GTT GAA CCT GCG G and ITS4: TCC TCC GCT TAT TGA TAT GC). The primers were added to the reaction mixture containing fungal DNA (~15ng) in a total volume of 50 µl of MasterMix (Invitrogen) and the PCR cycles were set at 94°C, 3 min (one hold), 94°C, 30 s, 53°C, 30 s, 72°C, 1 min (35 cycles), followed by a final extension step of 10 min, 72°C.

Sequencing

All PCR products were purified using the Chargeswitch ® PCR Clean-Up Kit (Invitrogen CS12000 http://products.invitrogen.com/ivgn/product/CS12000). The cleaned up putative PCR products were sequenced according to the protocol described in ABI Big Dye Terminator v.3.1 Cycle Sequencing Kit (https://products.appliedbiosystems.com/) (ABI, Warrington, UK) using the same primers, after which the products were purified by Sodium Acetate/Ethanol precipitation. They were then loaded onto an ABI 3100 DNA GENETIC ANALYSER for forward and reverse sequencing reactions. Sequences obtained were analyzed using Geneious Pro 4.8.3 software (http://www.geneious.com/). Sequences were submitted for comparison with those stored in Gen Bank using the BLASTn alignment software (http://www.blast.genome.ad.jp/) and the sequence homology identity was determined.

Protein gel electrophoresis

Protein gel electrophoresis is a simple way to separate proteins prior to downstream detection or analysis. We carried out a polyacrylamide gel electrophoresis (PAGE) using NuPAGE® Novex ® Bis-Tris MiniGels in an X-Cell SureLock Mini-Cell unit according to the manufacturer's instructions (http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Protein-Expression-and-Analysis/Protein-Gel-Electrophoresis/1D-Electrophoresis/Xcell-SureLock-Mini-Cell.html).

RESULTS

The antimicrobial activity of protein extracts supplied at 1 mg protein ml^{-1} of freeze-dried concentrates of plant and fungal sources against the four most common nosocomial pathogens is shown in Table 1. All four

Table 1. Inhibitory effects (zone of inhibition mm) on certain key notified hospital pathogens by protein extracts of locally collected fungi, plant species.

	E.coli	***B. cereus***	***C. krusei***	***A. fumigatus***
Anenome nemorosa	0	0	0	0
Filipendula ulmaria	0	0	0	0
Sambucus nigra	0	0	0	0
Shiitake Lentinula edodes	0	0	0	0
Ganoderma resinaceum	0	11	10	8
Mycena pura	9	8	16	10
Russula fragilis	0	10	8	0
Inocybe grammata	0	10	9	0
P-PER Kit Control	0	0	0	0
Ciprofloxacin	25	30	0	0

Table 2. Antimicrobial activities of locally growing wild type mushroom species on a range of nosocomial microorganisms.

	M. pura	***G. resinaceum***	**Ciprofloxacin**
E. coli	8	0	20
Staphylococcus	9	9	28
Salmonella poona	9	10	25
E.coli	9	0	25
S. aureus	9	0	18
S. epidermalis	8	0	20
C. glabrata	18	18	0
C. parapsilosis	15	12	0
A. flavus	12	12	0
C. albicans	18	18	0
C. krusei	10	9	0
A. niger	20	21	0
A. fumigatus	10	12	0

microorganisms were used in the Kirby-Bauer disc assay against protein extracts from well known medicinal plants viz., *Filipendula ulmaria* ('Meadowsweet', Rosaceae) used for stomach problems, heartburn ulcers, pain reliever for sore joints and muscles), *Anemone nemorosa* ('Wood Anenome', Ranunculaceae) used for various disorders including gastro-intestinal irritations etc, and *Sambucus nigra* ('Elderberry', Caprifoliaceae) known to have immune modulation effects. However, these respective protein extracts exhibited no zones of inhibition following overnight incubation of the cultures, and resembled those either exposed to discs impregnated with protein extraction kit blanks. In sharp contrast, all four locally wild growing mushrooms viz., Ganoderma resinaceum, *M. pura*, *I. grammata* and *R. fragilis* exhibited varying levels of inhibitory abilities but distinctly antagonistic against the four typical hospital pathogens tested. The wild mushroom *M. pura* was particularly versatile in exhibiting high levels of inhibition (evidenced by zone widths) against all microorganisms tested.

