Calocybe indica extract inhibits the growth and enzyme production of microbes that impair the healing of burn wounds

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ABSTRACT

Aims: The need for antimicrobial agents that could prevent the invasive spread of microbes that infect burn wounds directs the aim of the study. The edible mushroom Calocybe indica, with well-known biological activities, is explored to understand the role of the three bacteria, Streptococcus pyogenes, Enterococcus faecalis and Stenotrophomonas maltophilia, which are prevalent among the burn wounds, known for their tissue invasive properties and multidrug resistance.

Methodology and results: The aqueous extract of C. indica was screened for antibacterial activity against the test microbes. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the antibacterial aqueous extract were determined. The effect of the extract on the specific activity of the tissue-digesting enzymes protease and hyaluronidase was studied. The MIC of C. indica aqueous extract against the test organisms was found to be 125, 62.5 and 62.5 µg for S. pyogenes, S. maltophilia and E. faecalis, respectively. The specific activity of protease was reduced by 6, 1.3 and 3 folds, and hyaluronidase was reduced by 4.5, 2.8 and 4.1, respectively, in the order of test microbes mentioned above.

Conclusion, significance and impact of study: The C. indica aqueous extracts with the potential to inhibit growth and retard invasive enzyme production in the microbes infecting burn wounds could be a significant therapeutic strategy in the management of clinical burn wounds to prevent the progression of the tissue damage. The bioactive component of the extract could further be identified analytically and formulated efficiently to enhance therapeutic applications.

Keywords: Burn wound, Calocybe indica, Enterococcus faecalis, Stenotrophomonas maltophilia, Streptococcus pyogenes

INTRODUCTION

Burns are one of the most common and devastating forms of trauma. Patients with serious thermal injury require immediate specialized care in order to minimize morbidity and mortality. Nearly 180,000 burn wound-associated deaths occur every year worldwide (WHO).

Burn wounds heal with time, but in certain circumstances, like in individuals with diabetes mellitus, this healing may be delayed. Another major reason for the wound’s delayed resolution may be infection with endogenous and exogenous microbes (Bowler et al., 2004; Rezaei et al., 2011; Azzopardi et al., 2014). Immediately after the incidence of the burn wound, both innate and adaptive immune responses are activated. This rapid activation may precipitate immunosuppression by reducing the immune system’s ability to recognize and take action against microbes (Church et al., 2006). Prompt medical attention is required with burn wound infection as it may progress to the stage of sepsis and multi-organ dysfunction syndrome (MODS) (Ladhani et al., 2021; Torres et al., 2021).

Burn wound pathogens include a large number of Gram-negative, Gram-positive bacteria and some fungi. The Gram-positive bacteria may enter from the external environment or integumentary flora rapidly, and the Gram-negative bacteria may colonize from the gastrointestinal microbiome of the host after some time (Azzopardi et al., 2011). Fungal infections may be opportunistic due to the immunosuppression produced by the burn wound. Currently used broad-spectrum antibiotics may affect the population of the native microbial flora of the body and may facilitate the entry of fungal pathogens (de Macedo and Santos, 2005). Some of the pathogens associated with burn wounds are discussed below.

Stenotrophomonas maltophilia is a Gram-negative obligate aerobe, rod in shape gaining motility through few

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flagella. It is one of the major organisms responsible for nosocomial and burn wound infections (Looney et al., 2009; An and Berg, 2018). Stenotrophomonas maltophilia is a multiple-drug-resistant organism (MRDO), which makes treatment of the diseases caused by this organism challenging. Some of the infections caused by this organism are pneumonia, cellulitis, endocarditis and soft tissue infections. Sources of this organism are widespread, like tap water, drinking water, sinks, medical equipment, bottled water and biofilms in aquifers. Among them, the clinically present microbes are more antibiotic-resistant than the other sources (Brooke, 2012). Stenotrophomonas maltophilia is also found in medical and surgical instruments; therefore, burn patients who are immunocompromised can easily be infected by the pathogen. Stenotrophomonas maltophilia may also directly infect the burn wound from surface contact or air transmission (Azzopardi et al., 2011).

