CHAPTER 1

INTRODUCTION

Medicinal mushrooms have an established history of use in traditional oriental therapies. Modern clinical practice in Japan, China, Korea, and other Asian countries continues to relay on mushroom derived preparations. Edible mushrooms are among the best sources of many essential nutrients. Their nutritional value can be compared favourably with that of meat, eggs and milk (Breene, 1990). They are used as food stuffs containing both nutritive and medicinal values (Bonatti.M.P *et al.,* 2004, Agrahar-Murugkar.D *et al.,*2005). They play an important role in preventing diseases such as hypertension, hypercholesterolemia and cancer (Bobek.P *et al.,* 1999, Bobek P.L *et al.,* 1995). The occurrence of antibiotics in mushrooms is less documented and there are only few reviews that summarise the antibacterial activity from mushrooms (Gao *et al.,* 2003, Zjawiony, 2004). There are a large number of mushroom-derived compounds -cellular components and secondary metabolites that could be used to treat a variety of other disease states, such as compounds with potential hypoglycemic, antithrombotic, lowering cholesterol, antimicrobial, cytotoxic, antihypertensive, hepatoprotective properties, among others. Literature survey revealed that very little work has been carried out in this regard from India. Several mushroom species belonging to the Polyporaceae family are now being regarded as the next candidate producers of valuable medicines (Mizuno *et al.,* 1995). Mushroom “nutriceuticals" are bioactive compounds that are extractable from mushrooms, and they have nutritional and medicinal features that may be used in the prevention and treatment of diseases (Chang & Buswell, 1999). Several nutriceutical products have been isolated from medicinal mushrooms and three of these, which are

carcinostatic polysaccharide drugs, have been developed from mushrooms in Japan. The scientific community, in searching for new therapeutic alternatives, has studied many kinds of mushrooms and has found variable therapeutic activity such as anticarcinogenic, anti-inflammatory, immuno-suppressor and antibiotic. They are "Krestin" (PSK), from the cultured mycelium of Kawaratake (*Trametes versicolor*), "Lentinan" from the fruiting bodies of Shiitake (*Lentinus edodes*) and "Schizophyllan" (Sonifilan) from the culture fluid of Suehirotake (*Schizophyllum commune*) (Mizuno, 1993). In early studies performed by Anchel, Hervey and Wilkins (1941), diverse antibiotic activity was detected in basidiocarp or mycelia culture extracts of more than 2000 fungal species. Early studies proposed that most antibacterial components from basidiomycetous fungi were potent against Gram-positive bacteria only (Lee *et al.,* 1982) but more recent work showed that extracts were also active against Gram-negative organisms, *Proteus vulgaris* and *Escherichia coli, in vitro* (Yoon *et al.,* 1994). Some *Agaricus* species (*A. campestis, A. bisporus* and *A. arvensis*) have been shown to produce compounds active against both Gram-positive and Gram-negative bacteria (Wasser & Weis, 1999) and, extracts from *Lentinus edodes* have also been shown to be active against both types of bacteria (Hirasawa *et al.,* 1999).

Ganoderma species more often in combination with chemotherapeutic agents can be used to treat various bacterial diseases. Sheena *et al,* (2003) reported that methanol extract of *G.lucidum* showed remarkable antibacterial activity against *E.coli, Salmonella* species and *B.subtilis*. Zjawiony (2004) observed that 75% of polypore fungi that have been tested show strong antimicrobial activity. Hexiang *et al.,* (2006) isolated an antifungal protein *Ganoderm* in from *Ganoderma lucidum* and reported that it was sensitive to *Botrytis cinerea, Fusarium oxysporum, Phsalospora*

*piricola.*Klaus and Miomir (2007) have studied the influence of extracts isolated from *Ganoderma lucidum* on *E.coli, Bacillus, S.aureus* and *Salmonella* species. The aqueous fruiting body extract showed maximum zone of inhibition against *Bacillus* species while least zone of inhibition was reported for *E.coli* and *Salmonella* species.Smania *et al.*,(2007) observed maximum antibacterial activity of methyl australate, a derivative from *G.lucidum* against *E.coli* and *Psuedomonas aeruginosa* followed by *Staphylococcus.* Keypour *et al.,* (2008) investigated the antibacterial activity of a chloroform extract of *G.lucidum* from Iran. The results of disc diffusion tests showed that the chloroform extract had growth inhibitory effects on *B.subtilis and S.aureus*. Manjunathan. *et al.,* (2010) investigated the antibacterial activitly of *Lentinus tuberregium* (Fr) the exracts were more active to inhibit the growth of *Salmonella flexiner, Micrococcus luteus and Salmonella typhi.* Sadaf Quereshi *et al.,* (2010) reported that the acetone extract of *Ganoderma lucidum* possessed strong antibacterial activity which was most inhibitory against *Klebsiella pneumoniae, E.coli, B.Subtilis* and *S.typhi.* The water and ethanolic extract were found to be not much effective against all the strains. Arati *et al.,* (2010) reported that the tooth paste made of *Ganoderma lucidum* was found to be active against *Candida albicans*. Yihuai Gao *et al,* (2010) reported that the polysaccharides or terpenoids from *Ganoderma* showed activities against Herpes simple virus, Hepatitis B virus, HIV, and Epstein-Barr virus in vitro or in animal models. Sridhar, *et al*, (2011) reported the aqueous extract of *Ganoderma lucidum* showed maximum activity against *Salmonella typhi* and also reported that *Mucor indicus* and *Aspergillus flavus* was also sensitive to the extract of *Ganoderma lucidum.*

