



Original Research Article

Preliminary Phytochemical Analysis and Antibacterial Activity of *Ganoderma lucidum* collected from Dang District of Gujarat, India

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ABSTRACT

Keywords

Ganoderma lucidum;
antibacterial activity;
phytochemical screening;
MIC (Minimum Inhibitory Concentration).

G. lucidum has been used for hundreds of years as a health promotion and treatment strategy. The emergence of new infections, drug resistant bacteria, high costs of synthetic drugs and their voracious side effects has led to an increase in the use of traditional medicine globally. Hence an attempt has been made in this study to examine the antibacterial activity of various extracts of *G. lucidum* and whether it can be used as a potential antibacterial agent. A number of bioactive compounds have been revealed from *Ganoderma lucidum* which are found to be responsible for the pharmacological potential of the mushroom. Thus, the preliminary phytochemical screening was done to reveal the bioactive constituents of *G. lucidum*. The preliminary phytochemical screening of *Ganoderma lucidum* revealed that the extracts contain carbohydrates, glycosides, triterpenoids and phenolic compounds. The various extracts of *G. lucidum* were then subjected for the Minimum Inhibitory Concentration (MIC) determination with the help of micro-dilution bioassay. Different pathogenic organisms were selected against which the antibacterial activity of the extracts were determined. The result of the antibacterial activity showed that the methanolic extract of *G. lucidum* showed the strongest antibacterial activity among the four extracts against the bacterial strains.

Introduction

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources; many of these isolations were based on the uses of the agents in traditional medicine (Abraham and Thomas, 2012). Traditional medicines are used not only for primary health care of the poor in developing

countries, but also in countries where conventional medicine is predominant in the national health care system. The herbal medicines serve the health needs of about 80% of the world's population, especially for millions of people in the vast rural areas of developing countries; more than 65% of the global population uses medicinal plants as a primary health care modality (Kamaraj *et al.*, 2012).

Plants have been a major focus of investigations for novel biologically active compounds. However, filamentous fungi have been the producers of some of the most powerful secondary metabolites which have been developed into therapeutic agents (Roberts, 2004).

In recent years, many possible sources of natural antibiotics have been in use for several infectious diseases, mostly bacterial and fungal. In view of this, the searches for new anti-microbial agents from medicinal plants are even more urgent in the countries like India where infectious diseases of bacterial origin are not only rampant, but the causative agents are also developing an increasing resistance against many of the commonly used antibiotics (Kamaraj *et al.*, 2012).

There is a constant search for new antibiotics because the existing drugs have unwanted toxicity and their inappropriate and indiscriminate use have led to an increase in antibiotic-resistant strains. In search for new antibiotics, herbs and plants are being used.

Natural product medicines have come from various source materials including terrestrial plants, terrestrial microorganisms, marine organisms and terrestrial vertebrates and invertebrates. Numerous investigations have proved that medicinal plants as well as microorganisms contain diverse classes of bioactive compounds such as tannins, alkaloids, flavonoids, terpenoids, phenols, etc (Chitemerere and Mukanganyama, 2011). Thus the main aim of this work was to detect the various bioactive components present in *G. lucidum* and thus determine the antibacterial activity of *G. lucidum* to prove its use as a safe and potent antibacterial agent.

Materials and Methods

Preparation of extracts

The fruiting bodies of *Ganoderma lucidum* were obtained from Waghai village (20.7667°N, 73.4833°E), district Dang. The extraction method of Kamra and Bhatt, 2012 with certain modifications was used. The dried fruiting bodies were ground to a fine powder using a domestic blender. For preparing the extracts, methanol, ethyl acetate, chloroform, methanol:ethyl acetate (50:50, v/v) were used as solvents to obtain the pharmacologically active compounds from the mushroom.

For every 1 gram of powder, 50 ml of solvent was used and was subjected to extraction using a reflux apparatus. After the completion of extraction, the supernatant was filtered through Whatman #1 filter paper. All solvent extracted fractions were evaporated to dryness to obtain residues. For testing the antimicrobial activity, the residues were dissolved in Dimethyl Sulfoxide (DMSO) to obtain 10 mg/ml stock solutions. The extracts were stored at 4°C in air tight containers.