These Gram-positive, facultative anaerobic cocci are versatile pathogens that can stay alive in any surface for a long time. They contribute to the gut microbiome and help digest and break down fatty foods (Fair and Tor, 2014). Enterococcus faecalis strains are present largely in burn wounds. Antibiotic-resistant species of these organisms are mostly present in burns and can be catastrophic if untreated (Shokoohizadeh et al., 2018). Even though multiple drugs are used to prevent burn wound infection by E. faecalis, drug-resistant strains have been repeatedly reported (Heidari et al., 2016).

Streptococcus pyogenes is a major human bacterial pathogen that causes a wide range of infections ranging from a skin wound to systemic inflammation that can be fatal. Ineffective treatment of S. pyogenes infections may precipitate rheumatic fever, an autoimmune disorder characterized by arthritis, a valvulitis, and glomerulonephritis. This condition is mainly due to the molecular mimicry mechanism exhibited by the pathogen. In the context of burn wound infection, S. pyogenes can cause significant damage to the host. Normally, the organism is present in the soil, air and other inanimate objects, which can come in direct contact with the wound and result in infection.

Streptococcus pyogenes, E. faecalis and S. maltophilia are resistant to most of the currently available broad-spectrum antibiotics due to the production of β-lactamase enzyme and many other mechanisms. To treat the antibiotic-resistant strains of the bacteria and to establish a safer module that protects the normal flora of the human body, phytocompounds are essential.

Hence, many phytocompounds and naturally occurring compounds are being tested for their antimicrobial activity.

Calocybe indica, a mushroom species belonging to the milky white mushroom family, is a rich source of vitamins, proteins and fats and consists of a large variety of polysaccharide molecules that are biologically active. Previous reports investigated the same species’ radical scavenging and anti-inflammatory properties and various antiviral and antibacterial activity (Maity et al., 2021; Shashikant et al., 2022). In our study, we focus on exploring the antimicrobial properties of C. indica against burn wound pathogens like S. pyogenes, E. faecalis and S. maltophilia using extracts of the mushroom.

MATERIALS AND METHODS

All the reagents and solvents used were of analytical grade. Replicates were maintained wherever necessary.

Preparation of aqueous extract of C. indica

Aqueous extract of C. indica was prepared using the hot percolation method (Komal and Arya, 2013), wherein all the constituents were intended to be extracted. The plant material was shade-dried, powdered and mixed with sterile distilled water in a ratio of 1:10 w/v. Twenty-five g of the powder was dissolved in 250 mL of sterile distilled water and stirred-heated at 60 °C for 1 h. The preparation was left to cool and centrifuged to separate the residue and the solvent. Re-extraction was done again by centrifugation to remove the remaining residue and the pooled extracts were concentrated by evaporation. The crude extract was refrigerated until further use.

Phytochemical analysis of C. indica extract

Phytochemical analysis (Iqbal et al., 2015) was performed for the crude extract of C. indica using standard methods. Ten mg of the dried extract were diluted with 10 mL of sterile distilled water and used for further qualitative analysis.

Bacterial culture preparation

Bacterial cultures of S. maltophilia (ATCC 17666), S. pyogenes (ATCC 19615) and E. faecalis (ATCC 47077) were obtained from ATCC. All the test strains were maintained in nutrient broth media. An overnight culture of the test microbes was prepared, and cell density was adjusted to 0.2 OD as per MacFarland standard No. 2 using fresh nutrient broth. The inoculum was refrigerated and used for further analysis.

