Indian J. Applied & Pure Bio. Vol. 37(2), 464-477 (2022). **ISSN: 0970-2091**
A web of Science Journal

Biosynthesis of Silver nanoparticle from cultivated Mushroom (*Calocybe indica* P&C) and its Antibacterial activity

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Abstract

Environmental friendly green synthesis of silver nanoparticles from cultivated mushroom utilizing different spawn grains reduced by different substrates has shown essential benefits due to the fact that of potential therapeutic applications. The found AgNPs were categorized by UV - visible spectrum, FTIR and SEM which showed the reduction of Ag+ ions to Ag0. Further, the aqueous extract was analyzed for qualitative and quantitative phytochemical screening and statistical analysis (one- way ANOVA) were reported to check the significance over substrate and their harvest. The synthesized AgNPs shows more absorption UV spectrum (420nm) and FTIR vibrational peaks (3278, 2933, 1634, 1372, 1018.88 and 933cm^{-1}) respectively. SEM analysis was also performed. Antibacterial efficacy was studied against *Escherichia coli, Staphylococcus aureus, Bacillus subtilis* and *Pseudomonas aeroginosa* by disc diffusion method. The present study revealed that the synthesized AgNPs from *Calocybe indica* play a major role in developing the therapeutic drug against bacterial infectious disease and other health associated disorders.

Calocybe indica is consumable milky white mushroom and it's highly cultivated in India. The main advantage of edible mushroom are rich in protein content, grows even at high temperature and have long shelf life[13]. Cultivation of mushroom process has many benefits which includes low cost, less numbers of labors, large scale production done in small spaces and highly effect in biotechnological field[28]. Microbial method is useful for the maximum production of recycling agro waste in India[27].

Varieties of mushrooms are available, world -wide amount that only 2,000 species are edible, from that approximately 650species

contains medicinal values. Bioactive components are present in medicinal mushrooms includes phenolic compounds, polyketides, terpenes and steroids[12]. A mushroom has high amount of fiber, vitamins (thiamine, riboflavin, niacin, biotin, ascorbic acid and cobalamins) and minerals (potassium and phosphorus). It act as a good source for the immune system and highly effective against pathogens[22]. Secondary metabolism present in medicinal mushrooms shows best activity on antitumor, immune modulators, anti genotoxic, antioxidant, anti-inflammatory, hypocholesterolemic, antihypertensive, anti-platelet-aggregating, anti-hyperglycemic, antimicrobial properties[25]. It highly helps to heal the growth of cancer cells, depression, diabetes, nervous disorders and hypertension activities under pharmaceutical conditions[3].

In recent years researchers shows more interest on nanoparticles synthesis using medicinal plants, bacteria, fungi and yeast[26]. Silver nanoparticles (AgNPs) act as in capping agent, so it is highly effective to cure deadly pathogenic diseases. The synthesis of AgNPs plays an important role on increasing threats posed by antibiotic resistant microbes, when compared to other metals[1]. It is also used in several medical and pharmaceutical applications, such as best antimicrobial potential in medical purposes, silicone rubber gaskets, textile industries and wastewater treatment. Furthermore AgNPs is useful for different agricultural industries and food processing methods like food production and wrapping methods[19].

This study was mainly to evaluate the benefits of silver nanoparticle synthesis using edible mushroom (*Calocybe indica*) and also by cultivations. The approach of this study was analysed phytochemical screening and their antimicrobial potential of AgNPs using edible mushroom which was examined against four human pathogenic bacteria.

The pure culture of *Calocybe indica* (summer mushroom) was collected from Mycology research section in S.D.N.B. Vaishnav College, Chromepet, Chennai-600 044 (Fig-1) and plated on PDA by continuously sub culturing under Laminar air flow in sterile conditions and maintained in culture room. Culture was incubated at 28 ± 2°C in a BOD incubator.

Substrate selection :

Paddy straw and sugarcane bagassee were taken and immersed in cold water over night. The next day discard the water, paddy straw and sugarcane bagasee were transferred to polythene bag for autoclave at 121°C, 15 lbs for 15-20 min. After sterilization the substrates were air dried in shade. The substrate was maintained at 60% of moist condition. Then the selected substrates were used for cylinder preparation.