Yong *et al.,* (2008) reported that Exo biopolymer produced by *Ganoderma applanatum* inhibited Sarcoma-180 bearing mouse and increased the natural killer cell

activity. The phosphatase activity of macrophages was increased by 0.7 folds when compared to that of control. They also reported exopolymer contained glucose, serine, glycin and aspartic acid. Ai *et al.,* (2011), have reported that the phytochemical properties of the *Ganoderma applanatum* are the potential raw materials for nanotechnological engineering in clinical practice for the treatment of African trypanosomiasis. Akpera *et al.,* (2012) has reported that photochemical screening of aqueous extract of *Ganoderma applanatum* contain Saponins, flavonoids, cardiac glycosides and steroid and they have also reported the absence of alkaloids, tannins and anthraquiones.

T. giganteum has maximum antioxidant property and may be utilized as a promising source of Therapeutics Acharya *et al.,* (2005)have reported potent anticancer, hepatoprotective and antioxidant activities of *Tricholoma giganteum.* Subratha *et al.,*(2012*)* reported that methanolic extract of *T. giganteum* showed activity against *Staphylococcus aureus, Bacillus cereus, Bacillus subtilis, Proteus vulgaris, Escherichia coli, Pseudomonas aeruginosa, Candida albicans.* Gao *et al.,* (2003), Wang *et al.,* (1995) reported the presence of Trichogin a peptide molecule in *Tricholoma giganteum* is responsible for antifungal activity against *Fusarium oxysporum, Mycosphaerella arachidicola* and *Physalospora piricola,* as well as inhibitory effect on HIV-1 reverse transcriptase. Wang *et al.,* (1995) reported two lectins, TML-1 and TML-2, isolated from *Tricholoma mongolicum* by ion-exchange chromatography and gel filtration.. The hemagglutinating activities of TML-1 and TML-2 are sensitive to lactose inhibition and are stable between 10 and 80 degrees C. They exhibit antiproliferative activities against mouse monocyte-macrophage PU5-1.8 cells and mouse mastocytoma P815 cells in vitro. The two lectins differ in the content

of proline and tyrosine residues. Both are non-glycoproteins and have hydroxyproline residues.

Soumya *et al.*, 2001 elucidated the antioxidant capacities of *Tricholoma giganteum*

Krishnanendu *et al.*, (2012) reported the lowest yield for pharmacognostic study was obtained in actone and maximum in methanol for *Tricholoma giganteum* and they also reported the presence of antioxidant flavonoids and polyphenols which indicated the potent medicinal activities. Soumya *et al.,* (2001) explored the efficacy of Fa fraction of *Tricholoma giganteum* against Ehrlich's ascites carcinoma (EAC).

The aim of present work was to carry out invitro experiments to screen antimicrobial, antioxidant and anticancer activity of *Ganoderma applanatum, Tricholoma giganteum* and *Bolbitius vitellinus*. Since no much literature is available with regard to their activites of methanolic extract of basidiocarps and pure cultures of the above mentioned mushrooms. The present study reveals it biopharmaceutical properties.

OBJECTIVE OF THE STUDY

1. **To Identification and isolation of the pure cultures of the wild mushrooms.**
2. **To screen the Antimicrobial activity of some selected wild mushrooms.**
3. **To Screen the Antioxidant and reducing power of the mushrooms.**
4. **To Screen the Anticancer activity of the mushrooms.**

CHAPTER 2

MATERIALS AND METHODS

2.1 Collection of Mushrooms and identification

Basidiocarps collected from various localities in Bangalore, Singer (1972) classification concept was used in identification of Agaricales. For the identification of Ganoderma species keys provided by Steyaert (1972), Murill (1902) and Bhosle *et al.*, (2010)



Plate 1:Basidiocarp of *Bolbitius vitellinus.*



Plate 2:Basidiocarp of *Ganoderma applanatum*.



Plate 3: Basidiocarops of *Bolbitius sps.*



Plate 4: Gills of *Bolbitius sps.*

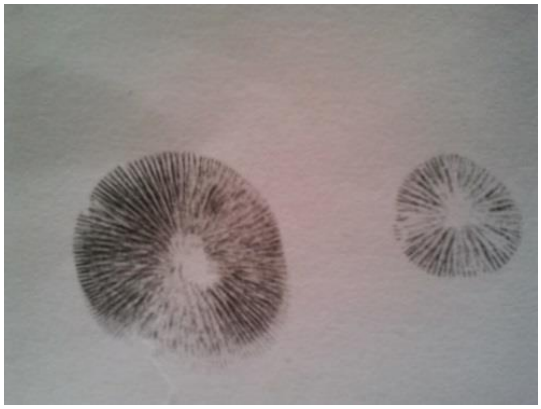


Plate 5: Spore print of *Bolbitius sps.*

2.2 Isolation of Pure culture of Mushrooms

The pure cultures of the mushroom was obtained by placing a portion of context of the pileus aseptically on sterilized Potato Dextrose Agar in a petiplate with the help of a sterile forceps and incubated at 25±1ºC for 6-7 days for initial mycelial growth. Pure cultures obtained were maintained in PDA slants and were used for further studies.



