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CULTIVATION AND CONSTITUENT SCREENING OF MILK MUSHROOM (CALOCYBE INDICA) AND ITS CYTOTOXICITY POTENTIAL AGAINST MCF 7 CELL LINES

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ABSTRACT
Calocybe indica, commonly known as milk mushroom, is a species of edible mushroom native to India. Mushrooms accumulate a variety of compounds, including carbohydrate, glycosides, phytosterols, phenol, tannins, flavanoids, terpenoids and saponins. Antioxidant compounds prevent oxidative damage related to aging and diseases, such as atherosclerosis, diabetes, cancer and cirrhosis. In this study, *Calocybe indica* was cultivated on three different substrates namely paddy straw, sugarcane bagasse and cocopeat: rice bran (1:1). Chemical constituents present in *Calocybe indica* were analysed and cytotoxicity were screened against cancer cell lines. The results showed that the cocopeat: rice bran (1:1) substrate resulted higher yield with high amount of Carbohydrate, Protein and Vitamin A than paddy straw and sugarcane bagasse as a substrate. Constituent analysis showed higher concentration of phenols, flavonoids, tannins, terpenoids, saponins and absence of glycosides and alkaloids. Different concentrations of *Calocybe indica* (1ng, 10ng, 100ng, 1µg, 10µg, 100µg) were evaluated against MCF 7 cell lines for cytotoxicity studies by MTT assay. In all cases crude extract of 100µg showed maximum cell apoptotic potential than the rest.

KEYWORDS: Cytotoxicity, *Calocybe indica*, MTT assay, coco peat, cancer cell lines.

1. INTRODUCTION
Mushroom has been defined as a macro-fungus with a distinctive fruiting body, which can be hypogeous or epigeous, large enough to be seen with the naked eye and to be picked by hand. It is considered as one of the important food items since ancient times and its consumption is being increased over the period for its significant role in human health, nutrition and diseases. Traditionally, people consume mushroom for its nutritional value, reducing obesity and lowering blood pressure (Obodai et al., 2014). The substrates used to grow mushroom contain cellulose, hemicellulose and lignin. Rice, wheat straw, cotton seed hulls, saw dust, bagasse.etc. were used to grow mushrooms (Hoa et al., 2015). Wheat straw leaves and sawdust have been used for the production of *Pleurotus ostreatus* (Shah et al., 2004). Paddy straw and wheat straw have been encouraged the growth of grey and pink oyster mushroom increasing their yield and nutritional value (Randive, 2012). Grass and cardboard when used on single and mixed beds not only gave better mycelial density compared to wheat straw and banana leaves but reduced the time required for pinhead formation and development of fruiting bodies (Tirkey et al., 2017). Wild mushrooms are reported to be rich in antioxidant compounds and vitamins B and D (Diez and Alvavez, 2001). The most widely distributed molecules with antitumor properties in mushrooms are sesquiterpenes, triterpenoids, glucans and glycoproteins (Reis et al., 2011). The major phenolic compounds that have been found in mushrooms are sterols, terpenes and ceramides (Vamanu and Nita, 2014). On the other hand, spent mushroom compost from white rot fungi possesses a great ability to degrade lignin-like pollutants. Mushroom-derived biopolymers also serve as model compounds in the development of suitable food and pharmaceutical products (Giavasis, 2014). Mushrooms have been reported as therapeutic foods useful in preventing diseases as they exhibit varied biological properties such as antibacterial, antimutagenic, antitumor and antiviral activities (Schillaci et al., 2013). *Calocybe indica* was first reported from India by Parkayastha and Chandra (1974). *Calocybe indica* has become the third largely grown mushroom utilised commercially in India

next to button and oyster mushroom (Beelman et al., 1989). The major compounds reported in the *Calocybe* extract are polysaccharides, phenols and flavonoids. The other important compounds are terpenoids, saponins and alkaloids. In addition, hypoglycemic, anti-hypercholesterolemic, immune-modulating, blood pressure preventing and atherosclerosis controlling compounds have been derived from mushrooms (Wasser and Weis, 1999). Antioxidants protect the living systems from free radicals which cause oxidative damage of proteins and nucleic acids. When immune system is weak, patients less protected against free radicals that may lead to cancer (Wong and Chye, 2009). Therefore antioxidant supplementation in food helps people to reduce the risk resulting from oxidative stress. Antioxidant and anticancerogenic properties of edible mushrooms in clinical studies have been reported (Espin et al., 2007). Keeping in view the increased nutritive and therapeutic values of *Calocybe indica*, the present study was conducted to enhance the fruit body production, screening of chemical constituent and cytotoxicity potential against MCF 7 cell lines.

