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Full Length Research Paper

Evaluation of phytochemical constituents and in vitro antibacterial activity of organic solvent fractions of *ganoderma lucidum* methanolic extract

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The phytochemical constituents and the antibacterial activity of different organic solvent extracts (*methanol, Ethyl acetate* and *n-butanol),* of the wild mushroom- *Ganoderma lucidum* was evaluated. Phytochemical analysis of the three soluble fractions of the *Ganoderma lucidum* methanolic extract indicates that methanol and n-butanol fraction extracts contain; flavonoids, carbohydrates, tanins, cardiac glycosides, saponins and terpenoids, while as in case of ethyl acetate fraction the result obtained indicate the presence of carbohydrates, cardiac glycosides, saponins and terpenoids. The inhibitory activity of the test extracts against some bacterial organisms was investigated by the disc diffusion method, using various concentrations of 200mgml^{-1}, 150mgml^{-1}, 100mgml^{-1}, 50mgml^{-1} and 25mgml^{-1}. Ethylacetate extract fraction exhibited antibacterial activity against both Gram positive and Gram negative bacteria. Resistance by the test microbes was observed against methanolic and n-butanolic fractions. However, most of them showed susceptibility to ethyl acetate extract fraction except *Streptococcus faecalis, Escherichia coli* and *Enterobacter aeroginosa* that showed resistance. Minimum Inhibitory Concentration (MIC) test showed *Corynebacterium pyogene, Bacillus subtilis* and *Klebsiela pneumoneae* strains were the most sensitive, showing susceptibility at lower concentrations of 12.5mg ml^{-1}, followed by *Pseudomonas aeroginosa* and *P. mirabilis.* Minimum Bactericidal Concentration (MBC) result showed that ethyl acetate soluble fraction has more bactericodal activity against *K. pneumoneae* at a concentration as low as 12.5mg ml^{-1}. The inhibitory effect of the test extract against the tested microbes was less than that of the standard antibiotic-Ampiclox^{R} (Ampicillin + Cloxacillin).The extract showed its potentials as an antibacterial agent that should be exploited.

Keywords: Phytochemical, Antibacterial, Organic solvent fractions, *Ganoderma lucidum* Extracts, Ampiclox.^{R}

INTRODUCTION

Medicinal plants are widely used in both developing and developed countries with 80% of the World's population relying mainly on traditional therapies which involves the use of plant extracts or their active substances (WHO, 1993). This shift to microbial herbal medication can be due to development of resistance to conventional antibiotics by some of these microbes (Ahmad *et al.,* 1998, Yang *et al.,* 2004) and toxicity in host organ cells (Prakash, 2006). besides, medicinal preparations of herbal origin eliminate side effects associated to synthetic drugs (Iwu *et al.,* 1999).Following the discovery of drugs from an array of plants (Farnsworth *et al.,* 1976),there has been serious investigations into other various plant extracts as remedies to variety of diseases (Geidam *et al.,* 2007,

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Jigna and Chanda 2007, Reddy and Jose 2010) with the aim of finding cheaper, readily available, effective and non toxic medications that are herbal based (Clark, 1996, Cordell, 2000, Rizwan *et al.,* 2012).

The extracts of the wild mushroom-*Ganoderma lucidum* is one of the healing medicinal agent of herbal base that has been used for over 4,000 years in Chinese Traditional Medicine (CTM) to treat wide range of illnesses including cancer (Anita *et al.,* 2007), diabetes (Wasser *et al.,* 1997), HIV/AIDs (McKenna *et al.,* 2002, Gao *et al.,* 2003), stress and coronary disorders (Anita *et al.,* 2006, Berger *et al.,* 2011), Allergies (Powell 2006, Stavinoha, 2011), and bacterial infections (Moradali *et al.,* 2006) with inhibitory activity against gram positive and gram negative bacteria (Yoon *et al.,* 1994, Suay *et al.,* 2000). The mushroom extracts have also been reported to posses the ability to strengthen human body immune system (Kim *et al.,* 2011).

These reports however, have not demonstrated the use of different partitioned organic solvent fraction of the extracts of *Ganoderma lucidum* against different bacterial pathogens; hence the need for using methanolic, ethyl acetate and n-butanol soluble extracts fractions of *Ganoderma lucidum* against some selected bacterial agents.

The aim of this investigation is to evaluate the phytochemical constituents and the antibacterial activity of some solvent fractions of *Ganoderma lucidum* methanolic extract.

