

## Original Research Article

# Phytochemical Screening and Antimicrobial Activity of Kenyan mushroom *Ganoderma lucidum*

### ABSTRACT

**Aim:** To screen the Kenyan *Ganoderma lucidum* for bioactive phytochemicals and to investigate its potential in management of some disease causing microbes.

**Study design:** Hexane, ethylacetate and methanol extraction; phytochemical study and antimicrobial activities were analysed on Kenyan *G. lucidum* mushroom.

**Place and Duration of Study:** Masinde Muliro University Science laboratories from January 2019 and March 2019.

**Methodology:** Sequential extraction of dried samples was done using hexane, ethylacetate and methanol. The crude extracts were assayed against *Escherichia coli*, *Klebsiella pneumoniae*, *Methicillin Resistant Staphylococcus aureus (MRSA)*, *Pseudomonas aeruginosa*, and *Creptococcus neoformans* using disc diffusion method. Ampicillin and nystatin were respectively used as controls for bacteria and fungi. Zones of inhibition measured signified the antimicrobial activity. ~~The following groups of compounds were present in the mushroom; steroids, triterpenoids, carbohydrates, phenolic compounds, glycosides, flavonoids, phytosterols and polyoses.~~

**Results:** The following group of compounds were present in the mushroom; steroids,

**Comment [GAM1]:** These are results and need not be included under methods

2 triterpenoids, carbohydrates, phenolic compounds, glycosides, phytosterols and polyoses.

3 Hexane and methanol extracts showed significant activity at (P= 0.022) against MRSA while  
4 ethylacetate extract was active against *Streptococcus pyogenes* (P=0.05). Bacteria strains  
*Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* and the fungi,  
*Cryptococcus neoformans* were insensitive to all the extracts.

**Conclusion:** Kenyan *Ganoderma lucidum* contains steroids, triterpenoids, carbohydrates,  
phenolic compounds, glycosides, flavonoids and phytosterols. Crude extracts (hexane,  
ethylacetate and methanol) were active against some pathogenic microbes MRSA, *S.*  
*pyogenes* and *C. neoformans*.

Keywords: *Ganoderma lucidum*, phytochemical analysis, antimicrobial

## 5 1.INTRODUCTION

6 *Ganoderma lucidum* is not only tropical equatorial but also worldwide medicinal mushroom that is either cultivated or  
7 grows naturally on the bark of trees. Polysaccharides, peptidoglycans and triterpenoids are the three major physiologically  
8 active constituents of *Ganoderma lucidum* [1]; [2]. It is known to contain more than 100 types of polysaccharides showing  
9 strong immunomodulating activities [3]. Terpenes have also been found to have anti-inflammatory, antitumorogenic, and  
10 hypolipidemic activities [4]. Among the reported chemical analyses of Chinese *Ganoderma lucidum* include the isolation of  
11 various Ganoderic acids [5]; [6]; [7], esters [7], alcohols [8] and lactones [9]. So far, there are no reports on either  
12 chemical constituents or biological activity of Kenyan *Ganoderma lucidum* and this made the researcher to carry out  
13 chemical and antimicrobial analysis on the Kenyan *Ganoderma* species.

Comment [GAM2]: Is this species different from the Chinese one in any way? Make this clear

## 15 2. MATERIAL AND METHODS

16 **2.1 Collection and Identification:** *Ganoderma lucidum* was cultivated and obtained from a mushroom farm in  
17 Kakamega county. The county lies at an altitude of 1500-1600m above sea level and is 358 km west of Nairobi. Kenya.  
18 The mushroom species was identified by a herbarium staff at the East African Herbarium National Museums of Kenya in  
19 Nairobi where a voucher specimen was deposited and identification number EAHNMK 261 assigned to the *Ganoderma*  
20 species.

### 21 2.2 Extraction of *Ganoderma lucidum*

#### 22 2.2.1 Preliminary Extraction

23 The collected mushroom was cleaned, air dried for 7 days, ground into fine powder using an electric grinder and stored  
24 under suitable conditions ready for analysis.

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## 2.2.2 Solvent Extraction and Analysis

A quantity of 1 kg of the powdered mushroom was soaked in 3L of distilled hexane at room temperature and pressure for 48 hours. The mixture was then filtered and the obtained filtrate concentrated under reduced pressure using a rotary evaporator, resulting in 6.20g of a dark gummy semi-solid, a 0.5% yield from the dry weight of the mushroom. The resulting residue was taken through sequential extraction with ethylacetate and lastly with methanol following the same procedure as with hexane. The resulting crude extracts were weighed and recorded. Ethyl acetate weighed 4.11g while methanol was 10.03g.