2. MATERIALS AND METHODS

The pure culture of mushroom *Calocybe indica* (Fig. 1a) was obtained from Malar Mushroom Farm, Puducherry, India. The pure culture was cultured on Potato Dextrose Agar (PDA) and Potato Dextrose Broth (PDB). The inoculum consisted of an agar block of 1 cm x 1 cm approximately, taken out of the culture slants with the help of inoculating needle. The inoculated pure culture was maintained by frequent sub culturing under sterile conditions and maintained at 25ºC in culture room (Chang and Hayes, 1978; Chang and Miles, 1989).

2.1. SUBSTRATE SELECTION

In order to find out best substrate for the cultivation of *Calocybe indica*, three different locally available substrates, namely, the paddy straw, and sugarcane bagasse and coco peat: rice bran (1:1) were taken. The substrates were soaked in tap water overnight. After draining the excess water, the substrates were filled in separate polythene bags, sterilized and autoclaved at 121ºC at 15 lbs for 15mins. After cooling, the substrates were shade dried and used for mushroom bed preparation.

2.2. SPAWN PRODUCTION

Mother spawn was produced from the pure culture of *Calocybe indica* under sterile conditions. Sorghum grains were washed and soaked in cold water for overnight and then the excess water was drained and supplemented with 2% of calcium carbonate ($CaCO_3$) to maintain the moisture and pH. The grains (200g) were packed in polypropylene bags (200×300 mm) with necks made from cut PVC pipes in place of the commercial plastic neck to hold the cotton plug, bags were autoclaved at 121ºC for 15mins. After sterilization, they were inoculated with the mother spawn of *Calocybe indica*. Inoculated bags were incubated at 27±2 ºC in darkness for 10 to 15 days until the mycelium fully covered the grains. The process of mixing spawn with grains is called spawning (Fig. 1b).

2.3. MUSHROOM BED PREPARATION

Cylindrical mushroom beds were prepared by following layer method of spawning. A layer of straw is laid in polypropylene bags (16×21cm) and sprinkled with one tablespoon full of spawn along the peripheral region of the straw. A second layer of processed straw is filled and spawned as above. Repeat the process until it covers 3/4th of the bag. Finally the bag is closed tightly with thread. The beds thus prepared are incubated in dark condition in a clean room for mycelial run at 30-35°C.

2.4. CASING

After the completion of mycelial run, the cylindrical mushroom beds were cut horizontally into two equal halves. Then casing soil was applied onto both halves of the mushroom bed to a height of 1-2 cm. The casing soil is prepared by steaming garden soil: vermicompost (1:1) (pH around 8.0) for one hour. The casing material should have high porosity and water holding capacity (Fig. 1 c & d).

2.5. FRUITING

After the casing procedure is over, the bags were kept in growth room with a high humidity of 80% and at a temperature of 28-30ºC. Water was sprayed on the top of casing with regular intervals for pin head initiation. The date of pin head initiation, fruit body harvest, number of fruit bodies, length of the stipe, and breadth of the pileus, biological efficiency and fresh weight of the fruit body were recorded.

2.6. Screening for Chemical Constituents

2.6.1. Preparation of aqueous extract of *Calocybe indica*

The basidiocarps of *Calocybe indica* were dried at room temperature for a week. The dried samples were ground in a blender using a 2 mm diameter mesh before the extraction. For every 1 gram of powder, 50 ml of water was used as solvent and subjected to extraction using a reflux apparatus. After the completion of extraction, the supernatant was filtered through Whatmann No.1 filter paper (Kamra and Bhatt, 2012). The freshly prepared extracts were subjected to standard chemical constituents screening to ensure the presence of following (Harborne, 1998; Adebayo and Ishola, 2009).