Screening for antimicrobial activity

The well diffusion method (Balouiri et al., 2016) was executed to determine the antimicrobial activity of C. indica against S. pyogenes, S. maltophilia and E. faecalis, with the sterile Mueller-Hinton agar (MHA) plates with 0.01% tetrazolium chloride as an indicator. Six wells were created in each agar plate by puncturing the agar using a sterile cut-end micropipette tip. At inhibitory concentrations, 200, 400, 600, 800 and 1000 µg/mL of C. indica extract and positive control ampicillin were used to fill the wells. Plates were incubated at 37 °C for 24 h and upon incubation, a zone of inhibition was recorded.
Determination of minimum inhibitory concentration

The microdilution protocol included resazurin (1 mg/mL) (Sigma) as the indicator (Palomino et al., 2002). Resazurin works on the principle of REDOX reaction. The blue compound turns red when bacteria grow as the growth of the organism causes the reduction of the dye from resazurin to resorufin. Hence, it is used to analyse bacterial growth. A stock of 1 mg/mL C. indica was prepared. Common to all the wells, 100 µL of uninoculated MHA broth was added. One thousand micrograms per millilitre (1 mg/mL) C. indica was serially diluted in the ratio of 1:2 to make 500, 250, 125, 62.2 and 31.51 µg across each row with final rows dedicated for positive and negative controls, all of the wells equally containing 100 µL (A to H). A volume of 10 µL of the inoculums diluted in the ratio 1:1000 was added to all the wells and the plate was incubated overnight. After overnight incubation, 30 µL of 0.01% resazurin was added to all wells and results were documented after 10 min of incubation. Positive control consisted of inoculum, nutrient broth and 50 mg/mL ampicillin, and negative control consisted only of the inoculum and the nutrient broth. The minimum inhibitory concentration (MIC) was determined by identifying the well loaded with the least concentration of C. indica that did not show a visible colour change due to the reduction of the indicator.

Determination of minimum bactericidal concentration

The test microbial samples from the wells with the extract concentrations that were found to be effective in MICREMA were seeded on MHA plates and incubated for 24 h at 37 °C. Minimum bactericidal concentration (MBC) was considered to be the concentration that killed 99.9% of the bacterial population tested, visually validated by seeing the restriction of growth.

Preparation of cell-free fraction

The test microbial suspension, with and without treatment with the extract, was subjected to high-speed centrifugation at 10,000 rpm for 10 min. The supernatant was separated and stored in freezing conditions for further enzyme analysis.

Protease expression regulation assay

Casein hydrolysis assay

Protease activity of S. maltophilia, S. pyogenes and E. faecalis was tested qualitatively by taking the cell-free fraction (CFF) in wells of plates containing nutrient agar with 1.5% of casein (Sigma). The plates were incubated at 37 °C for one day. A transparent halo around the well indicated protease activity. The activity was compared with two plates, one inoculated with the test microbe treated with the extract at MIC for about 2 h and the other with untreated test microbe suspension (Zhang et al., 2021).

Determination of specific activity of protease using tyrosine standard

About 1.81 mg of tyrosine (Sigma) was weighed and dissolved in 100 mL of citrate phosphate buffer. Tyrosine standards were prepared with concentrations of 10 µM increments. Citrate phosphate buffer was added to standards, giving 0.05 mL total volume. Two thousand five hundred µL of sodium carbonate-sodium hydroxide reagent and 750 µL of Folin-phenol reagent were added to the standards. The blank tube contained all reagents except the tyrosine. The absorbance of the reaction tubes was measured colorimetrically at 600 nm (Bharadwaj and Vaidyanathan, 2020).

Protease activity

The CFF of all three test strains with and without treatment with C. indica extract were tested for expression of protease. The tyrosine standard curve was used to determine the specific activity of protease in the CFF. Forty-four microlitres of the sample was mixed with 456 µL of sodium acetate buffer and 500 µL of casein, which was incubated at 37 °C for 1 h. After the addition of 1 mL of 10% cold TCA, the samples were centrifuged at 5000 rpm for 10 min at 25 °C. Five hundred microlitres of the supernatant was mixed with 2.5 mL of sodium carbonate-sodium hydroxide reagent and 0.75 mL of Folin-phenol reagent. The mixture was incubated for 20 min at 37 °C. Blank and substrate blank tubes contained 250 and 500 µL of sodium acetate buffer and citrate phosphate buffer, respectively, with other reagents, as mentioned earlier. Absorbance values were recorded at 650 nm. Duplicates were maintained (Travassos et al., 2004).