Spawn production and spawn running :

Spawn is used as a seed in propagation for mushroom production. It was isolated from fruiting culture of *Clocybe indica* in labouratary conditions. Different grains like Shorgum, Ragi and Pearl millet were taken to study the spawn run rate.

The grains were rinsed and filled with cold water incubated for 24h at room temperature.

Filtered and 2% of calcium carbonate ($CaCO_3$) was added; thereby moist level was maintained at 60%. Then grains (250 g) were packed in poly propylene bags (200 × 300 mm) and autoclaved at 121°C for 45 min. After sterilization it was cool for a day. The pure culture *(C. indica)* was inoculated in bags. The mushroom and grains were mixed well uniformly to spread the mycelium. The treated bags were again kept in dark for 17 to 23 days at 27 ± 2°C for the growth of mycelium completely covers the grains.

All the substrates selected for the study were spawned in polypropylene bags (16 x 21 cm) to make mushroom beds in cylindrical shape. The bags were tied at both ends with twine and placed shelves (made of iron) in spawn run room at 30±2°C in dark, till the completion of mycelia growth. After the growth were shifted to the cropping room (cemented floor).

Casing :

After the spawn run it should be covered using soil approximately 1-2 cm of thickness to promote fruiting. It should have large porosity to hold the water and the pH range must be 7 to 7.5.

Vermicomposting :

Vermicompost soil was prepared using earth worms, *Eisenia foetida* (red wigglers) to turn organic waste (kitchen waste) as an excellent source, nutrient-rich organic HYPERLINK "https://en.wikipedia.org/wiki/Organic_fertilizer" fertilizer. About 20-48 days were taken for complete vermicomposting process.

Fruiting :

The cased content was maintained in 80% of humidity at 25-28°C and 77-82°C. Frequently the water was sprinkled, initiation of pin head starts within 10-15 days. After the first harvest, slightly mix the casing then compact it again and continue spraying with water regularly. Soon, after the fruit body is harvested it is important to note the date of (pin head, number of fruit body, stipe length, pileus breadth, its fresh weight, image of the fruit body and biological activity).

Statistical analysis :

One-way ANOVA is applied to study the significance of different spawn with different substrate using the software SPSS 14.0 for Windows Evaluation Version.

Phytochemical analysis for qualitative methods :
Aqueous extract of cultivated mushroom :

The cultivated mushroom (*C.indica)* (paddy straw substrate inference and sugarcane bagassee substrate inference) powders were mixed with 50ml of sterile distilled water. The mixtures were kept in shaker for 24hrs. After centrifuged at 5000 rpm for 10min and the discard the pellet and supernatant was taken too analyzed for the phytochemicals screening[31].

Phytochemical analysis for quantitative methods:
Carbohydrate Estimation[9]:

One ml of each extract was taken and added 1ml of 5% phenol and 5 ml of concentrated

H_2SO_4. The tubes were shaken well and incubated for 10 minutes. The absorbance was measured at 490nm against a reagent blank in the spectrophotometer (ELICO SLIS9 UV VISIBLE). The different concentration of glucose was used as a standard.

Protein Estimation[6]*:*

1 ml of each extract was taken and added 5ml of protein reagent (Coomassie Brilliant Blue) shaken well. The absorbance was measured at 595nm against a reagent blank in spectrophotometer. The different concentration of Bovine serum albumin was used as a standard.

Vitamin Estimation[23] *:*

1 ml of the extracts were taken respectively, added 1ml of KOH solution to the sample tubes and kept the tubes in water bath at 60°C for 20 min, After cool the tubes and added 1 ml of xylene and cover the tubes and shacked well. The samples were measured at 560 nm. The different concentration of ascorbic acid was used as a standard.

Estimation of moisture content :

Five grams of cultivated mushrooms were taken in a petriplates and kept in hot air oven for 24 h at 67ºC. The percentage of moisture content was calculated using the formula as follows.

$$\% \text{ of Moisture content} = \frac{\text{(Fresh sample weight (g) – Dried sample weight (g)}}{\text{Fresh sample weight (g)}} \times 100$$

Biological potential :

The Biological potential is calculated by the formula given below
% of Biological potential = weight of fresh mushroom (g) / weight of dried substrate (g) × 100.