Preliminary phytochemical screening

The different Qualitative chemical tests can be performed for establishing a profile of given extract for its chemical composition. The extracts were then subjected to qualitative chemical tests for various phytoconstituents like alkaloids, flavonoids, carbohydrates, reducing sugars, tannins and phenolic compounds, cardiac glycosides, terpenoids, anthraquinones, saponins, volatile oils and steroids (Raaman, 2006), (De *et al.*, 2006) and (Shamaki *et al.*, 2012).

Detection of alkaloids

Mayer's Test

To a few ml of extract, one or two drops of Mayer's reagent were added by the side of the test tube. A white creamy precipitate indicated the test as positive.

Preparation of Mayer's Reagent

Mercuric chloride (1.358g) was dissolved in 60 ml of water and KI (5.0 g) was dissolved in 10 ml of water. The two solutions were mixed and made up to 100 ml with water.

Wagner's Test

To a few ml of extract, few drops of Wagner's reagent were added by the side of the test tube. A reddish brown precipitate confirmed the test as positive.

Preparation of Wagner's Reagent

Iodine (1.27 g) and KI (2 g) were dissolved in 5 ml of water and made up to 100 ml with distilled water.

Dragendorff's Test

To a few ml of extract, 1 or 2 ml of Dragendorff's reagent was added. A prominent yellow precipitate indicated the test as positive.

Preparation of Dragendorff's Reagent

Bismuth carbonate (5.2 g) and sodium iodide (4 g) were boiled for a few minutes with 50 ml glacial acetic acid. After 12 hrs the precipitated sodium acetate crystals were filtered off using a sintered glass funnel. Clear, red-brown filtrate, 40ml was mixed with 60 ml ethyl acetate and 1 ml water and stored in amber-coloured bottle.

Detection of Carbohydrates

a) Molisch's test

To 2 ml of extract, two drops of alcoholic solution of α -naphthol was added, the mixture was shaken well and 1 ml of conc. H_2SO_4 was added slowly along the sides of the test tube and allowed to stand. A violet ring indicated the presence of carbohydrates.

b) Fehling's Test

1 ml of extract was boiled on water bath. To this, 1 ml of Fehling solutions A and B were added. A red precipitate indicated the presence of sugar. Fehling's solution A: $CuSO_4$ (34.66 g) was dissolved in distilled water and made up to 500 ml using distilled water. Fehling's solution B: Potassium sodium tartarate (173 g) and NaOH (50g) was dissolved in water and made up to 500 ml.

c) Benedict's Test

To 0.5 ml of extract, 1 ml of Benedict's reagent was added. The mixture was heated on a boiling water bath for 2 mins. A characteristic coloured precipitate indicated the presence of sugar.

Benedict's Reagent

Sodium citrate (173g) and Na_2CO_3 (100g) were dissolved in 800 ml of distilled water and boiled to make it clear. $CuSO_4$ (17.3 g) dissolved in 100 ml distilled water was added to it.

d) Barfoed's Test

To 1 ml of extract, 1 ml of Barfoed's Reagent was added and heated on a boiling water bath for 2 min. Red Precipitate indicated the presence of sugar.

Barfoed's Reagent

Copper acetate (30.5) g was dissolved in 1.8 ml of glacial acetic acid.

Test for Glycosides

Legal's test

To the extract, few drops of 10% NaOH were added to make it alkaline. Then freshly prepared sodium nitroprusside was added to the solution. Presence of blue colouration indicated the presence of glycosides in the extract.

Keller-Killiani test (for cardiac glycosides)

To 2 ml of extract, 2 ml glacial acetic acid is added, followed by one drop of 5% FeCl₃. Conc. H₂SO₄ is added from the side of the test tube. Reddish brown ring appears at the junction of the two liquid layers indicating the presence of cardiac glycosides.

Detection of Proteins and Amino Acids

Millon's Test

To 2 ml extract, few drops of Millon's reagent were added. A white precipitate indicated the presence of proteins.

Biuret Test

An aliquot of 2 ml of extract was heated with 1 drop of 2 % CuSO₄ solution. To this 1 ml of ethanol (95%) was added, followed by excess of KOH Pellets. Pink colour in the ethanolic layers indicated the presence of proteins.

