EVALUATION OF DIFFERENT PROTEIN PRECIPITATES OF A WILD MUSHROOM, GANODERMA LUCIDUM FOR ANTIBACTERIAL ACTIVITIES AGAINST HUMAN PATHOGENIC BACTERIA

SUNDARAMOORTHY MARIMUTHU*, SABARIMANI KANDAN MAHENDRAN
Department of Zoology, Rajah Serfoji Government College, Thanjavur, Tamil Nadu, India. Email: drmsundartnj@gmail.com

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ABSTRACT

Objective: The objective of the present study was to isolate different antibacterial protein precipitates from Ganoderma lucidum against human pathogenic bacteria and to evaluate suitable precipitating agent.

Methods: The acid extract was prepared from the aqueous solution of the test mushroom. From separate aliquots of acid extract, antibacterial proteins were precipitated using five different concentrations (1.0–50%) of ammonium sulfate solutions, 10% trichloroacetic acid (TCA), 80% ethanol and methanol – Chloroform mixture (2:1 v/v). Protein quantification was performed in each stage of purifications. The as-prepared protein precipitates were subjected for antibacterial and hemolytic assays for identification of the active protein precipitate, which in turn was also checked for minimum inhibitory volume (MIV) for all test organisms.

Results: The quantity of each protein precipitated by different protein precipitating agents from the acid extract of the test mushroom was found in the range of 2.3–4.8 mg/g wet wt. Although all the precipitates showed different levels of antibacterial capacities, 10% TCA precipitate was considered as active protein as it yielded the maximum amount of protein (4.8 mg/g wet wt) as well as it exhibited burly bactericidal activities at lower volumes of protein solutions subjected (6.3 and 3.2 µl) on all bacterial strains tested with less hemolytic effects.

Conclusion: The protein precipitated by 10% TCA from the acid extract of the test mushroom could be developed as a drug candidate for treating infectious diseases caused by pathogenic microbes in human.

Keywords: Mushroom, Acid extract, Trichloroacetic acid, Minimum inhibitory volume, Hemolytic assay.

INTRODUCTION

The abuse of antibiotics results in the selection of multi-resistant bacterial strains and it is challenging to treat their infections. It is well-known evidence that Methylillin-resistant Staphylococcus aureus is resistant to multiple antibiotics rather than methicillin. Likewise, the bacteria Klebsiellasp. and Escherichiacoli present resistance to the third generation of cephalosporins [1,2]. To treat such infections, phytochemicals are widely recognized as good alternatives to classical antibiotics due to the fact that they are derived from natural sources existing in our environment. For instance, a recent study shows that the ethanolic extract of Trachyspermum ammi seed has strong antibacterial activity against Shigella spp. [3]. However, yet it is not a proven record for not developing resistant strains when treating infectious diseases by phytochemical drugs. For the past few decades, the small molecules of proteins (1–100 kDa) named antimicrobial proteins/peptides (AMPs) isolated from various parts of living entities have been extensively acknowledged as natural antibiotics as they assassinate the organism by direct cytolysis. This typical killing mechanism involves ionic interaction and never leads the organisms to develop resistance. They exhibit a broad spectrum of antimicrobial activities against bacteria, fungi, and viruses [4-10].

Our group [11-13] has also substantiated the presence of antibacterial proteins in different organs such as liver, heart, kidney, and muscles of fish, goat, and chick. Among these organs, only the heart tissues of all animals exhibited strong antibacterial activities against various human pathogenic bacteria. However, the bulk preparation of AMPs from such tissues could be a hard task due to the inconvenience involved in collection of such organs in kind enough quantities from slaughterhouses.

Mushrooms are supposed to be rich sources of various pharmaceutical compounds including AMPs [14-20]. Reishi mushroom (Ganoderma lucidum) is one of the most famous Oriental mushrooms having a long history of medicinal use in China, Japan, and Asian countries. It is a large, dark mushroom with a glossy exterior and a woody texture. Among edible mushrooms, G. lucidum has more pharmaceutical values rather than nutritional values. Varieties of commercial products of this mushroom are available in the world market in the forms of powders, dietary supplements, and tea [21]. Lindequeist et al. [22] have also reported the impending effects of different extracts of lingzhi on various bacterial strains. Although such medicinal properties of G. lucidum were explored to support its ancient claims, only less number of studies has been carried out on protein/peptide-based antibiotics of reishi mushroom. For example, a 45 kDa protein isolated from G. lucidum was found to have potential antibacterial and antioxidant activities [23]. Hence, in this paper, we have discussed our investigation concerning the presence of antibacterial proteins in G. lucidum against human pathogenic bacteria.