Synthesis of AgNPs[32] *:*

To 10 ml of aqueous extract of cultivated mushroom was mixed with 90 ml of 1mM aqueous $AgNO_3$ and $HAuCl_4 \cdot 3H_2O$ solution at room temperature to synthesis Ag nanoparticles respectively. Appearance of reddish brown colour established preliminary confirmation of Ag^+ ions reduced to Ag^0. The nanoparticles were centrifuged at 10000 rpm for 20 min. Pellet was rinsed with sterile water thrice, dried it. The obtained nanoparticle was further used for characterization and activity analysis.

Characterization of AgNPs :

AgNPs was characterized using spectrophotometer (ELICO SLIS9 UV VISIBLE). FTIR (Fourier transform infrared spectroscopy) results were carried out by Jasco 6300 spectrometer was read between 400–4000 cm^{-1}. Scanning electron microscopy (SEM) was used to view the size, surface morphology and shape of the nanoparticles (TESCAN VEGA3 SBU).

Antibacterial activity :

The pure cultures of both positive and negative human pathogens like *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas*

aeruginosa, *Staphylococcus aureus* were purchased in Royal Bio Research Centre, Chennai. Antibacterial property of AgNPs was analyzed by agar disc diffusion technique.

The pure culture of *Calocybe indica* was cultivated shown in Figure 1.

Combination of various substrates and spawns :

Paddy straw substrate with shorgum spawn gave significantly higher yield (159gm/ Kg) and the days required for spawn run ranges from 12 days to 15 days. Significantly, minimum yield were seen in sugarcane bagasse with shorgum (157.9gm/kg), paddy straw with ragi spawn(16gm/kg),sugarcane bagasse with ragi (26gm/k), Paddy straw substrate with pearl millet (50gm/kg),very poor yield was seen in sugarcane bagasse with pearl millet (11gm/kg) (Table-1).

Spawn run rate :

Among the three grains used, shorgum grains showed excellent spawn run with minimal days (12-15days). While, in ragi grains the complete spawn run days ranges from 13 days to 16 days and pearl millet grains takes above 15 days for complete spawn run and showed a fair report. (Table-2 and Fig. 2).

Pin head formation :

Significantly, minimum (8 days) from the date of complete spawn run were required for pin head formation in paddy straw substrate with shorgum spawn. Sugarcane bagasse substrate with shorgum spawns shown best in the next order in 10 days.

Flush formation :

Almost, 30-40 days required for first flush from the date of complete spawn run. Significantly, Minimum 30 days (*ie*., days counted continuously from the complete spawn run) required for paddy straw substrate with shorgum spawn for the first flush. Whereas, maximum days (above 40 days) required for sugarcane bagasse substrate with ragi and pearl millet spawn.

Stipe or stalk length :

The stalk length was significantly showed highest stalk in sugarcane bagasse substrate with shorgum spawn 15 cm. The moderate was seen in paddy straw substrate with shorgum spawn having 12cm. The least length was recorded in sugarcane bagasse with pearl millet spawn having 3 cm length.

Pileus breadth :

The pileus diameter was seen highly on paddy straw substrate with shorgum spawn 8.5cm. The next best in the order were sugarcane bagasse substrate with shorgum spawn 6cm and sugarcane bagasse substrate with pearl millet spawn 5 cm. similarly Kerketta[13] reported that the least breadth was seen in sugarcane bagassee with ragi spawn 3 cm.

Yield :

The overall yield ranged from 11g to 159 gm, appreciably more yield was seen in paddy straw substrate with shorgum spawn having 159 gm total yield which is in par with sugarcane bagasse substrate with shorgum

spawn having 157.9 gm total yield whereas, the lowest yield was seen in sugarcane bagasse substrate with pearl millet spawn having 11 gm as total yield.

Biological potential :

The biological potential of various substrates with different spawn was between 11% to 159%. The best biological potential was seen in paddy straw substrate with shorgum spawn having B.E. 159% which is in par with sugarcane bagasse substrate with shorgum spawn having B.E. 157.9 %.The next best order was seen in paddy straw substrate with pearl millet spawn having B.E 50%. Whereas, in sugarcane bagasse substrate with ragi having B.E 26%, paddy straw substrate with ragi spawn having 16%, sugarcane bagasse substrate with pearl millet spawn having B.E 11% respectively. Finally 9.795 tonne of several lignocellulosic substrates were utilized and 6.541 tonne of *C. indica* fruit bodies was produced by 42 farmers with an aggregate bio efficiency of 60.38% recorded[33].