2.6.2 Detection of Phenolic Compounds

2.6.2.1. Lead acetate test

To 5 ml of the extract, 4 ml of 10% lead acetate solution is added. Appearance of bulky white precipitate indicates the presence of phenolic compounds.

2.6.3. Detection of Flavonoids

2.6.3.1. Ferric chloride test

To 1ml of the extract, 1 ml of ferric chloride solution was added. Appearance of brown colour indicates the presence of flavonoids.

2.6.4. Detection of Alkaloids

2.6.4.1. Wagners' test

Wagner's reagent was prepared by dissolving Iodine (1.27g) and Potassium iodide (2g) in 5ml of distilled water. The solution is made up to 100 ml with distilled water. A brown precipitate forms when 1ml of extract is added with Wagner's reagent. Formation of brown precipitate indicates the presence of alkaloids.

2.6.5. Detection of tannins

2.6.5.1. Ferric chloride test

To 1ml of the extract, few ml of 5% ferric chloride was added. The development of dark bluish black colour indicates the presence of tannins.

2.6.6. Detection of Terpenoids

2.6.6.1. Liebermann-Burchard's test

To 2 ml of extract, few drops of acetic anhydride were added, boiled and cooled. Then 1ml of concentrated sulphuric acid was added on the sides of the test tube. Formation a brown ring at the junction of two layers and the formation of deep red colour indicate the presence of terpenoids.

2.6.7. Detection of Glycosides

2.6.7.1. Keller- killani test

Two ml of extract was dissolved in 2 ml of glacial acetic acid containing one drop of ferric chloride solution. The mixture was then poured into a test tube containing 1ml of conc. H_2S0_4. A brown ring at the inner-phase indicates the presence of a deoxyribose sugar, characteristic of cardenolides.

2.6.8. Detection of Saponins

2.6.8.1. Frothing test

One ml of filtrate was diluted with 4 ml of distilled water and the mixture was shaken vigorously. Formation of a persistent foam which lasts for at least few minutes indicates the presence of saponins.

2.7. Biochemical Analysis

2.7.1. Carbohydrate Estimation (Dubois method, 1956)

Dried mushroom powder (10g) was homogenized with 10ml of sodium phosphate buffer and subjected to centrifugation at 5000 rpm for 15 minutes. To 1ml of the supernatant, 1ml of 5% phenol and 5ml of concentrated sulphuric acid were added, mixed well and incubated for 10 minutes. The optical density of the sample was read at 490nm against a reagent blank in the (Elico Slis 9 UV visible) spectrophotometer. The amount of total carbohydrate present in *Calocybe indica* was calculated using a standard graph using D-glucose (10-100mg) as standard.

2.7.2. Protein Estimation (Bradford's method, 1976)

Ten grams of dried mushroom powder was homogenized in 5ml of 0.1M sodium phosphate buffer (pH-7) with the pre-chilled mortar and pestle. The homogenate was then centrifuged at 1000rpm for 20 minutes. To 100µl of the clear supernatant, 5ml of Coomassie Brilliant Blue reagent was added and mixed well. After 5 minutes, the optical density of sample was read out at 595nm against a reagent blank in the ELICO SL 159 UV Vis spectrophotometer. The amount of protein present in *Calocybe indica* was calculated by using a standard graph prepared with different concentrations of Bovine serum albumin [BSA-10-100µl].

2.7.3. Vitamin A Estimation (Rutkowshi et al., 2006)

One gram of sample was homogenized with 5ml of sodium phosphate buffer and subjected to centrifugation at 6000 rpm for 15 minutes. To 1ml of the above solution 1ml of KOH solution was added and shaken well for 1 min and then the tube was heated in water bath (60 ºC) for 20 minutes, it was cooled in cold water and 1 ml of xylene was added and the mouth of the tube was plugged. After shaking the tube for 1 min it was measured using ELICO SL 159 UV Visible spectrophotometer at 560nm.