Plate 6: Pure cultures of the mushrooms on Potato Dextrose Broth and PDA.

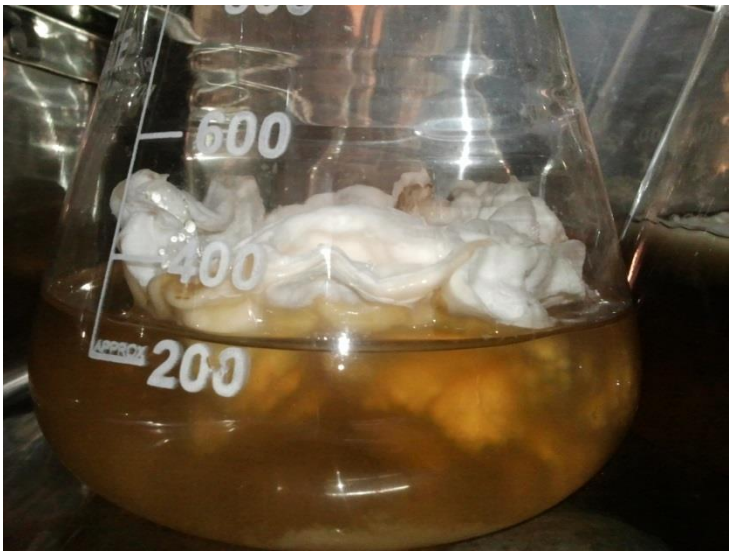


Plate 7: Mycelial growth of *Ganoderma applanatum* on Potato Dextrose Broth

2.3 Preparation of alcoholic extract from basidiocarps and pure cultures

In the present study, the dried basidiocarps and mycelium of above three mushrooms were used mushroom material was made into fine powder. 10 grams of mushroom powder was subjected to extraction using 100 ml of methanol at room temperature for 24 hrs and filtered through Whatman No. 4 filter paper. The extracts were recovered by filtration and kept at 40^0C in a rotary vacuum evaporator. The residue was collected and store at 4^0C for further use.

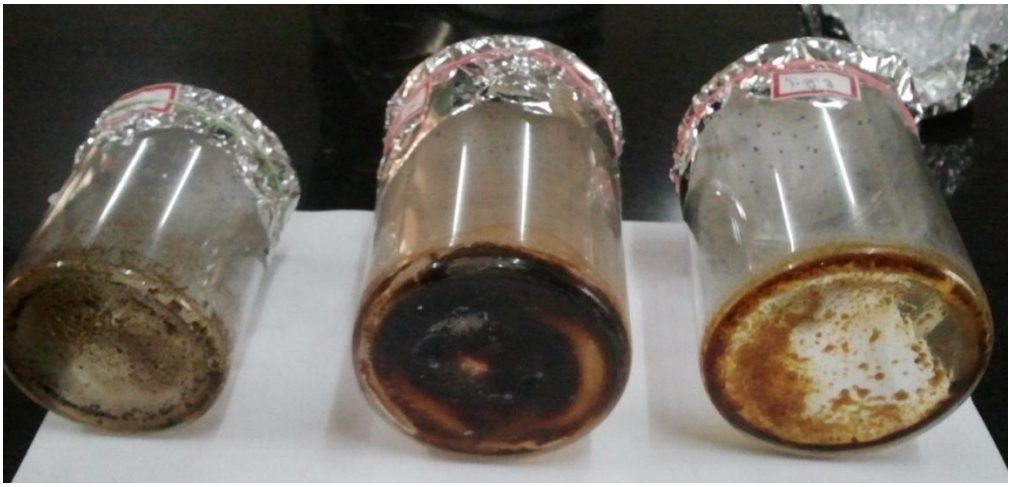


Plate 8: Methanolic extract of different mushroom samples

2.4 Screening of antibacterial and antifungal activity of mushroom extract

The antimicrobial activities of the extracts were determined by the Kirby-Bauer agar diffusion method according to NCCLS standards. (Bauer AW, 1966 NCCLS, 2000) All the bacterial cultures were grown in Nutrient broth and incubated at 37^0c for 24 hours. The fungal cultures were incubated at room temperature for 48 hours in Potato Dextrose Agar. Nutrient agar and Potato Dextrose Agar of about 20 ml were poured into each sterilized Petri plates aseptically and allowed to solidify. With the help of sterilized cork borer a well of 6mm diameter was made at the centre of the agar plates. The bacterial and fungal suspension was swabbed on the solidified media using sterilized swabs in the respective plates.

The mushroom extract was dissolved in Dimethylsulfoxide and the suspension was sterilized by filtration through a membrane filter (Turkoglu *et al.,* 2007). The mushroom extract of about 100 microliters was filled into the wells of the agar plates. The bacterial plates were incubated at 37^0 c for 24 to 48 hours. The fungal plates were incubated at 28^0c for 3-4 days. Inhibitory activity of DMSO was also tested. Nystatin was used as the reference disc for fungi. And Penicillin and Tetracycline was used as the reference disc for bacteria. After the period of incubation the zone of inhibition was measured, tabulated and compared with reference disc.