Statistical analysis :

ANOVA (one-way analysis of variance) was used to analyze the results between different substrate and different spawn run is found that P value is greater than 0.05 which is least significant (Table-3).

Phytochemical analysis :

Phytoconstituents of the samples were qualitatively analyzed. In both extracts flavanoids, saponins and cardioglycosides were present. Whereas phenol, tannin and alkaloid were absent in both extracts. Terpenoid was present in paddy straw substrate inference (Table-4). Sankaranarayanan[6] reported *Calocybe indica* using different solvent extracts investigated cardiac glycosides, alkaloids, proteins, flavonoids, saponins, tannins, phenols, coumarins, quinones, thiols, terpenoids, and steroids were present.

In quantitative phytochemical anazysed highly present in paddy straw substrate when compared to sugarcane bagassee showed in (table 52). Shyni *et al*.,[27] reported that the Acetone extract of *Calocybe indica* found to be the protein 6.89 mg/g and flavonoid 1.67 mg/g content.

Synthesis of silver nanoparticle :

Appearance of reddish brown colour established preliminary confirmation of Ag^{+} ions reduced to Ag^{0}. (Fig. 3)

UV–visible spectroscopy :

The maximum absorption peak was read at 420 nm (Fig 4) which confirms that Ag+ to Ag0 reduction. In recent years, (9) studied as King Oyster Mushroom (*Pleurotus eryngii*) of synthesized nanoparticles by surface plasmon resonance was read at 461 nm. Similarly Bansal *et al*.,[5] reported at 435nm using *Pleurotus florida.*

FTIR analysis :

AgNPs was analyzed by FTIR spectrum (Figure 5). The peaks were mentioned as 3278, 2933, 1634, 1372, 1018.88 and 933cm^{-1}, which is related to the functional groups of C–H stretch (alkenes) and C=C

Table-1. Evaluation of different substrate and different spawn for *Calocybe* production
PA = PADDY STRAW; SU = SUGARCANE BAGASSEE;
SH = SHORGUM; RA = RAGI;

Substrate & Spawn Combina-tion	Spawn Run Period 1 (days)	Pin Head Initiation (Days)	Harvest (Days)			Stipe Length (Cm)	Pileus Breadth (Cm)	Yield (G)	Biological Efficiency (%)
			I	II	III				
PA+SH	14	8	8	9	13	12	8.5	159	159
SU+SH	16	10	11	13	15	15	6	157.9	157.9
PA+RA	16	15	17	nil	nil	5	3	16	16
SU+RA	18	13	16	nil	nil	7	2	26	26
PA+PE	19	21	25	nil	nil	7.5	3	50	50
SU+PE	21	26	29	nil	nil	3	5	11	11

PE = PEARL MILLET.

Table-2. Different grains and spawn run rate

Different Grains	Number of Days taken for Spawn run							
Growth Day	1	3	5	7	9	11	13	15
Shorgum	–	Good	Good	Good	Very Good	Very Good	Excellent	Excellent
Ragi	–	Good	Fair	Good	Good	Good	Very Good	Excellent
Pearl Millet	–	Poor	Fair	Fair	Fair	Fair	Good	Good

POOR – Mycelium unspreaded, FAIR – Mycelium spreaded, GOOD – Mycelium partially spreaded, VERY GOOD – Mycelium well spreaded , covering completely over the grains and EXCELLENT – Mycelium well spreaded, giving the fuzzy, white appearance above grains.

Table-3. One way ANOVA Statistical data for Different substrate, spawn and harvest of *Calocybe indica*

Anova Values					
Content	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	732.000	2	366.000	7.262	.006
Within Groups	756.000	15	50.400		
Total	1488.000	17			

Table-4. Qualitative phytochemical analysis of aqueous extract of *Calocybe indica*

S.No	PHYTOCHEMICALS	Paddy straw substrate inference	Sugarcane bagassee substrate inference
1	Phenol	-	-
2	Flavonoid	+	+
3	Alkaloid	-	-
4	Tannin	-	-
5	Terpenoid	-	+
6	Glycoside	+	+
7	Saponin	+	+

Table-5. Quantitative phytochemical analysis of aqueous extract of *Calocybe indica*