2.8. Determination of moisture

A five gram sample of fresh mushroom was taken dried in an oven at 67ºC for 24 hours till constant weight was attained. The following formula was used to calculate the percentage of moisture.

$$\text{Moisture} = \frac{[(\text{Weight of original sample} - \text{weight of oven dried sample(g)}]}{\text{Weight of Original Sample (g)}} \times 100$$

2.9. Biological efficiency

Biological efficiency is a term frequently used in the mushroom industry to describe yield potentials of mushrooms from various agricultural by-products (straw, sawdust, sugarcane bagasse, banana fronds and coffee plant wastes, etc.). The biological efficiency is calculated using the following formula (Royse, et al., 2004).

$$\text{Bio-efficiency \%} = \frac{\text{Fresh weight of mushroom(g)}}{\text{Dry weight of substrate (g)}} \times 100$$

2.10. Cytotoxicity Assay on Cancer Cell Lines

Chemicals and reagents: MTT 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide Invitrogen, USA. Acridine orange Sigma, USA. All other fine chemicals were obtained from Sigma–Aldrich, St. Louis.

The MCF 7 cell line was obtained from NCCS (National Centre For Cell Science, Pune) and cultured in Rose well Park Memorial Institute medium (RPMI), supplemented with 10% fetal bovine serum, streptomycin (250 U/mL), gentamycin (100µg/mL) and amphotericin B (1mg/mL) that were obtained from Sigma Chemicals, MO, USA. All cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO2. Cells were allowed to grow to confluence over 24 hours before use. Cell growth inhibition was studied by MTT assay and cell viability was measured with the conventional MTT reduction assay, as described previously with a slight modification. Briefly, MCF 7 cells were seeded at a density of 5×10^3 cells/well in 96-well plates for 24 hours, in 200ul of RPMI with 10% FBS. Then culture supernatant was removed and RPMI containing various concentrations of test samples was added and incubated for 48 hours. After treatment cells were incubated with MTT (10µl, 5mg/mL) at 37ºC for 4 hours and then with DMSO at room temperature for one hour. The plates were read at 595nm on a scanning multi-well spectrophotometer. (Evelyn et al., 2012).

$$\text{Cell viability (\%)} = \frac{\text{Average test OD}}{\text{Control OD}} \times 100$$

3. RESULTS AND DISCUSSION

Calocybe indica was cultivated using different substrates. The result of different substrates used for *Calocybe indica* cultivation, days required for spawn run, pin head formation, fruit body formation, stipe length, pileus length and breadth, yield during harvest and biological efficiency were recorded.