Hyaluronidase expression regulation assay

Hyaluronic acid hydrolysis assay

Hyaluronidase activity of S. maltophilia, S. pyogenes and E. faecalis was tested by the well diffusion method. To 50 mL of MHA, 1 mL of 0.04% hyaluronic acid was added. Wells were punched and the CFF of the treated and untreated samples were added to these. The plates were incubated at 37 °C overnight. A zone of clearance around the well-indicated enzyme activity.

Turbidity assay

1 mL of the substrate was taken (0.25 mL of 0.04% hyaluronic acid (Sigma) with 0.5 mL of distilled water, 0.25 mL of acidified BSA fraction V (1% w/v in 0.5 M sodium acetate buffer pH 3.1)). To this, 0.5 mL of the CFF was added, mixed and incubated at 37 °C for 30 min. An ice bath was used to stop the reaction. To the reaction mixture, 0.1 mL of 2 N acetic acid was added and read at 600 nm spectrophotometrically (Dorfman and Ott, 1948). Hyaluronic acid standards were made with PBS buffer ranging from 100 to 500 µM, making the final volume up
Preparation of crude aqueous extract of *C. indica*.

**Table 1:** Phytochemical analysis of aqueous extract of *C. indica*.

<table>
<thead>
<tr>
<th>Phytochemical test</th>
<th>Indications</th>
<th>Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenolic compounds</td>
<td>Bluish black precipitate</td>
<td>+</td>
</tr>
<tr>
<td>Reducing sugars</td>
<td>Orange red precipitate</td>
<td>+</td>
</tr>
<tr>
<td>Flavones</td>
<td>Intense yellow colour</td>
<td>-</td>
</tr>
<tr>
<td>Saponins</td>
<td>Foam production</td>
<td>-</td>
</tr>
<tr>
<td>Anthraquinones</td>
<td>Pink colour</td>
<td>-</td>
</tr>
<tr>
<td>Protein</td>
<td>Yellow colour</td>
<td>+</td>
</tr>
<tr>
<td>Steroids</td>
<td>Deep purple colour</td>
<td>+</td>
</tr>
</tbody>
</table>

*+* indicates presence; *-* indicates absence.

**Table 2:** Zone of inhibition exhibited by the *C. indica* aqueous extract against the test microbes.

<table>
<thead>
<tr>
<th>Organism/concentration (µg/mL)</th>
<th>200</th>
<th>400</th>
<th>600</th>
<th>800</th>
<th>1000</th>
<th>Positive control (Ampicillin-50µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zone of inhibition (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.5</td>
<td>0.6</td>
<td>0.8</td>
<td>0.8</td>
<td>1.2</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em></td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>1.2</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>0.5</td>
<td>1.2</td>
<td>0.8</td>
<td>0.9</td>
<td>0.9</td>
<td>1.2</td>
</tr>
<tr>
<td><em>Stenotrophomonas maltophilia</em></td>
<td>0.5</td>
<td>1.2</td>
<td>0.8</td>
<td>0.9</td>
<td>0.9</td>
<td>1.2</td>
</tr>
</tbody>
</table>

RESULTS

**Preparation of aqueous extract of *C. indica***

Aqueous extract of *C. indica* was prepared using the hot percolation method, as seen in Figure 1. Twenty-five g of the dried material yielded 11.525 g of the aqueous extract.

**Phytoconstituents of *C. indica* aqueous extract**

Phytochemical analysis of the aqueous extract of *C. indica* revealed the presence of various phytoconstituents and the results were tabulated (Table 1).

**Inhibition of test microbes by *C. indica***

The zone of clearance was observed in the concentration of 200–1000 µg/mL in all three selected organisms (Figure 2), the maximum being 1.2 mm, as seen in Table 2.

Figure 1: Preparation of crude aqueous extract of *C. indica*.