MATERIALS AND METHODS

Sample collection, Identification and Preparation of Extracts

Fresh fruiting part of *Ganoderma lucidum* was harvested from Lafia, Nassarawa State in North-central Nigeria during the rainy season (August-September) and it was transported using a clean polythene bag. The mushroom was identified and authenticated at the Department of Botany, University of Maiduguri, Nigeria. It was then air-dried in the Laboratory. The dried mushroom was then ground to fine powder using clean pestle and mortar, and this powder was stored in an air tight glass jar at 4^0C until required for use.

The air-dried *Ganoderma lucidum* powder weighing 1.5 kg was introduced into soxhlet chamber extractor and 7.5 Litres of methanol added and extracted at 40^0C for 24h to obtain methanolic solvent extract fraction.

The filtrate was then evaporated within 24h using rotatory electric evaporator as described by Trease and Evans (1997). The test, colour and yield of the crude methanol extract were evaluated. This was properly labeled and stored in a glass jar at room temperature until use. The *in vitro* antimicrobial evaluation of *Ganoderma lucidum* was carried out using WHO guidelines (WHO, 2000).

Fractionation of the methanolic extract

The crude methanol extract residue obtained was then washed with 7.5 litres of Ethylacetate (Sigma-Aldrich, Germany) in a soxhlet chamber for 24 hour yielding filtrate containing ethylacetate soluble fraction, this was further filtrated using Whatman No. 1 filter paper and the filtrate obtained was evaporated with electric evaporator to produce a black coloured extract, that is not soluble in water, but soluble in ethyl acetate solvent, and weighing 12.86g (0.91%) was obtained. This was stored in a beaker sealed with foil paper at room temperature until needed for use. The same procedure was applied with 7.5 litres of n-butanol to the residue, now weighing 1407.5g, and a 12.76g (0, 91%) of n-butanol soluble fraction, chocolate in colour was obtained. Thus obtaining a clear soluble fraction of methanol, ethyl acetate and N-butanol extracts in sequence and based on their polarity as described by Motobashi *et al., (*2004)

Phytochemical analysis

A measure of 2g from different organic soluble solvent fraction of the mushroom extracts (Methanol and n-butanol) were dissolved in 5ml of distilled water, while the ethylacetate fraction was initially dissolved in 0.5ml ethylacetate and made up to 5ml by adding 4.5ml of distilled water. The solutions were then subjected to qualitative chemical analysis as described by Harbone (1976), to identify various active phytochemical constituents such as; alkaloids, flavonoids, carbohydrates, reducing sugars, tanins, phlebotanins cardiac glycosides, terpenoids, anthraquinones, saponins, volatile oils and steroids.

EVALUATION OF ANTIMICROBIAL ACTIVITY

Microbial cultures

Microbial cultures were obtained from human patients at the University of Maiduguri Teaching Hospital, and which includes Gram-positive (*Streptococcus faecalis, Staphylococcus aureus, Corynebacterium pyogene* and *Bacillus subtilis*) and gram-negative (*Salmonella typhi, Escherichia coli, Klebsiella pneumoneae, Pseudomonas aeruginosa, Proteus mirabilis* and *Enterobacter aeruginosa*) bacteria. The isolates were identified by biochemical test.

The cultures were propagated and stored on a nutrient agar plate in the Veterinary Medicine Laboratory, University of Maiduguri; the nutrient agar medium was obtained in dehydrated powder form (Oxoids Ltd, England) and reconstituted according to manufacturer's specification. All stock cultures were maintained in nutrient agar plates at 4^0C and sub-cultured in nutrient broth at 37^0C for 8hours prior to antimicrobial testing. One milliliter of the broth culture was then used to

inoculate the agar plates.

Extract preparation.

A 200mgml^{-1}stock solution of the different organic solvent soluble fraction extracts were prepared by dissolving 2g of each extract in 10ml of distilled water from which the following concentrations of each fraction was prepared ; 200mgml$^{-1,}$ 150mgml$^{-1,}$ 100 mgml$^{-1,}$ 50mgml^{-1} and 25mgml^{-1}. A standard antibacterial agent Ampiclox^{R} (Ampicillin+Cloxacillin-500mg, Cipla, Mumbai, India), at a prepared concentration of 25mg ml^{-1} was equally used with all the tested bacterial organisms and its inhibitory zones was compared with the tested organi solvent fraction extracts of Ganoderma lucidum.