**Comment [GAM3]:** Really? Why distilled hexane? Hexane is a distillate of petroleum but it is not termed distilled hexane as it is purchased ready for use and think you did the same.

## 2.3 Phytochemical screening

Standard procedures were as described by [10]; [11]; [12], [13]; [14]. These tests were based on visual observation of colour or precipitate formation after addition of specific reagents. Crude extracts of hexane, ethylacetate and methanol were analyzed for the presence of useful phytochemicals.

**Comment [GAM4]:** Chloroform extraction is mentioned in the method. Ammonia and caustic soda are also mentioned but are not there here, why?

**Salkowski test:** About 0.5g of hexane crude extract of Kenyan *Ganoderma lucidum* was placed in a clean test tube. A few drops of concentrated sulphuric (VI) acid were added to the extract in the test tube and shaken. On standing, if the lower layer turned red, it indicated the presence of steroids. If the lower layer turned golden yellow, it indicated the presence of triterpenoids. The procedure was repeated for ethylacetate and methanol extracts.

**Comment [GAM5]:** What was the colour change or outcome if both were present? Make this clear.

**Fehlings test:** About 0.5g of hexane extract was placed in a test tube and Fehling's A and B solution added to it and heated. If it gave an orange - red precipitate, reducing sugar would be present.

**Anthraquinone test:** About 0.5g of dry powdered mushroom material was extracted with either ammonia or caustic soda by filtration. If its filtrate or aqueous layer showed pink colour, it indicated the presence of glycosides.

**Comment [GAM6]:** Not included in the extraction method

**Keller – Killiani test:** This is for cardiac glycosides. The dry mushroom material was extracted with chloroform. Then glacial acetic acid, 2.0ml ferrous chloride and 0.5 ml of concentrated sulphuric (VI) acid were added. If acetic acid layer showed blue colour, it indicated the presence of glycosides.

**Comment [GAM7]:** Not mentioned as the reagents used for extraction above.

**Ammonia test:** A few drops of 1% NH<sub>3</sub> solution was added to an extract to be tested in a test tube. A yellow colouration was observed if flavonoid compounds were present.

**Ferric chloride test:** If alcoholic solution of the mushroom extract (Ethanol extract) reacted with freshly prepared FeCl<sub>3</sub>, it gave black fish green colour, it showed that flavonoids were present.

**Lead acetate test:** The reaction of alcoholic solution of the mushroom extract (ethanol extract) with 10% lead acetate in a test tube. If it gave white precipitate, this indicated the presence of flavonoids.

**Mayer's test:** 1.0 ml of Mayer's reagent (potassium mercuric iodide) was added to 1ml of the crude mushroom extract. Formation of white precipitate was a positive indicator for the presence of alkaloids.

56 **Tannins test;** 0.5g of powdered mushroom material was boiled in 20 mL of distilled water in a test tube and then filtered  
57 by gravity. Then 0.1% FeCl<sub>3</sub> was added to the filtered samples and observed for brownish green or a blue – black  
58 colouration. This colour change showed the presence of tannins.

#### 59 **2.4 Bioactivity tests of Crude extracts from *Ganoderma lucidum***

##### 60 **3.4.1 Culture of test micro-organisms**

61 The selected test microorganisms (*Escherichia coli*, *Klebsiella pneumonia*, *Streptococcus pyogenes* and Methicillin  
62 Resistant *Staphylococcus aureus* (MRSA) were resuscitated by culturing them on Mueller Hinton Agar (MHA) and  
63 incubated at 37°C for 18 hours. The microorganisms were later transferred to Mueller Hinton Broth and incubated at 37°C  
64 for 6 hours for a uniform and young culture for the antibacterial tests. The *Cryptococcus neoformans* was also cultured in  
65 Potato Dextrose Broth and incubated at 28°C for 5 days ready for the bioassay test.