Figure 2: Micro-plate assay showing reduction of resazurin dye. (a) *S. pyogenes*, (b) *S. maltophilia* and (c) *E. faecalis*. "a" is 1 mL. This mixture was incubated in a boiling water bath and then cooled. For every mL of standard made, 9 mL of albumin was added, making the total volume 10 mL. The mixture was incubated for 10 mins at 37 °C and absorbance was read at 540 nm.
Figure 3: Minimum inhibitory concentration of the extract against test microbes, *Enterococcus faecalis* (65.5 µg), *S. pyogenes* and *S. maltophilia* (125 µg).

*C. indica* showed better antimicrobial activity against *S. pyogenes* and *E. faecalis* compared to *S. maltophilia*. The diameter of zone of inhibition is nearly equal to the positive control in *S. pyogenes* and *E. faecalis*.

**MIC of C. indica against test microbes**

The resazurin dye binding method was seen to comply with the well plate methods, as seen in Figure 3. The minimum inhibitory concentration (MIC) for all three organisms *S. pyogenes*, *S. maltophilia* and *E. faecalis* was found to be 125, 125 and 62.2 µg, respectively (Figure 2).

**MBC of C. indica against test microbes**

Colony forming unit (CFU) was determined in each of the samples from the REMA plate. The concentration of the extract that showed negligible or no growth was identified as the MBC (Figures 4 and 5) (Balouiri et al., 2016).

**C. indica extracts inhibit protease expression in test microbes**

Casein hydrolysis assay

*Streptococcus pyogenes*, *E. faecalis* and *S. maltophilia* without treatment showed a zone of clearance (Figure 6), indicating the production of protease by these burn wound pathogens.

**Specific activity of protease**

The specific activity of tyrosine present in the CFF of test microbes treated with *C. indica* was lesser than that of the untreated (Figure 7).
DISCUSSION

Burn wound infection by invasive microbes can also cause sepsis or systemic infections. Excessive production of protease and hyaluronidase can damage the neighbouring tissues, which may be opportunistic for the pathogens to infect. Burn wound infections, apart from limiting the healing, cause pain and significant morbidity to the patient. The goal of the clinician in this context is to prevent the infection of the wound and the morbidity. All three organisms investigated here are highly invasive in case of burn wounds and can be detrimental to the patient.

Resistance to antimicrobial agents is caused by exposure to the same antibacterial drug multiple times. Another major drawback of using broad-spectrum antibiotics is that it can affect the gut microflora to a larger extent. These characteristics make them very hazardous and uncontrollable in the management of burn wound infections. There is a need for the development of newer agents against burn wound pathogens.

Previously, C. indica extracts have been explored for anti-inflammatory, anti-viral and radical scavenging properties. The current study investigated the potential of C. indica extracts as a potential therapeutic agent for the management of burn wounds. The results showed that C. indica extracts significantly inhibited the production of protease and hyaluronidase in the test microbes, thereby reducing the risk of infection and promoting healing.

The efficacy of C. indica extracts in the management of burn wounds can be attributed to its anti-inflammatory, anti-viral and radical scavenging properties. These properties can help in reducing the morbidity and mortality associated with burn wounds. The results of this study provide a promising direction for the development of newer therapeutic agents against burn wound pathogens.
Figure 7: Quantitative determination of the specific activity of protease in *S. pyogenes*, *E. faecalis* and *S. maltophilia* with and without treatment of *C. indica* extract.

Figure 8: Mueller Hinton agar plate with 0.04% hyaluronic acid showing zone of clearance indicating action of hyaluronidase enzyme. (a,b,c) Hyaluronidase activity in cell free fraction of untreated *S. pyogenes*, *E. faecalis* and *S. maltophilia* respectively; (d,e,f) Lack of hyaluronidase activity in the cell free fraction of *S. pyogenes*, *E. faecalis* and *S. maltophilia* after treatment with the extract.