Microorganisms tested: The pure culture of the microorganisms such as *Bacillus subtilis(2655), Proteus vulgaris(2813), Staphylococcus aureus(2127), Escherichia coli(2685), Micrococcus flavus(2376), Pseudomonas aeriginosa(5029), Salmonella abony(2257), Klebseilla pneumonia (2706), Pseudomonas fluroscence (clinical isolate), Cryptococcus sps(3349), Candida albicans (3100)* were used which obtained from NCL, Pune.

2.5 Screening for Antioxidant activity

Mushrooms have become attractive as functional foods and as a source of physiologically beneficial medicine (Chang, 1996, Chang 1999, Wasser, 1999 and Lindequist, 2005). Some advantages of using mushrooms over plants as sources of bioactive compounds are that often the fruiting body can be produced in much less time, the mycelium may also be rapidly produced in liquid culture and the culture medium can be manipulated to produce optimal quantities of active products.
Different wild mushroom species were reported to have antioxidant activity, which was mainly related to their phenolic content (Lee *et al.*, 2008 and Cheung, 2003)
The antioxidants found in mushrooms are mainly phenolic compounds (phenolic acids and flavonoids), followed by tocopherols, ascorbic acid and carotenoids.
Wild mushrooms are becoming more and more important in our diet for their nutritional (Crisan, 1978, Breene, 1990 and Manzi, 1999) and pharmacological (Bobek, 1991, Bobek, 1999, characteristics. The high protein and low fat/energy contents of wild edible mushrooms, reported by many workers (Aletor, 2005).

Oxidative stress

Free radicals are produced in the normal natural metabolism of aerobic cells, mostly in the form of oxygen reactive species (ROS). Once produced, most of the free radicals are neutralized by cellular antioxidant defences (enzymes and non-enzymatic molecules). Maintenance of equilibrium between free radicals production and antioxidant defences is an essential condition for normal organism functioning (Valko *et al.*, 2007)
Beneficial effects of ROS occur at low or moderate concentrations and involve cellular physiological roles of signalization and regulation (Freidovich *et al.*, 1999

and Fang *et al.,* 2002) Nevertheless, the equilibrium between ROS production and antioxidant defences might be displaced either by the overproduction of ROS or by the loss of the cell antioxidant defences (Machlin *et al.,* 1987) This disequilibrium is known as oxidative stress, and in this case, the excess ROS may oxidize and damage cellular lipids, proteins and DNA, leading to their modification and inhibiting their normal function (Fu *et al.,* 1998 and Ridnour *et al.,* 2005)

Oxidative stress might have natural causes such as extreme exercise or inflammation processes, or non-natural causes such as the presence of xenobiotics in the organism or situations related to several diseases. In fact, the noncontrolled production of free radicals has been related to more than one hundred diseases including several kinds of cancer (Valko *et al.,* 2006), diabetes, cirrhoses (Wei *et al.,* 2004) cardiovascular diseases (Shah *et al.,* 2004) neurological disorders (Moreira *et al.,* 2008) among others. The overproduction of ROS has also been related to the aging process (Halliwell, 1984, Halliwell, 1996 and Barja *et al.,* 2004)

Considering that 70% of the chronical diseases and related costs can be prevented, the knowledge about ROS and about their overproduction control is crucial (Fries *et al.,* 1993). This control can be achieved by the maintenance of good levels of antioxidants and free radicals scavengers, increasing diet quality (higher consumption of vegetables, leguminous and fruits) or avoiding behaviours that lead to a higher ROS production, such as tobacco, excessive exposure to environmental polluents and xenobiotics (Lachance *et al.,* 2001).

Free Radicals, Reactive Oxygen Species (ros) and Reactive Nitrogen Species (rns)

A free radical is defined as any atom or molecule possessing unpaired electrons in the outer orbit (Halliwell *et al.,* 1999 and Gutteridge *et al.,* 2000). They are generally unstable and very reactive. Free radicals derived from molecular Oxygen (O_2) are usually known by reactive Oxygen species (ROS) and represent the most important class of radical species generated in living systems (Miller *et al.,* 1990). In fact, despite the importance of O_2 to aerobic life, in some conditions it can be toxic. This phenomenon is called "oxygen paradox" (Gilbert *et al.,* 2000). Several ROS production pathways and the main endogenous antioxidant defences of the cell. The addition of one electron to molecular Oxygen forms the superoxide anion (O_2), which is considered the "primary" ROS. Superoxide anion is mostly produced in mitochondria, due to a small but continuous "leak" of the electrons in the mitochondrial electron transport system (ETS). These electrons generate superoxide anion instead of reducing oxygen to water. Measurements on submitochondrial particles suggest an upper limit of 1–3% of "leaking" electrons in the mitochondrial ETS (Cadenas *et al.,* 1998). Superoxide anion can also be produced by different endogenous enzymatic systems present in the cell like NADPH oxidases and xanthine oxidase (Gilbert *et al.,* 2000). O_2 have been implicated in several diseases (Kovacic *et al.,* 2005). Even though O2 is not a very active radical, it can interact with other molecules generating what are considered as "secondary" ROS, such as hydrogen peroxide (H2O2) and hydroxyl radical (OH•). Hydroxyl radical has a very short life time but is considered to be the most toxic among all ROS, being responsible for the attack to DNA molecules, damaging purins and pyrimidines and the structure of desoxyribose DNA (Halliwell *et al.,* 1990). Hydroxyl radical is the neutral form of the