S.No	Biochemicals	Paddy straw substrate estimation	Sugarcane bagassee substrate estimation
1	Carbohydrates	0.083 µg/ml	0.082 µg/ml
2	Protein	1.701 µg/ml	1.546 µg/ml
3	Vitamin	0.843 µg/ml	0.784 µg/ml
4	Moisture content	84.9 %	78.6%

Table 6. Antibacterial activity of silver nanoparticles of *Calocybe indica* against human pathogenic bacteria

Sample / Microorganisms	Zone of Inhibition in mm				
	Concentrations (µg)				S (20µg)
	125	250	500	1000	
Escherichia coli	10	12	14	15	20
Staphylococcus aureus	-	10	16	19	29
Pseudomonas aeruginosa	10	13	19	21	27
Bacillus subtilis	11	13	17	17	25

Fig. 1. *Calocybe indica* cultivation
A. Paddystraw substrate with Ragi spawn B. Sugarcane substrate with Ragi spawn
C. Paddystraw substrate with Pearl millet D. Sugarcane substrate with Pearl millet

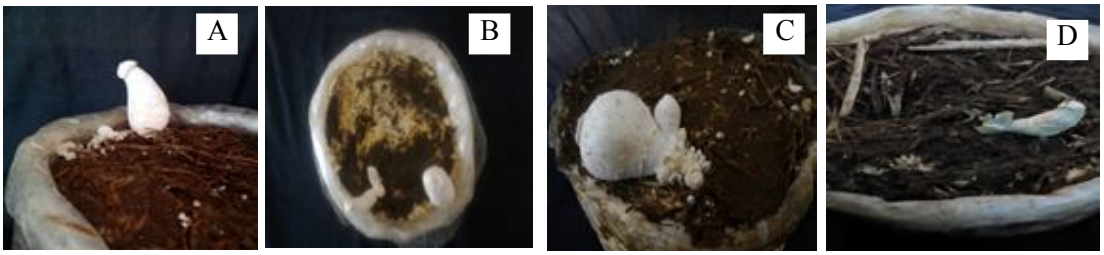


Fig. 2. Spawn run rate using different grains A. Shorgum grains
B. Pearl millet grains and C. Ragi grains

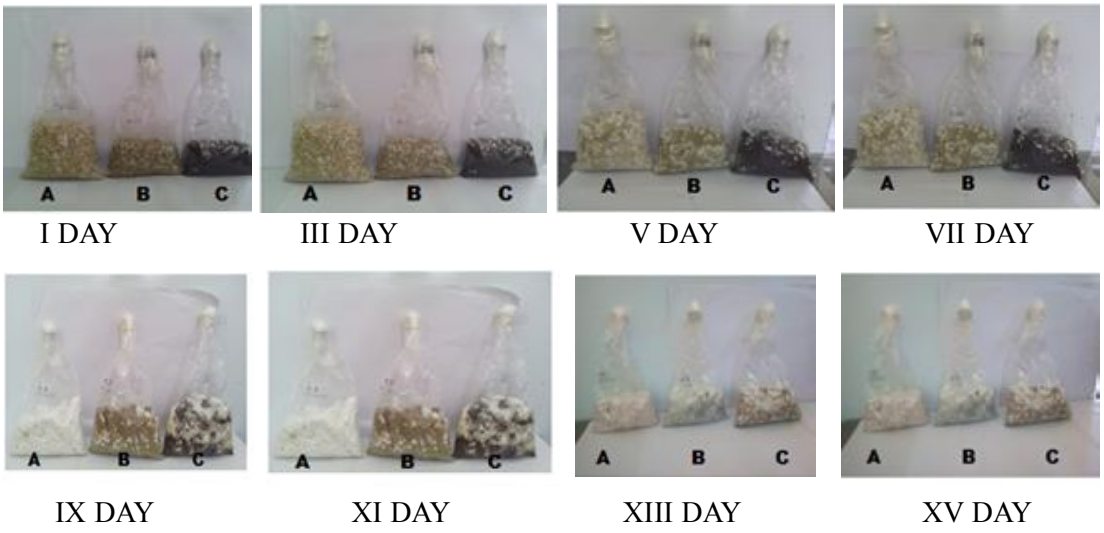


Fig 3. Synthesis of silver nanoparticles from the extract of *Calocybe indica*
A=Before synthesis of AgNPs B= After synthesis of AgNPs.