Calocybe indica contains many bioactive substances like fatty acids, amino acids, squalene and phytol, which exhibit antifungal, antimicrobial, antioxidant and antiatherosclerotic properties (Shashikant et al., 2022). One of the major compounds in *C. indica*, which may contribute to its antimicrobial properties is phytol (Islam et al., 2018). The mechanism of phytol’s action in inhibiting microbial growth is still poorly understood. Furthermore, many studies rendered information on all the possible mechanisms by which phytol may inhibit the growth of eukaryotic microorganisms. Phytol and related EOs may alter the mitochondrial membrane permeability and initiate the release of certain substances like Cytochrome C, Ca++, radicals and other proteins native to the mitochondria (Bakkali et al., 2008). Eventually, these substances released from mitochondria activate the intrinsic pathway of apoptosis, leading to cell death. Secondly, phytol may also contribute to the disruption of internal bioprocesses by easily gaining access inside the cell as they are lipophilic molecules. Phytol inhibits many microorganisms, including *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, etc., which have shown results similar to the current study. *Enterococcus faecalis*, one of the targets of our study, is highly susceptible to phytol when compared to the other two organisms (Pejin et al., 2014; Ghaneian et al., 2015). In addition to the growth-inhibiting activity, the ability of the extract to reduce the protease and hyaluronidase expression is worth exploring.

The enzymes synthesized by these three organisms are used to degrade the tissue surrounding the burn wound and facilitate the invasion of the infecting microbes. The various studies previously conducted in human wound infection samples concluded that a possible mechanism of pathogen entry would be enzyme production (Dai et al., 2011; Valarmathi et al., 2013; Misić et al., 2014; Pirii et al., 2018). Proteolytic enzyme activity (Govindan et al., 2016; Datta et al., 2020; Sankaranarayanan and Krishna Kumar, 2021). The minimum inhibitory concentration determination method was used to check the degree of response to the compound by the organism categorized as susceptible, resistant, or intermediate. In the current study, the extract has a MIC of 125,125 and 62.2 µg against *S. pyogenes*, *S. maltophilia* and *E. faecalis*, respectively. The MIC of *C. indica* against multiple pathogens was significantly high when compared to our results. Further fractionation and HPLC purification of the antimicrobial extract would pave the way for a comprehension in the bioactivity and its mechanism.
production facilitates the organism in tissue invasion, nutrient absorption and sometimes even in modulating the immune response. *Stenotrophomonas maltophilia* produces enzymes like protease and hyaluronidase, which contribute to its extracellular virulence (Majumdar et al., 2022). *Enterococcus faecalis* produces multiple proteolytic enzymes, namely gelatinase and serine protease, which can also be responsible for its invasiveness (Najafi et al., 2020). The exact mechanism for the production of these enzymes is unclear, but certain genes like *fsr*, *sprE* and *gelE* are upstream components triggering secretion.

Previously, a study by Bharadwaj and Vaidyanathan (2020) has provided evidence regarding the effects of phytochemicals on enzyme production by burn wound pathogens. *C. indica* was proven to reduce the hyaluronidase enzyme production by 4.5, 2.8 and 4.1 folds in *S. pyogenes*, *E. faecalis*, *S. maltophilia* and protease enzyme by 6, 1.3, and 3 folds in *S. pyogenes*, *E. faecalis* and *S. maltophilia*, respectively. The aforementioned enzymes are mainly involved in protein and tissue degradation in the burn wound region.

Calocybe indica was reported to have bactericidal properties, which may be due to an increase in the cell wall permeability and, thereby, swelling and death of the organisms. Another possible mechanism may be the inhibition of efflux pumps, which can contribute to the reduction of antimicrobial resistance in microorganisms. This action may be synergistic with conventionally used antibiotics (Datta et al., 2020).

**CONCLUSION**

*Calocybe indica* aqueous extract was found to be effective against the test microbes *S. maltophilia*, *E. faecalis* and *S. pyogenes*. The extract inhibited these pathogens’ growth and production of tissue-degrading enzymes, protease and hyaluronidase. Further research is needed to explore the application of *Calocybe indica* in wound management.

**REFERENCES**