We further extended our antimicrobial disc assay tests to 13 other commonly encountered nosocomial microorganisms (Table 2) using protein extracts obtained from two of the locally collected wild mushroom fungi viz., *G. resinaceum* and *M. pura*. The disc diffusion agar culture plate inhibitory assay results indicated that the putative protein extracts obtained from *M. pura* wild mushrooms exhibited strong inhibitory effects on all bacterial pathogens while *G. resinaceum* was inhibitory only towards the growth of *Staphylococcus* spp. and *Salmonella poona*. Interestingly, the peptide fractions of *M. pura* were overwhelmingly antagonistic against all nosocomial bacterial and fungal pathogens tested. The protein profiles obtained via PAGE (Figure 1) indicated sharp visual distinctions in the size and range of proteins present in the above two most potent wild fungi tested in this study. The major protein bands present in the PER Protein extracts of *M. pura* comprised low molecular mass peptides while in *G. resinaceum* there were multiple intense bands distributed over the entire size range. These were much larger in size (>14 kDa) and appeared to occur in pairs and in the size ranges around 3-8, 14-25, 35-38, and 49-62 kDa. There were a number

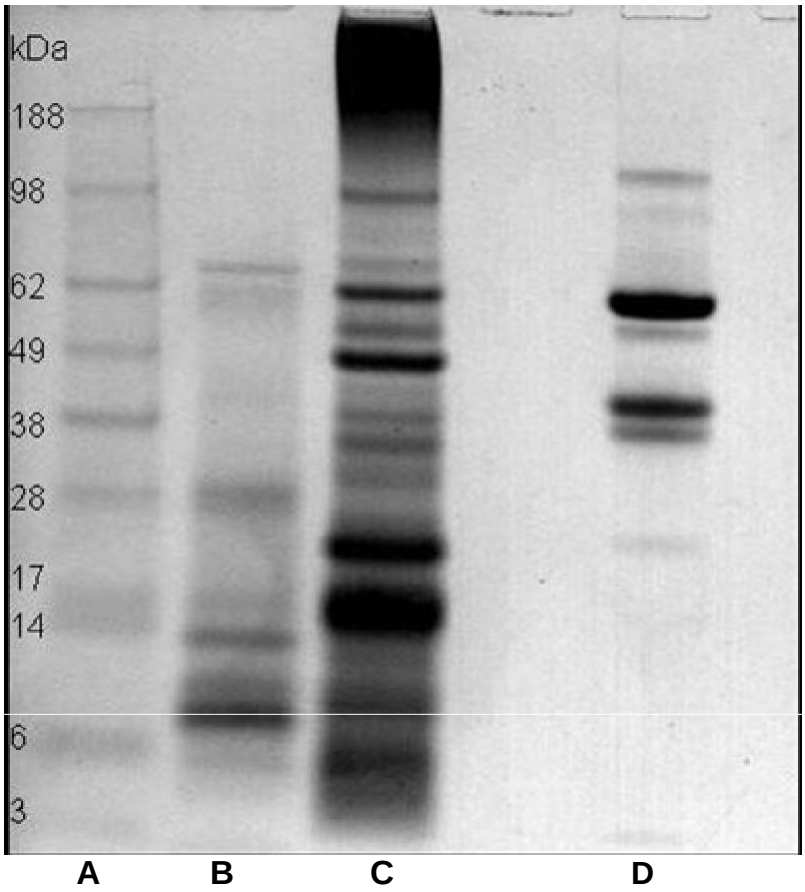

Figure 1. Polyacrylamide gel electrophoresis (PAGE) using Nupage protein gel (4 -12% Bis/Tris) in MES buffer for separation of putative proteins from wild fungi. A – Protein ladder, Invitrogen Nupage Seeblue Plus 2 ladder running in MES buffer; B – *M. pura*; C – *G. resinaceum*; D – Anion Exchange Protein Test Standard (Ovalbumin BSA, Applied Biosystems±) (https://products.appliedbiosystems. com)±

of less intense single band proteins in the intermediate ranges between these intense bands. The largest single most intensive protein band for *M. pura* was ~8kDa and that in *G. resinaceum* was ~16 kDa.

DISCUSSION

The protein extraction kit used in this study is well suited for rapid extraction and screening of antimicrobial peptides from wild mushroom fungi. The P-PER protein extracts of the three plant species and *L. edodes* (Shiitake mushroom) produced no zones of inhibition against any of the nosocomial microorganisms tested. This contrasts with an earlier study upon organic phase extracts of the same native plant species (Woods-Panzaru et al., 2009) and Shiitake mushrooms (Hearst et al., 2009), both of which had demonstrated antimicrobial properties against a range of hospital pathogens. It is probable that the organic phase extracts were virtually either free of or may have carried only minimal antimicrobial type of peptide residues from the plant or fungal samples. The organic phase extracts predominantly comprised aromatic secondary metabolites detected in the Shiitake mushrooms (Rao et al., 2009) and in the native herbal plants (Hearst et al., 2010) and these contributed to their antagonism towards hospital pathogens in our previous studies.