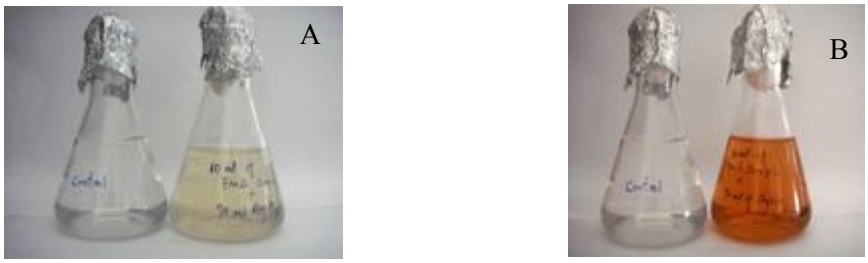


Fig. 4. UV analysis of the synthesized silver nanoparticles from aqueous extract of *Calocybe indica*

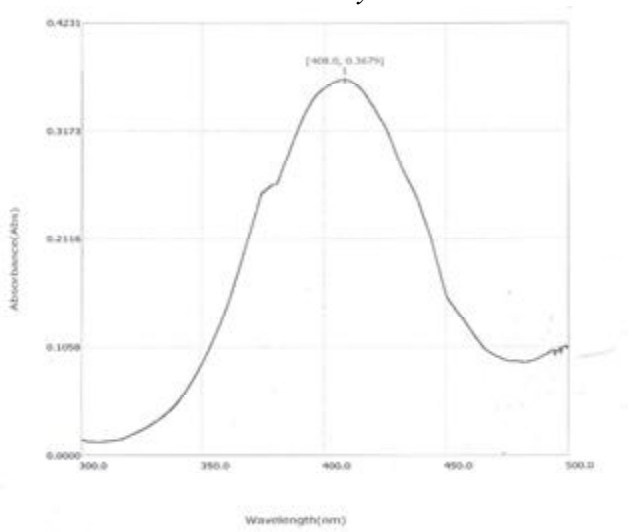


Fig 5. FTIR spectrum of the synthesized silver nanoparticles from aqueous extract of *Calocybe indica*

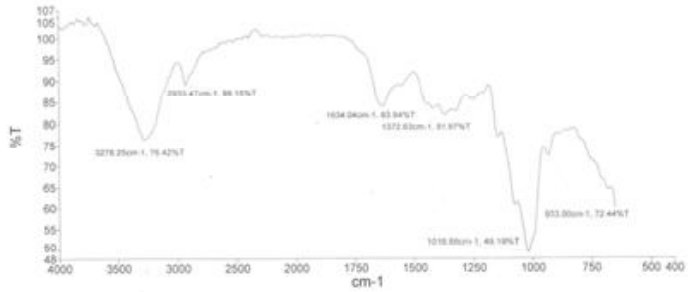
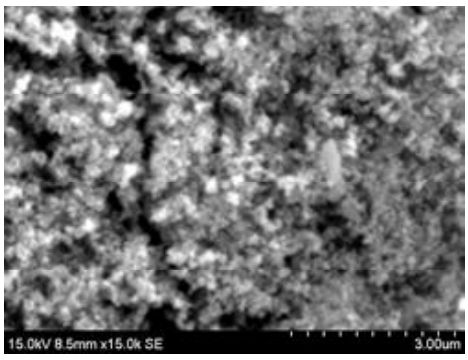


Fig 6. SEM image of the synthesized silver nanoparticles from the extract of *Calocybe indica*