hydroxide ion and it is formed by an electron transfer from transition metals to H_2O_2, and interacts with biomolecules immediately after generation. Eventual permanent damages in the genetic material caused by oxidative stress might represent the first step to mutagenesis, carcinogenesis and aging. Mitochondria are the most important source of ROS, but they are also the first targets of these radicals because ROS have an easy access to the membrane lipids, which are susceptible to free radicals attack. This attack is called lipid peroxidation and promotes the production of different types of ROS (Mehrotra *et al.,* 1991). The lipid peroxidation usually begins with the extraction of a hydrogen atom from a polyunsaturated lipid (LH) chain, through the action of reactive species such as HO•. This generates a highly reactive lipid radical (L•) that can react with O_2 to form a peroxyl radical (LOO•). If not neutralized by antioxidants defences, the peroxyl radical will react with other adjacent lipids producing hydroperoxides lipids (LOOH) that can easily be decomposed to form new L• radicals, initiating a process that is known as chain propagation reactions. This process when not stopped, can lead to much superior damage than the ROS that started the reaction (Abreu *et al.,* 2000 and McCord *et al.,* 2000).

2.5.1 2,2-diphenyl-1-picrylhydrazyl (DPPH) Assay

This is the oldest indirect method for determining AOA, it was firstly suggested in 1950s originally to discoverH-donors in natural materials. Later the test was quantified to determine the antioxidant potential of both individual phenolics and food as well as of biologically relevant samples. The DPPH test is based on the capability of stable free radical 2,2-diphenyl-1-picrylhydrazyl to react with H-donors including phenolics. As DPPH shows a very intensive absorption in the visible region, it can be easily determined by the UV–Vis spectroscopy, although ESR determination is also

acceptable. DPPH is likely more selective than ABTS_+ in the reaction with H-donors. For instance, the f value for series of tea catechins was found to be almost equal to the number of active OH-groups inherent in catechol and pyrogallol fragments (Nanjo, Mori, Goto, & Hara, 1999). In contrast to ABTS_+, DPPH does not react with flavonoids, which contain no OH-groups in B-ring (Yokozawa *et al.,* 1998) as well as with aromatic acids containing only one OH-group (von Gadov *et al.,* 1997).

The DPPH test was suggested in two versions, dynamic and static. In the first version, one measures the rate of DPPH decay observed after the addition of a phenolic-containing sample. In the static version, one determines the amount of DPPH scavenged by a sample tested. While the first assay characterizes the reactivity, the second determines the stoichiometry for the reaction of DPPH with H-donor for individual substance or the quantity of active OH-groups in complex mixture. In the dynamic version the reactivity is commonly characterized by the starting rate of DPPH decay (Da Porto, Calligaris, Celotti, & Nicoli, 2000; Nanjo *et al.,* 1999; von Gadov *et al.,* 1997; Yen & Duh, 1994).

The antioxidant activities of the methanol extract were measured on the basis of the scavenging activities of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical (Acharya *et al.,* 2005) Various concentrations of 1 mL of the tested extract were added to 4 mL of a 0.004% (w/v) methanol solution of DPPH. After 30 min of incubation period at room temperature, the absorbance was measured against a blank at 517 nm. Inhibition of free radical DPPH in percent (%) was calculated by:

I % = (Ablank – Asample / Ablank) x 100,

where Ablank is the absorbance of the control reaction (containing all reagents except the test compound), and Asample is the absorbance of the test compound. Extract

concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted inhibition percentage against extract concentration.

2.5.2 Reducing Power

The reducing antioxidant power of plant methanol extracts was determined by the method of Oyaizu (1986). Different concentrations of mushroom extracts in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50^0C for 20 min. Then, 2.5 ml of Trichloroacetic acid (10%) was added to mixture, which was then added with $FeCl_3$ (0.5 ml, 0.1%). The absorbance was measured at 700 nm against a blank using UV-Vis spectrophotometer. Increased absorbance of the reaction mixture indicates increase in reducing power.

2.6 Screening for Anticancer activity

Cancer can be defined as a rapid, abnormal, uncoordinated proliferation of aberrant cells in any tissue or organ of the body which may mass together to form a growth or tumor, or proliferate throughout the body indicating abnormal growth at other sites. If the process is not arrested, it may progress until it causes the death of the organism (Evans, 2002). Cancer is considered as one of the most fear some causes of morbidity and mortality in all over the world. Although the disease has often been regarded. principally as a problem of the developed world, more than half of all cancers occur in the developing countries (Stewart *et al.,* 2003). Unfortunately, currently available cancer chemotherapeutic agents insidiously affect the host cells especially bone marrow, epithelial tissues, reticulo-endothelial system and gonads (Mascarenhas, 1994). Most of the antineoplastic agents produce serious chronic or delayed toxicities that may be irreversible, particularly in heart, lungs and kidneys, thereby increasing morbidity (Nitha *et al.,* 2005).

Plants have a long history of use in the treatment of cancer. The approach for minimizing unwanted toxicity is to employ newer natural products that may act by different and distinct mechanism(s) and/or precipitate less serious adverse effects. A number of plant or other natural product extracts have been studied for anticancer activity leading to the development of several clinically useful anticancer agents (Da Rocha *et al.,* 2001). Hence, the natural products now have been contemplated of exceptional value in the development of effective anticancer drugs with minimum host cell toxicity.