Locally sourced rare, wild growing *G. resinaceum* showed that its protein extracts possess inhibitory abilities against three of the four test microorganisms (enlisted in Table 1), whilst *M. pura* was highly inhibitory against all microbes tested. *R. fragilis* and *I. grammata* were only active against two of the microorganisms, but nonetheless potential antimicrobial peptides were present in both these wild mushroom species. In light of this discovery, *G. resinaceum* and *M. pura* were assayed on a wider range of test microorganisms, primarily yeasts and moulds (Table 2). Furthermore, *G. resinaceum* protein extract displayed antimicrobial activity, against 8

of the 13 organisms, primarily the yeasts and mould species tested - suggesting there are potentially antimicrobial peptides present in that species of wild mushroom fungus. This may be of immense interest to dental and skin care disorders. By contrast, *M. pura* protein extract, although carried only low molecular mass peptides seen as less intense bands in Figure 1, showed antimicrobial activity against all of the test micro-organisms and thus may be attractive for complementary therapies involving broad range antibiotic cover and this particular wild mushroom could be further explored for novel antimicrobial peptides. The results also demonstrated that intensity and numbers of protein bands might not be the true indicators of efficacies of antimicrobial potentials. Since we were examining the antimicrobial activity of P-PER protein extracts for the first time, it was necessary to confirm that the kit constituents were not imposing artefact results. The negative P-PER kit control showed no sign of inherent antimicrobial activity, which though is an expected result, nevertheless proved that the reagents used during the protein extraction had no inhibitory characteristics and therefore any inhibition occurring was due to the extracts. Ciprofloxacin control discs provided the expected outcome and showed distinct activity against *E. coli* and *Bacillus cereus*, but showed no activity against the yeasts and moulds. The prospective peptides in the protein extracts (Figure 1) of the wild mushrooms particularly *G. resinaceum* and *M. pura* that show antimicrobial activity against a range of nosocomial pathogens in the fractions will be isolated and characterised. It is too premature to fully justify the pharmacological potency of antimicrobial peptides that may be present within the protein fractions of the wild fungi tested. However, this present pilot study revealed the occurrence of clinically significant antimicrobial peptides in local, wild mushroom fungi that are strongly inhibitory against a range of menacing hospital pathogens and thus urgently warrants isolation of these potent protein fractions via protein separation and identification techniques. On-going work at our Venomics laboratory (http://www.science.ulster. ac.uk/pbrg/) using either HPLC-QT-of or MALDI-Tof techniques and obtaining 3-D conformational analyses for further understanding its structure-function activities may help fully characterize the potent antimicrobial peptides in the mushroom fungi tested in this study.

ACKNOWLEDGEMENT

The authors wish to thank the Agri-Food and Biosciences Institute (AFBI) and the University of Ulster (UU) and the Nuffield Science Foundation for their support and assistance in the research. MH was supported by a Nuffield Science Bursary administered by Sentinus. J.E.M was partly supported by Department of Health and Social Services and Public Safety (DHPSS) Research Development Office Grant (RRG 9.9), Belfast, UK.

REFERENCES

Hearst C, McCollum G, Nelson D, Ballard LM, Millar BC, Goldsmith CE, Rooney PJ, Loughrey A, Moore JE, Rao JR (2010). Antibacterial activity of Elder (Sambucus nigra) flower or berry against hospital pathogens. J. Med. Plants Res., 4(17): 1805-1809.

Hearst R, Nelson DWVA, McCollum G, Millar BC, Maeda Y, Goldsmith CE, Rooney PJ, Loughrey A, Rao JR, Moore JE (2009). An examination of the antibacterial and antifungal properties of constituents of shiitake (Lentinula edodes) and oyster (Pleurotus ostreatus) mushrooms. Compl. Ther. Clin. Pract., 15(1): 5-7.

Phillips R, Shearer L (1981). In “Mushrooms and Other Fungi of Great Britain and Europe” - A Pan original (Derek Reid and Ronald Rayner, Eds) Pan Books, pp. 72, 99, 153, 237.

Rao JR, Smyth TJ, Millar BC, Moore JE (2009). Antimicrobial properties of shiitake mushrooms (Lentinula edodes). Int. J. Antimicrob. Agents, 33: 591-592.

Thompson AH, Bjourson AJ, Orr DJ, Shaw C, McClean S (2007). A combined mass spectrometric and cDNA sequencing approach to the isolation and characterization of novel antimicrobial peptides from the skin secretions of Phyllomedusa hypochondrialis azurea Peptides, 28: 1331-1343.

Woods-Panzaru S, Nelson DWVA, McCollum GC, Ballard LM, Maeda Y, Goldsmith CE, Loughrey A, Rao JR, Moore JE (2009). An examination of antibacterial and antifungal properties of constituents described in traditional Ulster cures and remedies. Ulster Med. J., 78(1): 13-15.