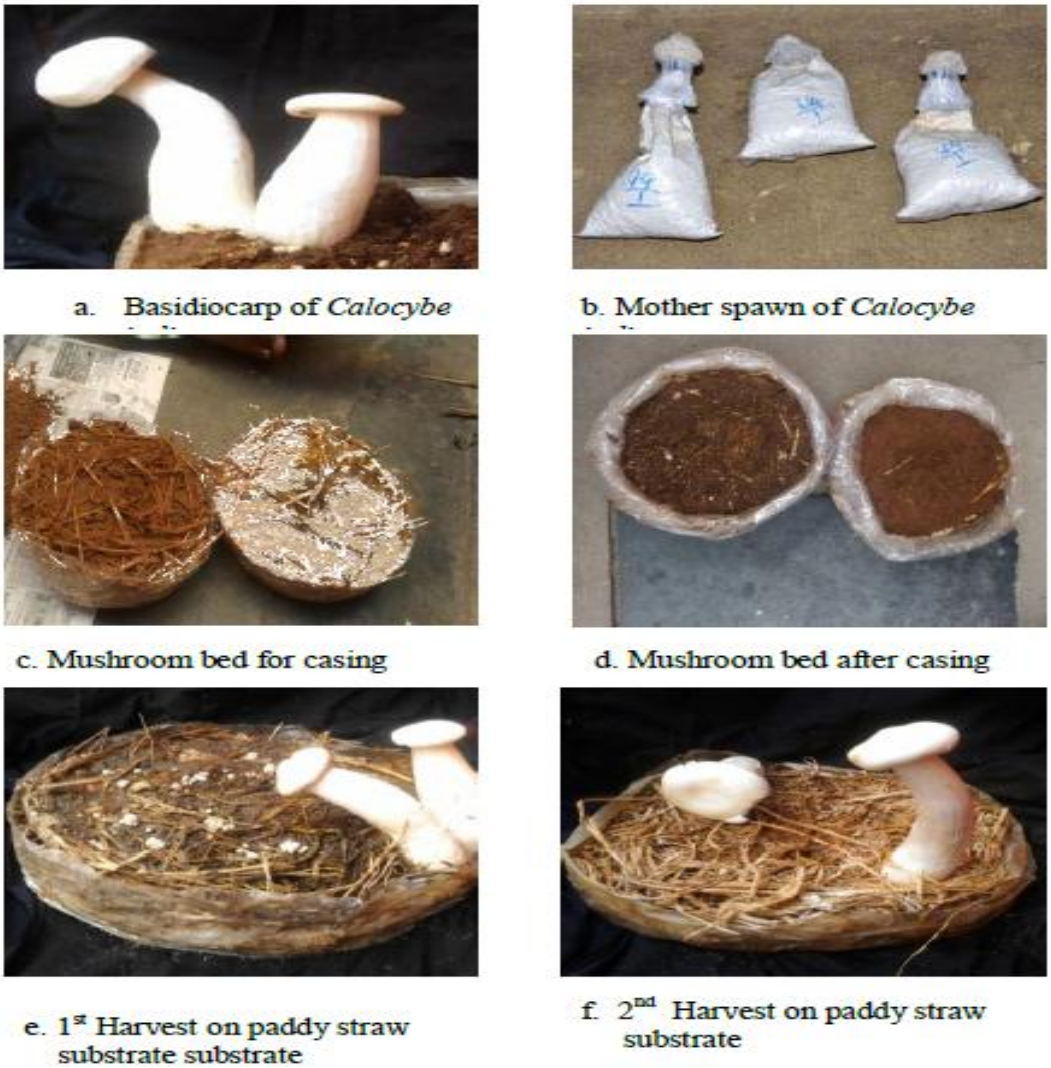

Figure 1.

a. 1st Harvest on Cocopeat and rice bran substrate

b. 2nd Harvest on Cocopeat and rice bran substrate

c. 1st Harvest on Sugarcane baggase substrate

d. 2nd Harvest on Sugarcane baggase substrate

Figure 2

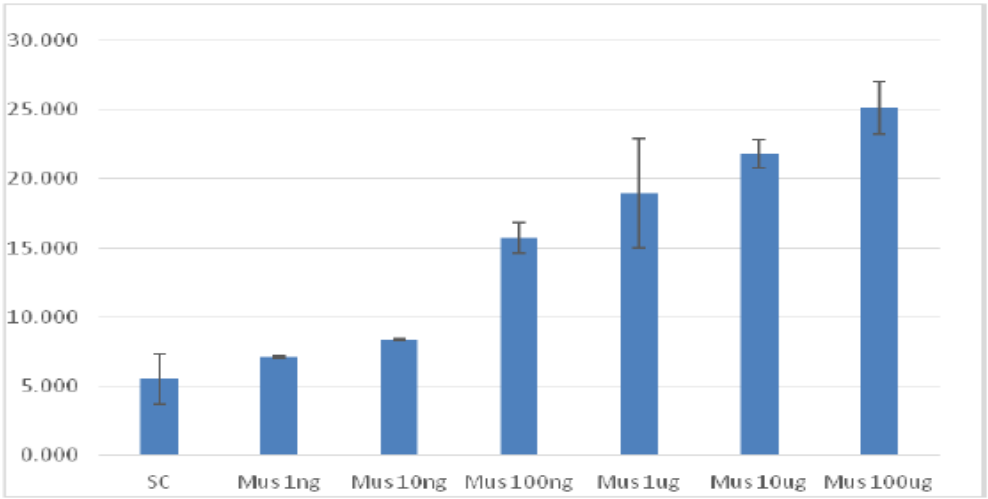

Figure 3: MTT Assay for MCF7 Cell.