METHODS

Collection and identification of test mushroom

The reishi mushroom (G. lucidum) was collected from a local dry land after capturing its picture. It was brought to the laboratory and manually matched with images of the list of Indian mushrooms and confirmed the same. Their properties and medicinal values were also checked with the help of appropriate articles (Fig. 1).

Tissues preparation

The fruiting body of the mushroom is slashed and washed well with tap water followed by distilled water. Appropriate weight of the tissue was taken and minced into small pieces and again washed well. Then, it was finely ground in an appropriate volume of deionized water with the help of mortar and pestle so as to prepare 10% (W/V) aqueous solution. The aqueous sample solution was stored in the refrigerator until use.
Preparation of acid extract
The aqueous solution of *G. lucidum* was subjected to acid extraction adopting the procedure of Matute *et al.* [24] with a slight modification. And the acid extracted protein solution was collected in a separate sterile vial and stored in the refrigerator until use.

Precipitation of ABPs using different precipitating agents
The acid extracted proteins were precipitated using different protein precipitating reagents such as 10–50% of ammonium sulfate solutions (five concentrations), 10% trichloroacetic acid (TCA), 80% Ethanol and Methanol – Chloroform mixture (2:1 ratio) following the procedure of Sundaramoorthy *et al.* [25].

Protein estimation
The protein concentration of stepwise protein extraction of the test mushroom was estimated by adopting the procedure of Lowry *et al.* [26].

Collection of bacterial isolates
Seven bacterial isolates *E. coli*, *Proteus mirabilis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Salmonella paratyphi* A, and *Pseudomonas aeruginosa* procured from Microbiology Laboratory, K.A.P. Viswanatham Government Medical College, Tiruchirappalli, were sub cultured periodically and stored in glycerol semi solid media. These pure isolates were inoculated in 1% Mueller-Hinton Agar media and sub cultured periodically and stored in glycerol semi solid media. These pure isolates were inoculated in 1% Mueller-Hinton Agar media and incubated at 37°C for overnight.

Antibacterial assay
The antibacterial assay of the test samples was performed in microtiter plate using resazurin as an indicator of cell growth adopting the procedure of Sarker *et al.* [27]. The organisms were diluted to 200 times by 1% MHA. A sterile 96 well plate was labeled as per the protocol designed. A volume of 100 µl of test protein was pipetted out into the appropriate columns of the plate. Following this, the microwells of the respective rows were filled with 10 µl of the diluted bacterial suspension (5×10⁴ CFU/ml). Finally, to each well, a 10 µl of resazurin indicator solution (270 mg resazurin diluted in 40 ml of distilled water) was added. The plate was covered with the lid provided and incubated for 24 h at 37°C. The color change was then observed visually, and the change of color from purple to pink or colorless was recorded as the presence of bacterial growth whereas the retaining of purple as such indicated the growth inhibition (Table 1). The experiments were done in triplicate for concordant results.

Hemolytic assay
The minimum hemolytic volume (MHV) of all the eight protein precipitates was carried out in a sterile microwell plate following the method of Zhu *et al.* [28] with a slight modification. The human blood sample was gifted by a local clinical laboratory. The sediment of red blood cells was repeatedly washed with 0.9% NaCl and made into 4% suspension with normal saline. All the test wells were filled with 100 µl of normal saline. The first wells of the marked column for protein samples were filled with 100 µl of the respective protein solutions and serially diluted to ensure that each first well had 50 µl of the protein sample in serially descending concentration. Then, 100 µl of 4% hRBCs was added in the wells of the respective rows. The 4% hRBCs alone and 4% hRBCs with 10% SDS were used as 0% (negative control) and 100% (positive control) hemolytic controls, respectively. After 2 h of incubation, the button formation or color change was recorded as absence or presence of hemolysis, respectively. The MHV of a protein is defined as the lowest volume of protein at which 100% hemolysis occurs. The experiments were done in triplicate for concordant results.

Minimum inhibitory volume (MIV)
The active protein sample (10% TCA precipitate) was chosen for MIV based on three criteria which include (i) potential bactericidal activity on all test organisms, (ii) less hemolytic effects on hRBC, and (iii) less quantity of reagent consumption for precipitating the appropriate protein. The active protein was tested for MIV for the same organisms subjected to antibacterial activity. The method adopted for MIV determination was also the same as that of antibacterial assay except for the serial dilution of the test protein solution. All the wells designed for the protocol were filled with 100 µl of 1% nutrient broth. About 100 µl of the protein solution was pipetted into each well of the first column of the plates marked for seven different bacterial strains. Then, a serial dilution was made to ensure that the first wells each row had 50 µl of the test proteins, and the succeeding wells had half of the volume of protein solution from the previous well in serially descending concentrations. Thereafter, 10 µl of bacterial suspension followed by the same volume of resazurin indicator were added in each and incubated in the same way similar to that of antibacterial assay performed adopting the method of Sarker *et al.* [27]. The MIV values were determined by visual observation of color change, as mentioned above. The experiments were done in triplicate for concordant results.