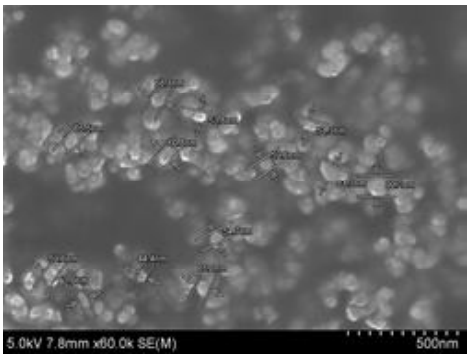
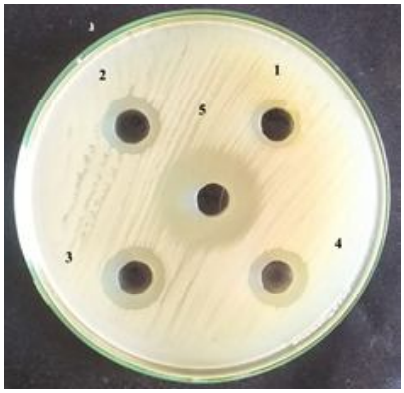
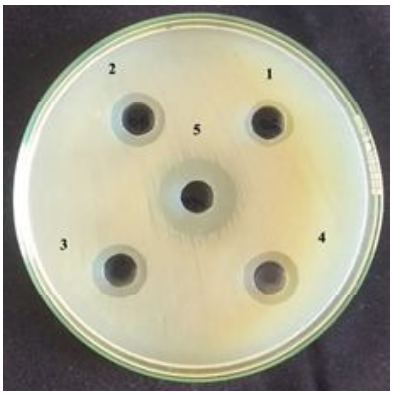


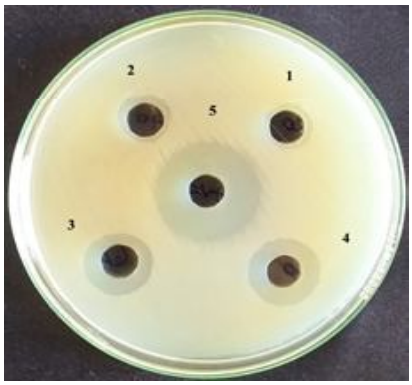
Fig. 7. Antibacterial activity of *Calocybe indica* AgNPs against human pathogenic bacteria

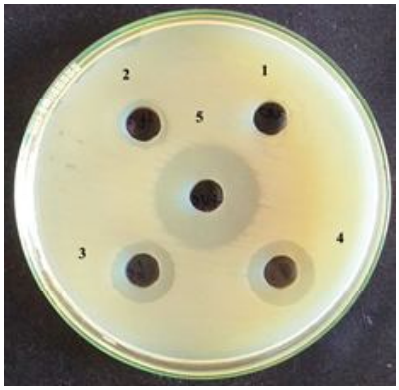
Bacillus subtilis *Escherichia coli*





Pseudomonas aeruginosa *Staphylococcus aureus*





(alkenes) stretching. (8) reported that the absorption peak at 3433 cm^{-1} O-H stretch (alcoholic groups), 1742 cm-1 C=O, and 1629 cm^{-1} compared to N-H bending vibrations of the amide I and II, which in-dicates proteins present in the extract of *G. lucidum*.

SEM analysis :

AgNPs was analyzed using SEM which revealed the image of polydispersed spherical shape and size of the particles ranges at 51.3 nm to 99.2 nm (figures 6). *Agaricus bisporous, Pleurotus ostreatus, Ganoderma lucidum* and *Calocybe indica* AgNPs showed to be spherical in ag-gregated form and its size range at 50-100 nm reported by (8).

Antibacterial efficacy of AgNPs :

The silver nanoparticle was treated against the urinary tract and wound infection causing bacteria includes *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*. The results were

given in (Table-1 and Fig. 7). Among the tested organisms, *Bacillus subtilis* shown good antibacterial activity when compare to other bacteria. Silver nanoparticles have moderate activity against *Pseudomonas aeruginosa* and showed lowest efficacy against *Staphylococcus aureus* and *Escherichia coli*. Aghizion *et al*.,[3] Studied that *Escherichia coli,* showed high efficacy for both AgNPs of fresh *Calocybeindica* and *P. ostreatus* (12mm and 12 mm) respectively. For dried sample (*P. florida*) showed high activity (9mm). *Staphylococcus aureus* showed the best activity for fresh *A. bisporus* (21mm), moderate activity for *P. florida* (19mm) and *P. ostreatus* (15mm). Dried *C. indica* (7mm) and *P. florida* (7mm) shows moderate zone of inhibition. Tetracyclin, streptomycin and ampicillin used as positive control.

The current research work was revealed that the AgNPs showed an effective activity which helps as a major advantage in the field for the therapeutic importance. The green synthesized nanoparticles using cultivated mushroom with paddy straw and sugarcane bagassee substrate inference acts as a good capping agent and high activity. Moreover it contains number of secondary metabolisms in each substrate respectively and best antibacterial property.

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