Antimicrobial Sensitivity testing

In vitro antibacterial activity of the three organic solvent fraction extracts of *Ganoderma lucidum* was determined against ten bacterial species by using disc diffusion method, as described by the National Committee of Clinical Laboratory Standards (1993). A set of disc (6mm in diameter) were prepared using whatman's No.1 filter paper, each disc was impregnated with specific concentrations of the extracts. The discs were then dried at 50^{0}C.

Culture of each bacterium was diluted using sterile normal saline to give an inoculum size of 10^{6} cfu ml^{-1}. The inoculum were spread on the surface of the dried nutrient agar plates with cotton wool swabs soaked with diluted suspension of the test organism. These plates were incubated at 37^{0}C for 30 minutes before the discs were applied aseptically. This treated plate was incubated at 37^{0}C for 24h. The same procedure was carried out using Ampiclox^{R} at 25mg mL^{-1}as a positive control. A plate with no antibiotic or the extracts was also prepared as the negative control experiment.

Zones of inhibition above 6 mm diameter of each isolate were used as a measure of susceptibility to the extracts and these values were compared to that of the standard antibiotic.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The minimum inhibitory concentration (MIC) of the methanol, ethyl acetate and n-butanol soluble fraction of the crude methanol extract of *Ganoderma lucidum* were determined using the method described by Greenwood (1989). Six sterile test tubes were arranged in five rows in a test-tube rack, each row for one of the five microorganisms used for the test. Half a milliliter of sterile nutrient broth was pipetted into all the tubes , and 0.5ml of the ethyl acetate soluble fraction of the extract containing 200mg ml^{-1} was pipetted into the first test tube of five rows to obtain a concentration of 100mg ml^{-1}, from which a serial dilution of the extract in each row was made to obtain a concentration of 50mg ml^{-1}, 25mg ml^{-1}, 12.5mg ml^{-1}, 6.25mg ml^{-1} and 3.13mg ml^{-1} respectively. The test organism (0.5ml) were pipetted into each of the test-tubes and incubated at 37^{0}C for 24h. T his was repeated for methanolic and n-butanol fractions. The MIC was recorded as the least concentration of the extract that completely inhibits the growth of the test organism. The content of the test-tubes were further sub cultured for 24h to determine bactericidal or bacteriostatic activity of the of the extract. Absence of growth on the subculture medium after MIC determination indicates bactericidal effect.

RESULTS

The result obtained from methanolic extraction of *Ganoderma lucidum obtained* a bitter tasting, chocolate coloured, gelatinous methanol extract which can dissolve in water to produce a greenish-yellow colour, and the yield of the extract was calculated to be 79.5g (w/w) which represents (5.3%), (Table 1). Phytochemical analysis of the three soluble fractions of the *Ganoderma lucidum* extract indicates that methanol and n-butanol fraction extracts contain; flavonoids, carbohydrates, tanins, cardiac glycosides, saponins and terpenoids, however, the ethyl acetate fraction showed the presence of carbohydrates, cardiac glycosides, saponins and terpenoids. This study also indicated that the phytochemicals, carbohydrates, cardiac glycosides and terpenoids were present in the three organic solvent fractions in considerable amount. (Table 2)

The result of the gradient extraction gave the following yields (w/w %) methanol, (5.3%) ethyl acetate (0.91%) and n-butanol (0.91%) soluble fractions respectively (Table 1).

The *in vitro* sensitivity studies indicates no inhibition of bacterial growth by methanol and n-butanol soluble fraction extracts against all the tested bacteria when used against both Gram positive and Gram negative bacterial organisms tested (Table 3 & 5) however, a concentration dependent growth inhibitory activity of the ethyl acetate fraction extract was observed on some of the test organisms such as *S. aureus* and *S. typhi* which showed more susceptibility at a concentration of 100-200mg ml^{-1}, while there was resistance at 50mg ml^{-1}.

However, *C. pyogene, B. subtilis, K. pneumoneae, P. aeruginosa* and *P. mirabilis* equally showed concentration dependent susceptibility to ethyl acetate soluble fraction extract, with all their growth inhibited at higher concentration of 200mg mL^{-1}, moderately at 150mgml^{-1} and 100 mg ml^{-1}and less at 50mg ml^{-1}.