Detection of Flavonoids

Shinoda test (Magnesium Hydrochloride reduction test)

To the test Solution, few fragments of Magnesium ribbon were added and concentrated Hydrochloric acid was added drop wise, pink scarlet, crimson red or occasionally green to blue color appears after few minutes.

Alkaline reagent test

To the test solution few drops of sodium hydroxide solution was added; formation of an intense yellow color, which turned to Colourless on addition of few drops of dil. acid, indicated the presence of flavonoids.

Detection of Phytosterols

Liebermann – Burchard's Test

To the extract, 3 ml of acetic anhydride was added and mixed. To this one or two drops of concentrated H₂SO₄ were added slowly along the sides of the test tube. An array of colour change showed the presence of phytosterols.

Test for Triterpenoids and Steroids

Liebermann – Burchard's Test

Extract was treated with few drops of acetic anhydride, boiled and cooled. Conc. Sulfuric acid was added from the sides of the test tube, showed a brown ring at the junction of two layers and the upper layer turning green showed the presence of Steroids and formation of deep red colour indicated the presence of triterpenoids.

Salkowski test

Extract was treated with few drops of conc. sulfuric acid, shaken well and allowed to stand for some time, red color at the lower layer indicated the presence of Steroids and formation of yellow colored lower layer indicated the presence of Triterpenoids.

Detection of Phenolic Compounds and Tannins

Ferric Chloride Test

To the extract, few drops of neutral 5% ferric chloride solution were added. A dark green colour indicated the presence of phenolic compounds.

Lead Acetate Test

To the extract, 3 ml of 10% lead acetate solution was added. A bulky white precipitate indicated the presence of phenolic compounds.

Test bacteria used

A total of eight bacterial species were tested. The Gram positive species were *Staphylococcus aureus* (MTCC-96) and *Bacillus cereus* (MTCC-430) and Gram negative species were *Escherichia coli* (MTCC-1687), *Klebsiella pneumoniae* (MTCC-3384), *Proteus mirabilis* (MTCC-425), *Pseudomonas aeruginosa* (MTCC-2453), *Salmonella typhi* (MTCC-733), *Enterobacter aerogenes* (MTCC-111).

Culture media and inoculum preparation

Twenty-four hour old pure cultured bacteria grown in Mueller Hinton broth were used to prepare the inoculum. For

each bacterial species tested, turbidity was adjusted to a scale of 0.5 on the MacFarland standard. To standardize the inoculum density, the 0.5 McFarland turbidity standard was set by mixing 99.5 ml of 0.18M H₂SO₄ with 0.5 ml of 0.048M of BaCl₂. The inoculum size of the test strain was prepared to a density of 1×10⁸ cfu/ml.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC was determined as the lowest concentration of the extract which inhibited the growth of the tested bacteria. A broth micro-dilution bioassay in 96-well micro titer polystyrene plates was used to determine MIC. The method of Yakob *et al.*, 2012 was followed with modifications. The wells of each column (1-12) were filled with 50 µl of sterilized Mueller Hinton broth (except the first well of each column). 100 µl of the extracts (Methanol, ethyl acetate, chloroform, methanol: ethyl acetate) having a concentration of 10 mg/ml dissolved in DMSO was added to the first well of columns 4-12. Serial twofold dilutions were made of the 10 mg/ml extract with the broth in the 7 consecutive wells of the columns. The concentration of the extracts ranged from 5 to 0.0781 mg/ml. Next, 50 µl of the bacterial inocula (10⁸ cfu/ml) were added to each well so that the final volume of each well was 150 µl. The first column of the plate served as the positive control. Streptomycin (1 mg/ml) was used as the antibiotic control and was serially diluted. The second column of the plate served as the negative control (50 µl of DMSO+ 50 µl broth + 50 µl bacterial inoculum). The third column of the plate was made the sterility control (containing 150 µl of broth). The tests were done in triplicates for each bacteria used. The plates were

covered and then incubated at 37°C for 24 h. After 24 hrs, 40 µl of 0.2 mg/ml iodinitrotetrazolium chloride was added to each well and the plates were further incubated at 37°C for 30 min. Bacterial growth in the wells was indicated by development of red-pink color, while growth inhibition was indicated by no change in the colour of cell suspensions. The MIC of each extract is defined as the lowest concentration inhibiting the growth of the bacteria and was recorded.