**RESULTS**
The total protein contents of the aqueous extract of *G. lucidum* were found to be 39.28 mg/g wet wt. After acid extraction, the protein Table 1: Antibacterial activities of proteins precipitated from acid extract of *Ganoderma lucidum* with different protein precipitating agents against human pathogenic bacteria

| Protein precipitates versus pathogens | Ammonium sulfate precipitates of different percentages | 10% TCA | 80% Ethanol | Meth-chloroform mixture (2:1) |
|--------------------------------------|------------------------------------------------------|---------|-------------|-------------------------------|
|                                      | 10% | 20% | 30% | 40% | 50% |                        |                        |                             |
| *Eschereria coli*                    | +   | +   | +   | +   | +   | -            | -            | +/-                         |
| *Proteus mirabilis*                  | ±   | ±   | ±   | ±   | ±   | -            | -            | ±                           |
| *Klebsiella pneumoniae*              | ±   | ±   | ±   | ±   | ±   | -            | -            | ±                           |
| *Pseudomonas aeruginosa*             | ±   | ±   | ±   | ±   | ±   | -            | -            | ±                           |
| *Staphylococcus aureus*              | ±   | ±   | ±   | ±   | ±   | -            | -            | ±                           |
| *Salmonella typhi*                   | ±   | ±   | ±   | ±   | ±   | -            | -            | ±                           |
| *Salmonella paratyphi A*             | ±   | ±   | ±   | ±   | ±   | -            | -            | ±                           |

*Interpretation:* Bacterial Growth (+), Growth Inhibition (−) and Partial Growth Inhibition (±). TCA: Trichloroacetic acid
Table 1 reveals the antibacterial activity of different protein precipitates obtained from the acid extract of test mushroom. The growth of all test organisms except E. coli was found susceptible in partial or whole by the test proteins. However, the 10% TCA precipitate exhibited a strong growth inhibition on all test bacteria.

Table 2 shows the MHV of the protein precipitates on hRBCs. Protein precipitates obtained by 10–40% ammonium sulfate solution caused severe hemolysis even at the lowest volume of protein solution (1.56 µl) where the remaining four protein precipitates exhibited no hemolytic effects up to the higher volume of 12.5 µl.

Table 1

| Concentration of protein precipitates versus hemolysis | 50 µl | 25 µl | 12.50 µl | 6.25 µl | 3.12 µl | 1.56 µl | C1 | C2 |
|------------------------------------------------------|-------|-------|---------|---------|---------|---------|----|----|
| Ammonium sulfate precipitates at different percentages (%) | H⁺ | H⁺ | PH⁺ | H⁻ | H⁻ | H⁻ | H⁻ | H⁻ |
| 10 | H⁺ | H⁺ | PH⁺ | H⁻ | H⁻ | H⁻ | H⁻ | H⁻ |
| 20 | H⁺ | H⁺ | PH⁺ | H⁻ | H⁻ | H⁻ | H⁻ | H⁻ |
| 30 | H⁺ | H⁺ | PH⁺ | H⁻ | H⁻ | H⁻ | H⁻ | H⁻ |
| 40 | H⁺ | H⁺ | H⁺ | H⁻ | H⁻ | H⁻ | H⁻ | H⁻ |
| 50 | H⁺ | H⁺ | H⁺ | H⁻ | H⁻ | H⁻ | H⁻ | H⁻ |

Interpretation: Hemolysis (H⁺), Absence of hemolysis (H⁻), Partial Hemolysis (PH⁺), C1 - (Negative Control – 0% Hemolysis) and C2 – (Positive Control – 100% Hemolysis), TCA: Trichloroacetic acid

DISCUSSION

The health benefits of different mushroom species have been documented many decades ago. A few literatures in this regard have been addressed here. Antioxidant, antimicrobial, and antiproliferative activities of Agaricus sp. and Cantharellus lusibarbus have been studied [29,30]. In earlier years, it was thought that the compounds responsible for the bioactivities might be only secondary metabolites. Later studies have shown that certain higher molecular weight peptides and proteins of mushroom species are also taking part in bioactivity. Various antifungal proteins from different fungi such as Clitocybe sinopica, Aspergillus giganteus, Aspergillus niger, Zygosaccharomyces bailii and Tricholoma giganteum [19,31-34] have been reported. An immune modulatory protein LZ-8 from G. lucidum was also proposed to have therapeutic effects on cancer and autoimmune disease [35]. Subsequent to this finding, in the present work, the presence of antibacterial proteins in the acid extract of the mushroom, G. lucidum was verified using different protein precipitating agents.