##### 66 **3.4.2 Antibacterial tests**

67 19 g of Mueller Hinton Agar (MHA) media was dissolved in 500ml of distilled water in a 500ml conical flask and heated to  
68 boiling to dissolve the media completely. Mueller Hinton Agar media was then sterilized by autoclaving at a temperature of  
69 121°C and pressure 15psi for 15 minutes. The media was then left to cool to 45 °C and dispensed into sterile plates of 22  
70 mm diameter of 20mL per plate and the agar allowed to solidify. Using a 6 mm diameter cork borer, wells were then  
71 made through the agar in the dishes (well diffusion method) [15]. Concentrations of 100 mg/ml for crude, isolated  
72 compounds of hexane, ethyl acetate and methanol solvents and 10 mg/ml for control (Ampicillin) were dissolved in  
73 dimethylsulphoxide (DMSO). The reconstituted fractions were serially diluted in tenfold ratios. 100µl from each aliquot was  
74 dispensed into each well using micropipettes and left at 4°C for 4 hours for the sample to completely diffuse into the  
75 media. The test microorganisms (*Escherichia coli*, *Klebsiella pneumonia*, *Streptococcus pyogenes* and Methicillin  
76 Resistant *Staphylococcus aureus* (MRSA) were then uniformly spread on each plate and incubated at 37°C for 24 hours.  
77 Each plate had a similar concentration but with different bacteria streaked on each. Zones of bacterial growth inhibition  
78 were observed and diameter of each was measured in millimeters to assess the antimicrobial activity. The values  
79 obtained were used to determine the antibacterial activity by comparing the test and standard microorganisms.

##### 80 **3.4.3 Antifungal tests**

81 9.8 g of Potato Dextrose (PDA) agar media was dissolved in 200ml distilled water and placed in a 500ml conical flask.  
82 The mixture was heated to boiling to dissolve the media completely. The PDA agar media was sterilized by autoclaving at  
83 121°C at 15psi for 15 minutes. Sterilized media was left to cool to 45°C and dispensed into petri dishes. The media was  
84 then left petri dishes so as to solidify on the petri dishes. Nystatin was used as control for the fungi. Concentrations of 100

**Comment [GAM8]:** This a common method and need not be described here in full like this.

**Comment [GAM9]:** There are no results of this tenfold dilution or mention at what concentration the extracts were used.

85 µg/ml for both crude extracts, isolated compounds and 10mg/µl for the control (Nystatin) were prepared by dissolved in  
 86 one milliliter of dimethylsulphoxide (DMSO). The prepared concentrations (100mg/ml) of crude extracts (hexane, ethyl  
 87 acetate and methanol), isolated compounds and control (Ampicillin) were varied by tenfold serial dilutions for each sample  
 88 using micropipettes. The fungus (*Cryptococcus neoformans*) was introduced into the petri dishes by streaking with a  
 89 sterilized wire loop. They were allowed to stay untouched for about four hours to diffuse in the media. All the inoculated  
 90 petri dishes were then taken into the incubator at 28°C. They were allowed to stay there for about 24 hours before  
 91 observations were made.

### 92 3. RESULTS AND DISCUSSION

#### 93 3.1 Phytochemical results

94 Crude extracts weighed as follows; hexane 6.18 g, ethyl acetate 4.11g while methanol was 10.03g. Crude extracts from  
 95 *Ganoderm alucidum* were used in phytochemical screening and antimicrobial activities. The phytochemical screening  
 96 performed on the mushroom *Ganoderma lucidum* crude extracts was based on visual observation of coloured complex  
 97 formation according to [10];[11]; [12]; [13]; [14].

98 **Table 1: Phytochemical screening of Kenyan *Ganoderm alucidum***

No.	Secondary metabolites	Test Name	Hexane	Ethyl acetate	Methanol
1.	Steroids	Salkowski test	+	+	+
2.	Triterpenoids	Salkowski test	+	+	+
3.	Saponins	Foam test	-	-	-
4.	Alkaloids	Ferric chloride test	-	-	-
		Mayer's test	-	-	-
		Hager's test	-	-	-
5.	Carbohydrates	Fehlings test	+	+	+
6.	Phenolic compounds (tannins)	Braemer's test	-	+	+
7.	Glycosides	Keller – Killiani test	+	+	+
		Anthraquinone test (Caustic soda)	+	+	+
		Anthraquinone test (ammonia)	+	+	+
8.	Flavonoids	Ferric chloride test	+	+	+
		Lead acetate test	+	+	+
9.	Phytosterols	Salkowski test	+	+	+
10.	Polyoses	Molisch test	-	+	+

99 Key: Positive result is represented as (+), negative result is represented as (-)

100 From the table above, steroids, triterpenoids, saponins, carbohydrates, glycosides, flavonoids and phytosterols were  
 101 present in all the three extracts. Phenolic compounds and polyoses were present in ethylcetate and methanol extracts but  
 102 absent in hexane extract. Carbohydrates in form of polysaccharides have also been isolated from other *Ganoderma*

**Comment [GAM10]:** These need be separated. There is no need to lump two disticnt section into one as though space was runing out.