Results and Discussion

Qualitative phytochemical screening

The preliminary phytochemical screening of *Ganoderma lucidum* revealed that the extracts contain carbohydrates, glycosides, triterpenoids and phenolic compounds. Methanol and the Methanol: ethyl acetate extract were found to extract the maximum active components being solvents that have low polarity. This result was in accordance to the previously reported literature (Shamaki *et al.*, 2012) and is represented in Table-1.

Determination of Minimum Inhibitory Concentration (MIC)

The antimicrobial activity was determined using four extracts of *G. lucidum* against a wide range of bacterial strains. The MIC was determined by the broth micro-dilution bioassay in 96-well micro titer polystyrene plates and was taken as the lowest concentration of the extract which inhibited the growth of the tested microorganism.

The MIC values of the extracts against the tested bacteria are represented in Table-2. The MIC value of the extracts ranged from 0.625 to 2.5 mg/ml.

The result of the antibacterial activity showed that the methanolic extract of *G. lucidum* showed the strongest antibacterial activity among the four extracts against the bacterial strains. This indicates that the active principle which inhibits the growth of susceptible bacteria may dissolve better in methanol than in the other solvents.

The MIC of the methanolic extract was found to be 0.625 mg/ml against *Pseudomonas aeruginosa*. The MIC of the ethyl acetate, chloroform and methanol: ethyl acetate extract was found to be 1.25 mg/ml against *Bacillus cereus*, 0.625 mg/ml against *Bacillus cereus* and 1.25 mg/ml against *Bacillus cereus* respectively. The methanolic and chloroform extract were found to have the lowest MIC (0.625 mg/ml) against the test organisms.

Bacillus cereus and *Enterobacter aerogenes* were found to be the most susceptible bacterial strains as all the four extracts inhibited their growth. For *Bacillus cereus*, the chloroform extract was found to be the most effective, for *Staphylococcus aureus* and *Enterobacter aerogenes*, all the extracts were equally found to be effective, whereas for *Escherichia coli* and *Pseudomonas aeruginosa*, only the methanol extract was found to be effective. The antibacterial activity of the methanolic extract against *Escherichia coli* and *Pseudomonas aeruginosa* is of great importance as they are emerging pathogens.

Klebsiella pneumoniae, *Proteus mirabilis* and *Salmonella typhi* were found to be the least susceptible bacterial strains as none of the extracts could inhibit their growth. However, a similar study showed lower MIC (31.25 µg/ml) of the methanolic extract for *Pseudomonas aeruginosa* (Kamra and Bhatt, 2012).

In one study, it was found that the sesquiterpenoid extract of *G. lucidum* exhibited extra strong activity against *Escherichia coli*, *Proteus mirabilis* and *Bacillus subtilis* (Bhosle *et al.*, 2010). Another study showed that the acetone extract of *G. lucidum* was most inhibitory against *K. pneumoniae* having the lowest MIC value of 4.33 ± 0.33 mg/ml, followed by *E. coli* (8.17 ± 0.48 mg/ml) and *Bacillus subtilis* (14.00 ± 0.46 mg/ml); moderate values in case of *S. aureus* (19.00 ± 0.00 mg/ml) and the highest MIC values were exhibited against *Ps. aeruginosa* (21.30 ± 0.34 mg/ml) and *S. typhi* (20.80 ± 0.87 mg/ml) (Quereshi *et al.*, 2010), which were higher than the MIC reported in the present study. The lower the MIC, the more promising and sensitive the extract.

According to one study, the methanolic extract from *G. lucidum* was found to possess efficient antibacterial activity against the Methicillin Resistant *Staphylococcus aureus* (MRSA). The petroleum ether extract at very low concentration exhibited incomplete inhibition, but at high concentration showed complete inhibition against MRSA (Prasad and Weseley, 2008). In another study, the sesquiterpenoid extract of *G. lucidum*, *G. praelongum* and *G. resinaceum* from fruiting bodies and mycelium showed a low range of MIC from 0.390 to 6.25 mg/ml and 1.256 to 12.5 mg/ml respectively (Ameri *et al.*, 2011). The results above and many more have proved that *G. lucidum* can be used to combat stubborn organisms.