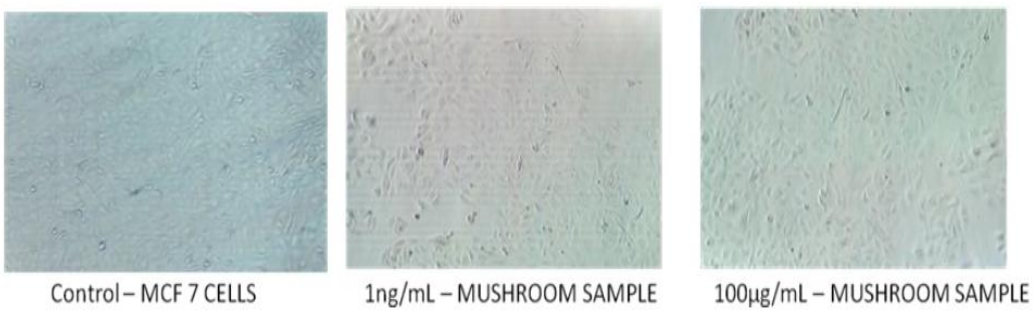

Figure 4: MTT Assay – MCF7 Cell Lines – Microscopic View.

Table 1: Evaluation of Different Substrates for Calocybe Indica.

Diferent Substrate	Spawn Run Period (Days)	Pin Head Initiation (Days)	Harvest (Days)		Stipe Length (Cm)	Pileus Breadth (Cm)	Biolo-Gical Yield (Gram)	Biolo-Gical Effici-Ency %	Diferent Substrate
			I	II					
CP+RB	17	11	26		23	13.3	7.2	562	116.5
PADDY STRAW	16	13	27		28	12.6	7.1	512	102.5
SUGARCANE BAGASSE	19	19	30		32	11.6	6.7	320	64

CP-COCOPEAT RB-RICE BRAN

Table 2: Biochemical Analaysis.

Biochemicals	Coco Peat + Rice Bran (Mg/G)	Paddy Straw Substrate Estimation (Mg/G)	Sugarcane Substrate Estimation (Mg/G)
Carbohydrate	0.089	0.083	0.082
Protein	1.751	1.701	1.546
Vitamin A	0.748	0.843	0.784
Moisture content	80.8%	84.9%	78.6%

Table 3: Qualitative Analysis of Extracts of *Calocybe Indica.*

Component	Name of the test	Results
Phenols	Lead acetate test	+++
Flavanoids	Ferric chloride test	+++
Tannins	Ferric chloride test	+++
Saponin	Frothing test	+++
Terpenoids	Libermann-Burchard's test	++
Glycosides	Keller-Killani test	-
Alkaloids	Wagners test	-

+++ denotes high concentration of component
++ denotes positive result
- denotes negative result

3.1. Spawn Run Rate

Sorghum grains were used for the preparation of spawn which took 12-15 days for spawn colonization.

3.2. Different Substrate and Their Spawn Run Rate

Among the conventional substrates used, coco peat: rice bran (1:1) substrate gave marked higher yield (562g) and a spawn run period of 16 to 17 days followed by paddy straw (512 g) which took 16 days for complete spawn run. Comparatively lower yield was observed in sugarcane bagasse (320g) that took 19 days for spawn run. Concomitant to the present report Chakrabathy et al. (2016) reported a similar spawn run in paddy straw as substrate whereas for cocopeat: rice bran (1:1) showed better result when compared to only cocopeat as substrate (Table 1). Evidently the results of Krishnamoorthy and his co-workers showed that paddy straw was colonized more quickly by the milky white mushroom when compared to black gray hay, soybean hay, maize stalks and finger millet straw (Krishnamoorthy et al., 1997). In contrast to these results, Amin et al. (2010) reported duration of 91 days for the spawn run in rice straw.

3.3. Days for Pinhead Formation

Among the various types of substrates used for *Calocybe indica* production, cocopeat: rice bran (1:1) substrate took a minimum of 11 days from the date of complete spawn which was followed by paddy straw substrate (13 days) and sugarcane bagasse (19 days). However, Vijayakumar et al., (2014) recorded 28 days for wheat straw and 31 days for coconut coir pith.

3.4. Days for Harvest

A minimum of 26 days were required for the first harvest of mushrooms when cocopeat: rice bran (1:1) were used as substrate and 28 days for the second harvest, followed by 27 days for first harvest and 28 days for the second harvest when grown on paddy straw and a maximum of 30 days for the first harvest and 31 days for the second harvest. Similar duration (27 and 35 days) for first flush has been reported by Mohit et al., (2018) and Dhakad et al., (2015) accordingly.