103 *lucidum* species. Polysaccharides have been known to have strong immunomodulating activities [3]. Kenyan *Ganoderma*  
 104 *lucidum* gave positive test for carbohydrates. If tested for immunomodulating activity, it could be a source of medicine.  
 105 Triterpenoids have also been confirmed in other *Ganoderma lucidum* species to be useful anti-inflammatory,  
 106 antitumorogenic and hypolipidemic activity. Kenyan *Ganoderma lucidum* has again tested positive for triterpenoids. This  
 107 again shows that triterpenoids from this mushroom can be used as a good source of medicine.

108 **Table 2: Phytochemical screening of Indian *Ganoderma lucidum* from Waghai village**

No.	Phytochemical	Test	EtOAc	MeOH	MeOH:EtOAc
1.	Steroids	Salkowski's test	+	+	+
2.	Triterpenoids	Salkowski's test	+	+	+
3.	Saponins	Foam test	-	-	-
4.	Alkaloids	Mayer's test	-	-	-
Wagners test		-	-	-	
Dragendorf test		-	-	-	
5.	Carbohydrates	Molisch test	+	+	+
Fehlings test		+	-	-	
Benedict test		-	-	-	
6.	Phenolic cpds/ tannins	Ferric chloride test	+	+	+
Lead-acetate test		+	-	+	
Keller-Killiani test		+	+	+	
7.	Glycosides	Keller-Killiani test	+	+	+
8.	Flavonoids	Alkaline reagent test	-	-	-
9.	Phytosterols	Liebmann-Burchards reagent	-	-	-

**Comment [GAM11]:** You cannot use these results in your discussion as part of your results. Mention and give citations only and not tables like this here. Remove the table and contents.

109 **Key:** Positive result is represented as (+), negative result is represented as (-)

110 From the table above, it shows that Indian *Ganoderma lucidum* has similar group of phytochemicals as those found in the  
 111 Kenyan species; steroids, triterpenoids, carbohydrates, phenolic compounds or tannins and glycosides [16]. The only  
 112 difference is that flavonoids and phytosterols are present in the Kenyan species while the same compounds are absent in  
 113 the Indian species. Unlike the Kenyan species, the Indian variety lack flavonoids and phytosterols [16]. *Ganoderma*  
 114 *lucidum* from foot hills of Marudhamalai, India have been known to contain triterpenoids, steroids, flavonoids and  
 115 alkaloids. The mushroom lacks saponins, glycosides and carbohydrates [17]. Phytochemicals from *Ganoderma lucidum*  
 116 mushrooms all over the world have been known to be medicinal. The most vital phytochemicals have been triterpenoids  
 117 and polysaccharides. Hexane extracts from Russian *Ganoderma lucidum* contains terpenoids only while ethylacetate  
 118 extracts from the same mushroom contains flavonoids, terpenoids and phenols [18].

### 119 3.2 Bioactivities of crude extracts and controls against antimicrobial tests

120 All crude extracts and controls were subjected to various micro-organisms. Commercial antimicrobials ampicillin and  
 121 nystatin acted as controls for bacteria and fungi respectively. Results are shown in the table below.

122 **Table 3: Antimicrobial statistical data for crude extracts, isolated compounds and controls**

Micro-organism	Amp/Nys	Hexane extract	EtOAc extract	MeOH extract	P-value
<i>E.coli</i>	0	0	0	0	
<i>P. aeruginosa</i>	0	0	0	0	
<i>K. pneumoniae</i>	0	0	0	0	
<b>MRSA</b>	<b>31±0.33</b>	<b>13.3±0.33</b>	<b>10.3±0.33</b>	<b>10.3±0.33</b>	<b>P= 0.022</b>
<b>S. Pyogenes</b>	<b>40.3±0.33</b>	<b>10.3±0.33</b>	<b>11.7±0.33</b>	<b>0</b>	<b>P = 0.05</b>
<b>C. neoformans</b>	<b>30.7±0.33</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>P = 0.05</b>

Key: Amp(Ampicillin), Nyst(Nystatin), MeOH (Methanol), EtOAc (Ethylacetate)

The results above showed that all extracts showed inhibition against *MRSA* (*Methicillin Resistance Staphylococcus aureus*). *Streptococcus pyogenes* was inhibited by all the tests applied except methanol extract. None of the extracts inhibited *P. aeruginosa*, *E. coli* and *K. pneumoniae*. Increasing the concentrations of crude extracts and compounds isolated could cause high inhibition against test organisms. Screening results are shown in table 2. The results in the same table showed that hexane and methanol extracts exhibited significant inhibitions against *MRSA* ( $p=0.022$ ) and ethyl acetate against *Streptococcus pyogenes* ( $p=0.05$ ). *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* did not respond to any of the extracts including the controls used. *E. coli*, *P. aeruginosa*, *Klebsiella pneumoniae* did not respond to all the crude extracts (hexane, ethylacetate and methanol) as shown in table 2. Non responsive effects of micro-organisms against crude extracts (hexane, ethyl acetate and methanol), and controls does not mean that they cannot respond totally to the tests applied. Synergy could have been a factor that might have led to the negative effect. Concentrations of bioactive compounds in the crude extracts and controls might have been low. Another factor is that there might be complete absence of compounds that could cause inactivity.