The total protein content of the aqueous extract of the test mushroom, G. lucidum was found to be 39.28 mg/g.wet.wt. This quantity is significantly relevant to the range of other mushroom (Pleurotus ostreatus, Volvariella volvacea, Agaricus campestris, and T. Hemii) protein levels, i.e., 33–38% [36]. The antibacterial proteins precipitated by different protein precipitating agents from the acid extract were found in the range of 2.3–4.8 mg/g.wet.wt. Among the precipitates, 10% TCA solution has yielded the highest quantity of protein precipitates (4.8 mg/g.wet.wt), as shown in Fig. 2.

Except for the growth of E. coli, all other organisms’ growth has been inhibited by the test proteins at different levels. However, the 10% TCA precipitate has exhibited a strong growth inhibition on all test bacteria. The presence of antimicrobial proteins in mushrooms and fungus is in agreement with some previous studies. Antifungal proteins and peptides have been isolated from diverse fungal species [31–33,37]. A mixture of antibacterial proteins against Gram-positive and negative bacteria
was extracted from white button mushroom (Agaricus bisporus) using ultrasonication technique by Kayitha and Damodharan [38]. A protein bound polysaccharide called polysaccharopeptidase, exhibiting strong antimicrobial activity has also been isolated from Coriolus versicolor fungi [39].

The 10% TCA precipitate of the test mushroom chosen for MIV had potential bactericidal activities on test organisms at two minimum volumes, as shown in Fig. 3 (6.3 and 3.2 µl). Similarly, the bacterial growth inhibition at micromolar concentration of different mushroom AMPs has also been investigated earlier. For instance, the proteins purified from Clitocybe sinopica had potential anti-fungal activity toward Agrobacterium tumefaciens at 0.14–2.7 µM [19]. These proteins had also a broad spectrum of activities against a number of plant pathogenic bacteria including strains of Agrobacterium and Xanthomonas.

Although the AMPs isolated from natural sources like mushrooms have a potential killing capacity of different pathogens, most of them present toxic effects to the host cells. Unless making some essential modifications in their sequences, their toxicity could not be prevented or reduced. Instance, fowlcidins (~1 and ~2) of chicken displayed potent salt-independent activities against a range of Gram-negative and Gram-positive bacteria, including antibiotic-resistant strains with hemolytic property [40]. The hemolytic activity of both fowlcidins was reduced significantly by 10% PBS. Similarly, Trp/Pro-rich short peptides consisting only 13 residues such as tritritcin and indolicidin having a broad spectrum of antimicrobial activities showed relative toxicity toward eukaryotic cells. Hence, much effort has been taken to decrease the cytotoxicity and to increase the cell selectivity of these two peptides [41,42]. Auspiciously, the test proteins of G. lucidum have exhibited less toxicity on hRBCs. The test proteins yielded by 40 and 50% ammonium sulfate, 10% TCA and 80 Ethanol and Methanol–Chloroform mixture were found to be nontoxic to the human erythrocytes from 1.6 µl to 12.5 µl. The higher volumes caused distinct hemolysis, as found in Table 2.

The active protein precipitate showed strong antibacterial activity at the lowest volume of protein solution subjected (3.2 µl) and not in higher volumes (Fig 3). Logically, the drug concentration and level of bioactivity should be in the same line. However, it is found contradictory in this study. Although the exact reason for this is not known, this might be due to the mode of action of the tested protein. In the lower concentration, the protein sequence would be completely open, and the active domain could be accessible to act with the receptor molecule of the bacterial cell wall. In case of a higher concentration, the sequential crow would lead several folding and which may hide the active site on the receptor molecule. In contrast to the bactericidal capacity, the lowest volume of the test protein, i.e. 1.6 µl showed no hemolysis. Hence, further deep studies in this regard could help to develop them as drug candidates by solving the above-discussed problem. The development of such natural antibiotics could also be affordable in the pharmaceutical market.

CONCLUSION

It is well-known fact that mushrooms have plenty of medicinal properties with their secondary metabolites. However, the study on antimicrobial properties of mushroom proteins is still infancy. In this circumstance, this study has substantiated that the protein precipitated by 10% TCA from the acid extract of the mushroom, G. lucidum has strong antibacterial effects against human pathogenic bacteria with less hemolytic effects. Hence, this protein precipitate could be considered for the development of natural antibiotics after kind enough further studies.

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AUTHORS’ CONTRIBUTIONS

The first author’s contributions are work idea, execution of the work plan, the arrangement of materials, and drafting the manuscript. The second author carried out experiments.

CONFLICTS OF INTEREST

Authors declare that they have no conflicts of interest.

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