2.6.1 Passaging of Cancer Cell Lines

The media was removed from the t-25 flask and was then rinsed with Phosphate Buffer Saline, the cells are trypsinized with 0.25% trypsin to remove the adherent cells into the suspension, after this five ml of freshly media was filtered and added into the trypsinized flask with combination of antibiotics to resist the growth of Bacteria and Viruses. These flasks were then incubated at 37^0C in co_2 incubator with 5% co_2 for 5-7 days for the monolayer formation on the surface of t-25 flask.

Media

HeLa: Human; Cervix

Hep G2: Human; Liver

Minimum essential medium (DMEM) with 2mM L-glutamine and Earle's BSS adjusted to contain 1.5g/L Na bicarbonate, 0.1mM non-essential amino acid, and 1.0mM Na pyruvate, 90%; fetal calf serum, 10%.

ZR-75-30: Human; Mammary gland; Epitelial; Ascites; Ductal carcinoma

RPMI-1640 medium with Na pyruvate, 90% adjusted to contain 1.5g/L Na bicarbonate; fetal calf serum, 10%.

2.6.2 Freezing/Preservation of Cancer Cell Lines

The media was removed from the t-25 flask and was then rinsed with Phosphate Buffer Saline, the cells are trypsinized with trypsin to remove the adherent cells into the suspension, after this three ml of 10% Fetal Bovine Serum was filtered and added into the trypsinized flask with combination of antibiotics to resist the growth of Bacteria and Viruses. From this one ml of the suspension media containing the cells

are transferred into 1.5 ml of cryovials, to this vial 0.5ml of sterilized and filtered DMSO is added. The cryovials are then stored in Liquid Nitrogen or -80^{0}C. These cell lines can be stored for years and revived back when needed by adding the Cell line media.

2.6.3 Determination of The Cell Viability by Trphan Blue Staining

The viability of the cell lines was done by trphan blue staining method, where in 10µl of the cell suspension was taken and mixed with 10µl of trphan blue stain, this was mixed and loaded on to hemocytometer and the cell count of the viable cell was calculated in the WBC cell counter chamber.

The viable cells were seen hollow under the microscope where as the dead cells took up the trphan blue strain.



Plate 9: Cancer cell lines under inverted microscope

2.6.4 Colorimetric MTT (tetrazolium) assay

Colorimetric MTT (3-(4, 5-dimethythiazol-2-yl)- 2,5-diphenyl tetrazolium bromide) assay was carried out as described by Mosmann (1983). 10µl of MTT solution (5mg/ml) was added to all wells of 96 wells micro plate followed by 4h incubation at 37°C. Acid isopropanol was added to all wells for dissolving the dark blue crystals. The microplate plate was then read on an ELISA reader at wavelength 570nm within 1h after adding isopropanol.

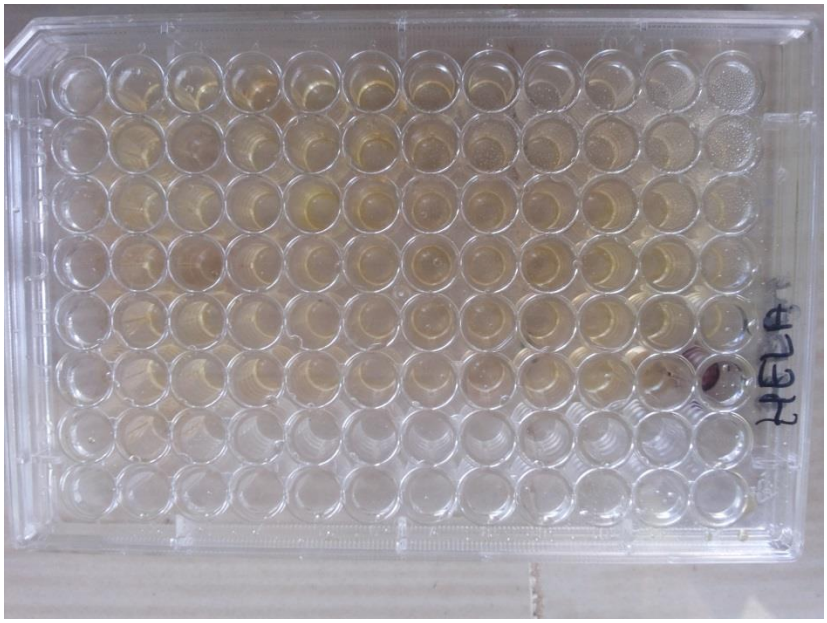


Plate 10: MTT assay For HeLa in 96 well microtitre plate

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Collection of Mushrooms and identification

The wild mushrooms were obtained in and around Bangalore and subjected to identification and were identified as *Ganoderma appalantum, Tricholoma giganteum* and *Bolbitius vitellinus*, and the mushrooms were used in this study.

Ganoderma applanatum: (Pers.) Pat.

Kingdom: Fungi
Division: Basidiomycota
Class: Agaricomycetes
Family: Ganodermataceae
Genus: Ganoderma
Species: applanatum

Ganoderma applanatum is a bracket fungus with a cosmopolitan distribution. The spore bodies are up to 30–40 centimetres (12–16 in) across, hard, woody textured, and inedible; they are white at first but soon turn dark red-brown. It is a wood-decay fungus, using primarily dead heartwood, but also as a pathogen on live sapwood, particularly on older trees. It is a common cause of decay and death of beechand poplar, and less often of several other tree genera, including alder, apple, elm, buckeye and horse chestnut, maple, oak, walnut, willow, western hemlock, Douglas Fir, and Spruce. A peculiarity of this fungus lies in its ability to be as a drawing medium for artists. When the surface is rubbed or scratched with a sharp implement, it changes from light to dark brown, producing visible lines and shading.