Ampicillin, an antibacterial control acted against Methicillin Resistant *Staphylococcus aureus* (*MRSA*) ( $p = 0.022$ ) and *Streptococcus pyogenes* ( $p = 0.05$ ) while nystatin, an antifungal control acted against *Creptococcus neoformans* ( $p = 0.05$ ). Ethylacetate extract from Indian *Ganoderma lucidum* acted against *MRSA* at 25 mm while methanol extract from the same Indian mushroom was against *E.coli* and *P. aeruginosa* at 1.25mg/ml and 6.25mm respectively [16]; [17].

**Table 4: Antimicrobial statistical data for crude extracts of Indian *Ganoderma lucidum***

Organism	Minimum Inhibitory Concentration (mg/ml)		
	Ethyl-acetate	Methanol	MeOH:EtOAc
<i>Staphylococcus aureus</i>	2.5	ND	2.5

**Comment [GAM12]:** Use the same styles as in your methods. These were not introduced anywhere in the text and should not be included

**Comment [GAM13]:** Indicate at what dilution was the sensitivity since serial dilutions were made of each extract. Results are incomplete without showing the outcomes of the serial dilutions

**Comment [GAM14]:** Table 3

**Comment [GAM15]:** 3

**Comment [GAM16]:** What does it mean? Results are clear and their implication is that they are totally insensitive to the extracts

**Comment [GAM17]:** Synergy is working together. How can that bring negative results? The mushroom is used as a whole

**Comment [GAM18]:** Controls were commercial drugs how could they not have active ingredients? You used drugs that the microbes were resistant to which shows a flaw in experimental design. You should have used drugs with known sensitivity to the bacteria before running experiments and have true positive and negative controls.

**Comment [GAM19]:** Not your work. Cite and do not include in the discussion verbatim. It is called plagerism.

<i>Escherichia coli</i>	ND	1.25	ND
<i>Pseudomonas aeruginosa</i>	ND	0.625	ND

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142 **4. CONCLUSION**

143 Phytochemical analysis of crude extracts (hexane, ethylacetate and methanol) showed that steroids, triterpenoids,  
 144 carbohydrates, phenolic compounds, glycosides, flavonoids and phytosterols were present in Kenyan *Ganoderma*  
 145 *lucidum*. ~~Saponins and alkaloids were not identified in this extracts.~~ Crude extracts (hexane, ethylacetate and methanol)  
 146 were active against some antimicrobials MRSA, *S. pyogenes* and *C. neoformans*. ~~However, *E. coli*, *P. aeruginosa*,  
 147 *Klebsiella pneumoniae* did not respond to all the crude extracts under investigation.~~

148 **5. RECOMMENDATIONS**

149 Bioactive responsible compounds should be isolated from the extracts of *Ganoderma lucidum*. The mushroom could  
 150 produce compounds that could have medicinal value. Antimicrobial and bioactivities should also be done to the isolated  
 151 compounds.

152 Limitations:153 **REFERENCES**

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**Comment [GAM20]:** Do not discuss negative findings or state them as conclusive findings. Concentrate on the positive findings only unless all results were negative.

**Comment [GAM21]:** Your results did indicate this and it should have ended at result section.

**Comment [GAM22]:** You did not report on the serial dilutions. Possibly they were all negative but there need to report on them as you mentioned them in the methods section. For study that was done somewhere else, there is a possibility of a copy and paste aspect as you also discussed results of the Indian mushroom.

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194  
195 **DEFINITIONS, ACRONYMS, ABBREVIATIONS**

196 *C. neoformans* – *Creptococcus neoformans*

197 DMSO - Dimethylsulphoxide

- 198 *E. coli* – *Escherichia coli*  
199 EtOAc- Ethyl acetate  
200 *G. lucidum* – *Ganoderma lucidum*  
201 *K. pneumonia* – *Klebsiella pneumonia*  
202 MeOH - Methanol  
203 MHA – Muller Hinton Agar  
204 MRSA – Methicillin Resistant *Staphylococcus aureus*  
205 *P. aeruginosa* – *Pseudomonas aeruginosa*  
206 PDA – Potato Dextrose Agar  
207 *S. pyogenes* – *Streptococcus pyogenes*

UNDER PEER REVIEW