Preliminary phytochemical screening

The preliminary phytochemical screening of *Ganoderma lucidum* revealed that the

extracts contain carbohydrates, glycosides, triterpenoids and phenolic compounds. Methanol and the Methanol: ethyl acetate extract were found to extract the maximum active components being solvents that have low polarity.

Antibacterial activity

The antimicrobial activity was determined using four extracts of the mushroom against a wide range of bacterial strains. The MIC was determined by the broth micro-dilution bioassay in 96-well micro titer polystyrene plates and was taken as the lowest concentration of the extract which inhibited the growth of the tested microorganism. The result of the antibacterial activity showed that the methanolic extract of *G. lucidum* showed the strongest antibacterial activity among the four extracts against the eight bacterial strains. This indicates that the active principle which inhibits the growth of susceptible bacteria may dissolve better in methanol than in the other solvents.

The extracts could inhibit Gram positive as well as Gram negative bacteria. This shows the presence of active ingredients with broad spectrum of antimicrobial activity. Preliminary phytochemical analysis showed the presence of carbohydrates, glycosides, triterpenoids, phenolic compounds and tannins. The presence of these phytoconstituents in the extract could be the reason for the antibacterial activity. This study justifies the claimed uses of *G. lucidum* in the traditional system of medicine to treat various infectious diseases caused by the microbes.

New bacterial infections have increased to a great extent in the last few years.

Table.1 Qualitative phytochemical analysis of *Ganoderma lucidum* from different organic solvents

Phytochemical	Test	Observation			
		Methanol	Ethyl Acetate	Chloroform	Methanol: Ethyl Acetate
Alkaloids	Mayer's test	-	-	-	-
	Wagner's test	-	-	-	-
	Dragendorff's test	-	-	-	-
Carbohydrates	Molisch's test	+	+	+	+
	Fehling's test	+	-	-	+
	Benedict's test	+	-	-	+
	Barfoed's test	-	-	-	-
Glycosides	Legal's test	+	+	+	+
	Keller-Killiani test	+	+	+	+
Proteins and Amino acids	Millon's test	-	-	-	-
	Biuret test	-	-	-	-
Flavonoids	Shinoda test (Magnesium Hydrochloride reduction test)	-	-	-	-
	Alkaline reagent test	+	-	-	+
Phytosterols	Libermann – Burchard's test	-	-	-	-
			(Triterpenoids)		
Triterpenoids and Steroids	Libermann – Burchard's test	+	+	+	+
	Salkowski test	+	+	+	+
Phenolic Compounds and Tannins	Ferric Chloride test	+	+	+	+
	Lead Acetate test	+	-	+	+

Table.2 Minimum inhibitory concentration (MIC) (mg/ml) values of the extracts against the tested organisms

Minimum Inhibitory Concentration (mg/ml)				
Organism	Methanol	Ethyl acetate	Chloroform	Methanol: Ethyl acetate
<i>Bacillus cereus</i> (430)	1.25	1.25	0.625	1.25
<i>Staphylococcus aureus</i> (96)	ND	2.5	2.5	2.5
<i>Enterobacter aerogenes</i> (111)	2.5	2.5	2.5	2.5
<i>Escherichia coli</i> (1687)	1.25	ND	ND	ND
<i>Pseudomonas aeruginosa</i> (2453)	0.625	ND	ND	ND

This along with the severe problem of antibiotic resistance due to indiscriminate use of antibacterial drugs and dangerous side effects of some commercially available antibacterial drugs has led scientists to depend on natural sources and semi-synthetic derivatives of natural products. The presence of antimicrobial substances in the higher plants and filamentous fungi is well established. Hence the antibacterial activity of *G. lucidum* is described in this study.

The extracts showed antimicrobial activity against certain tested bacteria. *Bacillus cereus*, *Enterobacter aerogenes*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* were found to be inhibited by the various extracts of *G. lucidum*. The presence of important phytoconstituents like carbohydrates, glycosides, triterpenoids, phenolic compounds and tannins could be

responsible for the antibacterial properties. The extracts could inhibit Gram positive as well as Gram negative bacteria indicating that the active ingredients are broad spectrum compounds. However, thorough research needs to be done in order to recognize the phytoconstituents responsible for the antibacterial activity before being used for the development of any drugs.

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