Resistance to ethyl acetate fraction extract was observed with *S. faecalis, E. coli and E. aeroginosa.* Ampiclox^{R}, the standard antibacterial agent used as

Table 1: Phytochemical yield of Ganoderma lucidum from different organic solvent agents

Organic solvent	Yield (%)
Methanol	5.3
Ethyl acetate	0.91
N-butanol	0.91
All percentage were calculated from 2g of the extract	

Table 2: Qualitative phytochemical composition of *Ganoderma lucidum* from different organic Solvent soluble fraction

Phytochemical.	Tests.	Observations from different organic solvent soluble fractions		
		Methanol.	Ethyl acetate.	N-butanol.
Alkaloids	Dragendorff's reagent	+	+	+
	Meyer's reagent	+	+	+
Flavonoids	Shinoda's	–	–	–
	Ferric chloride	+	+	+
	Lead acetate	–	–	–
	Sodium hydroxide	+	+	+
Carbohydrates	Molisch's	+	+	+
	Bartoed (monosaccharide)	+	+	+
	Fehling's	+	+	+
	Combined reducing sugars	+	+	+
	Ketones	+	+	+
	Pentose	+	+	+
	Soluble starch	+	+	+
Tannins	Ferric chloride	+	+	+
	Lead acetate	–	–	–
	Hydrochloric acid	+	+	+
Phlebotanins	Phlebotanins test	+	+	+
Cardiac glycosides	Salkowski's	–	–	–
	Lieberman-Bucharnard's	+	+	+
Anthraquinone	Free anthraquinones (Berntragent's)	+	+	+
	Combined anthraquinones	+	+	+
Saponin glycosides	Frothing	–	–	–
	Fehling's solution	+	+	+
Terpenoids	Test forTerpenoids	+	+	+

+ Present, - Absent

positive control in this study, inhibited all the test organisms at 25mg ml^{-1}. Zones of inhibition produced by this agent were greater than that of the ethyl acetate organic soluble fraction in this study. (Table 4)

The minimum inhibitory concentration (MIC) of ethyl acetate soluble fraction from *Ganoderma lucidum* crude methanol extract for five (5) microorganism tested (Table 6,) showed *C. pyogene, B. subtilis* and *K. pneumoneae* were the most sensitive, showing susceptibility at lower concentrations of 12.5mg ml^{-1}, followed by *P. aeroginosa* and *P. mirabilis*

Subculture of the test tube contents above the MIC showed bacterial growth (Table 4) and provided results of minimum bactericidal concentration (MBC). The result showed that ethyl acetate soluble fraction has more bactericidal activity against *K. pneumoneae* at a concentration as low as 12.5mg ml^{-1}. *C. pyogene and B. subtilis* showed growth at concentrations of 12.5mg mL^{-1}, while *P. aeruginosa* and *P. mirabilis* showed growth at 25mg ml^{-1}. (Table 7)

DISCUSSION

The present study strengthens reports of antibacterial properties of *Ganoderma lucidum* by Wasser and Weis (1997), Stamets (2000) and Gao, *et al.,* (2003). This also agrees with the claims of folkloric beliefs and herbal practices for the use of extracts of this

Table 3: *In vitro* antibacterial activity of methanol fraction extract of *Ganoderma lucidum*

Test organisms	Extract concentration (mg ml$^{-1)}$/ Zone of inhibition				Antibiotic (Ampiclox^{R})
	200	150	100	50	25mg ml^{-1}
S. faecalis	-	-	-	-	15mm
S. aureus	-	-	-	-	20mm
C. pyogene	-	-	-	-	40mm
B. subtilis	-	-	-	-	45mm
S. typhi	-	-	-	-	26mm
E. coli	-	-	-	-	16mm
K. pneumoneae	-	-	-	-	32mm
P. aeruginosa	-	-	-	-	18mm
P. mirabilis	-	-	-	-	42mm
E. aeruginosa	-	-	-	-	18mm

- = Resistance

Table 5: *In vitro* antibacterial activity of N-butanol fraction extract of *Ganoderma lucidum*

Test organisms	**Extract concentration (mg ml^{-1})/ Zone of inhibition**				**Antibiotic (Ampiclox^{R})**
	200	150	100	50	25mg ml^{-1}
S. faecalis	-	-	-	-	15mm
S. aureus	-	-	-	-	20mm
C. pyogene	-	-	-	-	40mm
B. subtilis	-	-	-	-	45mm
S. typhi	-	-	-	-	26mm
E. coli	-	-	-	-	16mm
K. pneumoneae	-	-	-	-	32mm
P. aeruginosa	-	-	-	-	18mm
P. mirabilis	-	-	-	-	42mm
E. aeruginosa	-	-	-	-	18mm