Plate 11: basidiocarp of *Ganoderma applanatum*

Tricholoma giganteum Massee, *Bull. Misc. Ihf. Kew* 1912:254. 1912.

Basionym: Macrocybe gigantea (Massee) Pegler & Lodge, Mycologia 90: 497. 1998.

Kingdom: Fungi

Division: Basidiomycota

Class: Agaricomycetes

Order: Agaricales

Genus: Tricholoma

Species: giganteum.

Pileus 7-32 cm diam, conicoconvex then expanding; pale orange(5A4) to greyish orange (5B4),smooth, glabrous, paler towards the margin, glabrous and silky smooth but cracking on drying; margin slightly incurved, scurfy, often cracking. Lamellae adnate, sinuate, straw yellow (3B4), ventricose, densely crowded, with lamellulae of four lengths. Stipe 15-18 × 7 cm, cylindrical, often elongate, solid finally fistulose; surface concolorous with pileus, fibrillose-striate. Context up to 3 cm thick at disc, white. Spore deposit white. Spores 5.7-7 × 3.5- 4.3 Q = 1.6, ovoid to short ellipsoid,

hyaline, inamyloid, thinwalled. Basidia 25- 40 × 5-8 µm, narrowly clavate to sub cylindrical, tetrasporic; Lamellar edge fertile; cystidia absent. Hymenophoral trama regular, 2.4-5.6 µm diam, Pileipellis a compact, repent cutis of interwoven hyphae, 2.8-7 µm diam. All hyphae with clamp connections.

Bolbitus Fr.In Epicrisis:253 (1888)

Type species: *bolbitius vitellinus* (Pers. Ex Fr.) Fr.

Kingdom: Fungi
Division: Basidiomycota
Class: Agaricomycetes
Order: Agaricales
Genus: Bolbitus
Species: vitellinus

Basidiocarp coprinoid, ephemeral. Pileus thin, viscid, striate towards the margin. Lamellae ascending to free, rapidly dellquescent after spore discharge. Stripe central, hollow, usually white; caulocystidia present. Spore print color brown. Spores ellipsoid, smooth, thick walled, with truncate germ pore. Basidia 2 or 4 spored. Cheilocystidia always present, non capitates. Pleurocystidia present or absent. Hymenophoral trama regular. Context thin. Pileal surface an epithelium of piriform elements; pileocystidia present. Hyphae with or without clamp connections. On soil, wood and dung.



Plate 12: *Bolbitus vitullinus* in natural habitat

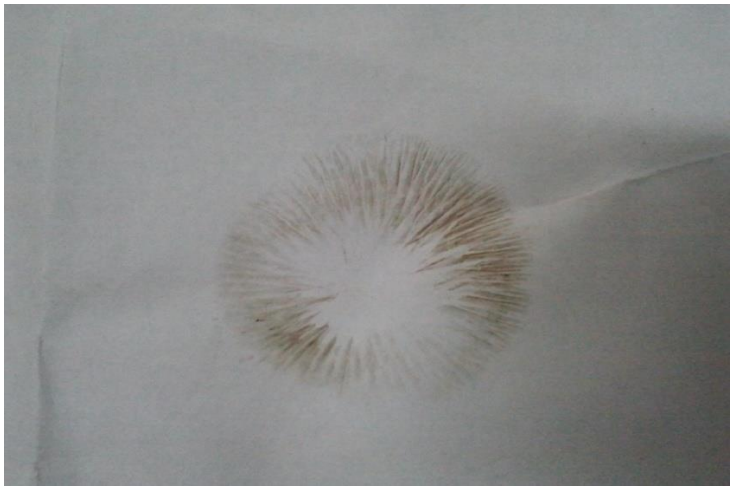


Plate 13: spore print of *Bolbitus vitullinus*

3.2 Isolation of Pure Cultures of Mushrooms

The mycelia mat cultured from the context of the mushroom appeared white on the potato dextrose agar plate. These on further culturing in potato dextrose broth formed a mycelia mat which was used for further studies.



Plate 14: Pure cultures



Plate 15: Pure culture of *Ganoderma applanatum* on potato dextrose broth

3.3 Preparation of Alcoholic Extract From basidiocarps and Pure Cultures

The dried 10grams of basidiocarp were grinded and were mixed in methanol for extraction. The evaporated methanolic extract obtained in the range between 1 to 1.7grams for each 10grams of the sample. The methanolic extract of *Ganoderma applanatum* was found to be dark brownish black in color. The extract of *Tricholoma giganteum* appeared to be brown in color and *Bolbitus vitellinus* was lightish brown with orange tinch.

The weight of the mycelia mat obtained from the Potato dextrose broth ranged from 12-15grams, each of the 10grams sample of the mycelia yielded 0.9-1.4grams of the methanolic extract. The methanolic extract of *Ganoderma applanatum* appeared to be brownish in color, whereas *Tricholoma giganteum* appeared to be light brown and *Bolbitius vitellinus* appeared to be lightish yellow in color and this may be due to the use of Potato dextrose broth.