3.5. Length of Stipe and Breadth of Pileus

Highest length of stalk was recorded in cocopeat: ricebran (1:1) (13.3cm) which is followed by paddy straw substrate (12.6 cm) and sugarcane bagasse (11.6 cm). The diameter of pileus was recorded highest on

cocopeat: ricebran (1:1) (7.2cm) followed by paddy straw (7.1 cm) and sugarcane bagasse (6.7 cm). Amin et al. (2010) obtained a maximum of 9cm using paddy straw. A length of 6.8cm was reported by Suman et al., (2018) on paddy straw. A length of 6.8cm was reported by Suman et al., (2018) on paddy straw.

3.6. Yield

The overall yield ranged from 300g to 600g maximum yield was seen in cocopeat with rice bran substrate that showed an yield of 562g (Fig. 2 a & b) followed by paddy straw (512g) (Fig. 1 e & f) and sugarcane bagasse (320g) (Plate 2 c & d). Maximum yield of 420g/bed (Senthil Nambi et al., (2011) 600g/kg dry substrate (Singh et al., 2018) have been achieved using various substrate.

3.7. Biological Efficiency

The biological efficiency of different substrate ranged from 60% to 120%. The highest biological efficiency of 116.5% was seen in coco peat: rice bran (1:1) substrate which is in par with paddy straw substrate, the biological efficiency of which was 102.5% and the lowest biological efficiency was recorded in sugarcane bagasse (64%). Amin et al., (2010) reported a similar result of biological efficiency using the same combination of the two different substrates (coco peat : rice bran).

3.8. Biochemical Analysis

The range of total carbohydrates, protein, vitamin A, moisture content of *Calocybe indica* were 0.082 mg/g to 0.089 mg/g, 1.546 mg /g to 1.751 mg /g, 0.748 mg /g to 0.843 mg /g, 78.6 to 84.9%, respectively (Table 2). *Calocybe indica* was found to be in rich in protein, vitamin and carbohydrate conforms the well established fact that provides highly superior nutritional quality (Subbiah and Balen, 2015). This result is nearly similar to the report of Nuhu Alam et al., (2007). However, there is a slight difference in biological analysis this may due to different compost and its composition (Nuhu Alam et al., 2007) (Table 2).

3.9. Screening of Chemical Constituents

The aqueous extract of *Calocybe indica* showed marked levels of phenols, tannins, flavanoids, saponins and terpenoids. However the extract showed negative result for alkaloids and glycosides (Table 3). Presence of these metabolites indicates that Indian medicinal mushrooms like *Calocybe indica* are potential sources of anti-tumor and antioxidant activities. These results corroborate with those reported by Prabu and Kumuthakalavalli (2014).

3.10. Cytotoxicity Assay on Cancer Cell Lines

Different concentrations of *Calocybe indica* (1ng, 10ng, 100 ng, 1µg, 10µg, 100µg) were evaluated against MCF 7 cell lines for cytotoxicity studies. Different concentration of extracts exhibited different levels of absorbance. From the results, it is clear that an increase in concentration (1ng, 10ng, 100ng, 1µg, 10µg, 100µg) showed increase in cell apoptosis. In all cases crude extract of 100µg showed maximum cell apoptotic potential than the rest (Fig.4). By comparing with the standard, all the extracts showed a significant cytotoxicity potential against cancer cell lines (Fig. 3). Anticancer activities of *Calocybe* have also been determined using A5449 human lung cancer cell line (Nisha and Kumuthakalavalli, 2016) in Delton's lymphoma ascites induced mice (Ganapathy, 2014) and T24 urinary bladder cancer cell line (Selvi, 2011).

4. CONCLUSION

Calocybe indica was cultivated using different substrates of which Coco peat : rice bran (1:1) showed higher spawn run rate, faster pin head formation, noticeable fruit body formation, stipe length, pileus length and breadth, yield and biological efficiency. Biochemical analysis shows *Calocybe indica* was rich in protein, vitamin, and carbohydrate. Studies on chemical constituents indicate the presence of phenols, tannins, flavanoids, saponins, terpenoids and absence of alkaloids and glycosides. The anticancer study clearly indicates *Calocybe indica* possess profound antioxidant and antitumor activity. Cytotoxicity assay on cancer cell lines using the crude extract of *Calocybe indica* (100 µg) showed considerable cytotoxicity potential against cancer cell lines.

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