- = Resistance

Table 4: *In vitro* antibacterial activity of ethyl acetate fraction extract of *Ganoderma lucidum*

Test organisms	**Extract concentration (mg ml^{-1})/ Zone of inhibition**				**Antibiotic (Ampiclox^{R})**
	200	150	100	50	25mg ml^{-1}
S. faecalis	-	-	-	-	15mm
S. aureus	13mm	10mm	9mm	-	20mm
C. pyogene	15mm	13mm	12mm	11mm	40mm
B. subtilis	14mm	13mm	12mm	11mm	45mm
S. typhi	13mm	11mm	10mm	-	26mm
E. coli	-	-	-	-	16mm
K. pneumoneae	14mm	13mm	13mm	12mm	32mm
P. aeruginosa	12mm	11mm	9mm	8mm	18mm
P. mirabilis	15mm	13mm	12mm	10mm	42mm
E. aeruginosa	-	-	-	-	18mm

R- Resistance. mm- Milliliters (zone of inhibition).

mushroom to treat bronchitis and other unspecified mushroom to treat bronchitis and other unspecified bacterial organisms infection (Anonymous, 2011), by acting on both gram positive and gram negative bacteria, the ethyl acetate fraction have depicted broad spectrum of activity with better antibacterial activity than

Table 6: Minimum inhibitory concentrations (MIC) of *Ganoderma lucidum* ethyl acetate extract

Test organisms	Extract concentration ($mgml^{-1}$)				
	50	25	12.5	6.25	3.13
C. pyogene	-	-	-	+	+
B. subtilis	-	-	-	+	+
K. pneumoneae	-	-	-	+	+
P. aeruginosa	-	-	+	+	+
P. mirabilis	-	-	+	+	+

-= indicate absence of bacterial growth , += indicate presence of bacterial growth.

Table 7: Minimum bactericidal concentrations of ethyl acetate extract of *Ganoderma lucidum*

Test organism	Extract concentration ($mg\ ml^{-1}$)				
	50	25	12.5	6.25	3.13
C. pyogene	-	-	+	+	+
B. subtilis	-	-	+	+	+
K. pneumoneae	-	-	-	+	+
P. aeruginosa	-	+	+	+	+
P. mirabilis	-	+	+	+	+

-= indicates bactericidal effect. += indicates no bactericidal effect

methanol and n-butanol extracts. However, contrary to findings by Wang and Ng (2006), ethyl acetate fraction in this study showed no activity against *E. coli*, equally showing resistance are *S. faecalis and E. aeruginosa*, all other tested organism showed a concentration dependent susceptibility to the ethyl acetate fraction. Thus diseases that these organisms are implicated in can be managed with this extract. This stresses the importance of subjecting herbal preparations to fractionation for better biological activity. (McLaughlin, 1991).

The antibacterial activity of this extract may be attributed to the presence of polysaccharides that are reported to bind to leucocytes surfaces or serum specific proteins leading to activation of macrophages, T-helper, Natural killer (NK), and other effector cells (Mueller *et al.,* 2000). However, carbohydrates can facilitate the growth of bacterial organism; therefore, can antagonize the antibacterial activity of the active principles (Cushnie and Lamb, 2011). This can explain the reason for resistance of some of the tested bacterial organisms with ethyl acetate fraction, and complete resistance to other extract fractions in this study. Besides, Flavonoids, Tanins, Terpenoids and Saponins also found in this study, are reported to possess antibacterial activities (Narayana *et al.,* 2001, Cushnie and Lamb, 2005; Ray-Sahelian, 2005). In addition, Saponins are also reported to have cytotoxic effect with surfactant properties on cell membrane (Ray-Sahelian, 2005), this can assist in destruction of invading micro organisms. The *in vitro* susceptibility of gastrointestinal microbes such as *S. typhi, K. pneumoneae, P. aeroginosa* and *P. mirabilis* to this extract further indicates the antidiarroeal property of the extract from this mushroom.

In conclusion, this study has shown that the ethyl acetate soluble fraction of *Ganoderma lucidum* have broad spectrum antibacterial activity against a variety of gram positive and Gram negative bacteria, thus stressing the importance of exploiting its use in the management of diseases in which these organisms are implicated.

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