Plate 16: Methanolic extract of the samples

3.4 Screening of Antibacterial and Antifungal Activity of Mushroom Extract

The antimicrobial activity for the wild mushrooms are given in the table 01 and figure 01. Where in the zone of inhibition is given for each sample in millimeter.

	zone of inhibition in mm				
Organisms	*Ganoderma applanatum* Basidiocarp (100µg/ml)	*Tricholoma giganteum* Basidiocarp (100µg/ml)	*Bolbitius vitellinus* Basidiocarp (100µg/ml)	Tetracyclin (30µg/ml)	Nystatin (30µg/ml)
B.subtilis	42	**51**	14	17	0
P.vulgaris	**50**	41	25	16	0
S.aureus	**38**	32	9	20	0
E.coli	**40**	25	32	8	0
M.flavus	32	**35**	14	20	0
P.aeriginosa	0	**35**	16	8	0
S.abony	**35**	30	14	11	0
K.pneumonia	**45**	36	19	5	0
P.fluroscence	15	15	0	10	0
Cryptococcus	6	0	0	0	21
C.albicans	3	7	12	0	18

Table 1: Screening of Antimicrobial activity of wild mushrooms.

The methanolic extract of the wild mushroom showed zone of inbition for both bacteria and fungal species, where in *Ganoderma applantum* showed clear zone of

inhibition except for *P.aeriginosa, Tricholoma giganteum* showed a moderate zone of inhibition when compared to the extract of *Ganoderma applanatum*. *Bolbitius vitellinus* also had an impact on the growth on pathogen microorganism but to smaller extent, but all the mushroom samples showed greater zone of inhibition when compared to the standard of tetracyclin.

The highest zone of inhibition was seen for *B.subtilis* with a diameter of 51mm when incubated with *Tricholoma gigantium* extract.

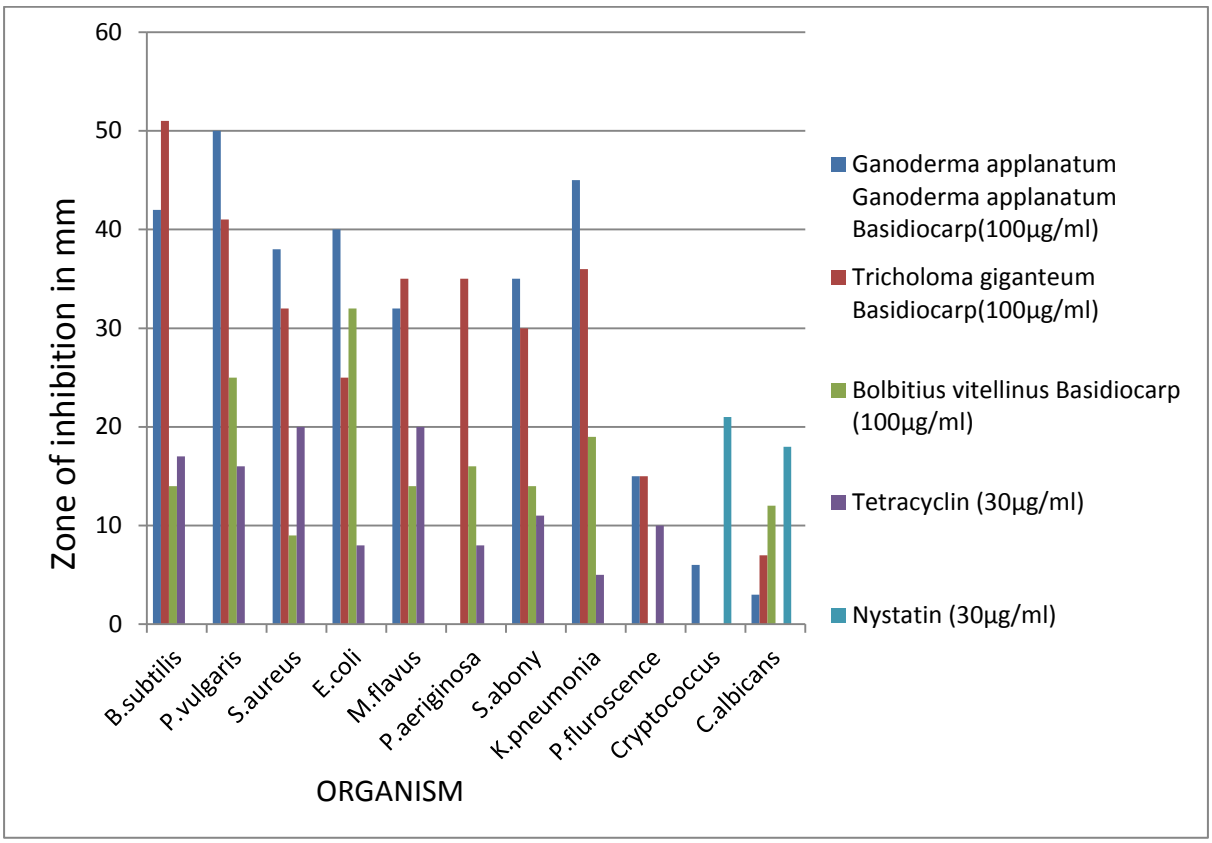


Figure 1: Screening of Antimicrobial activity of wild mushrooms.

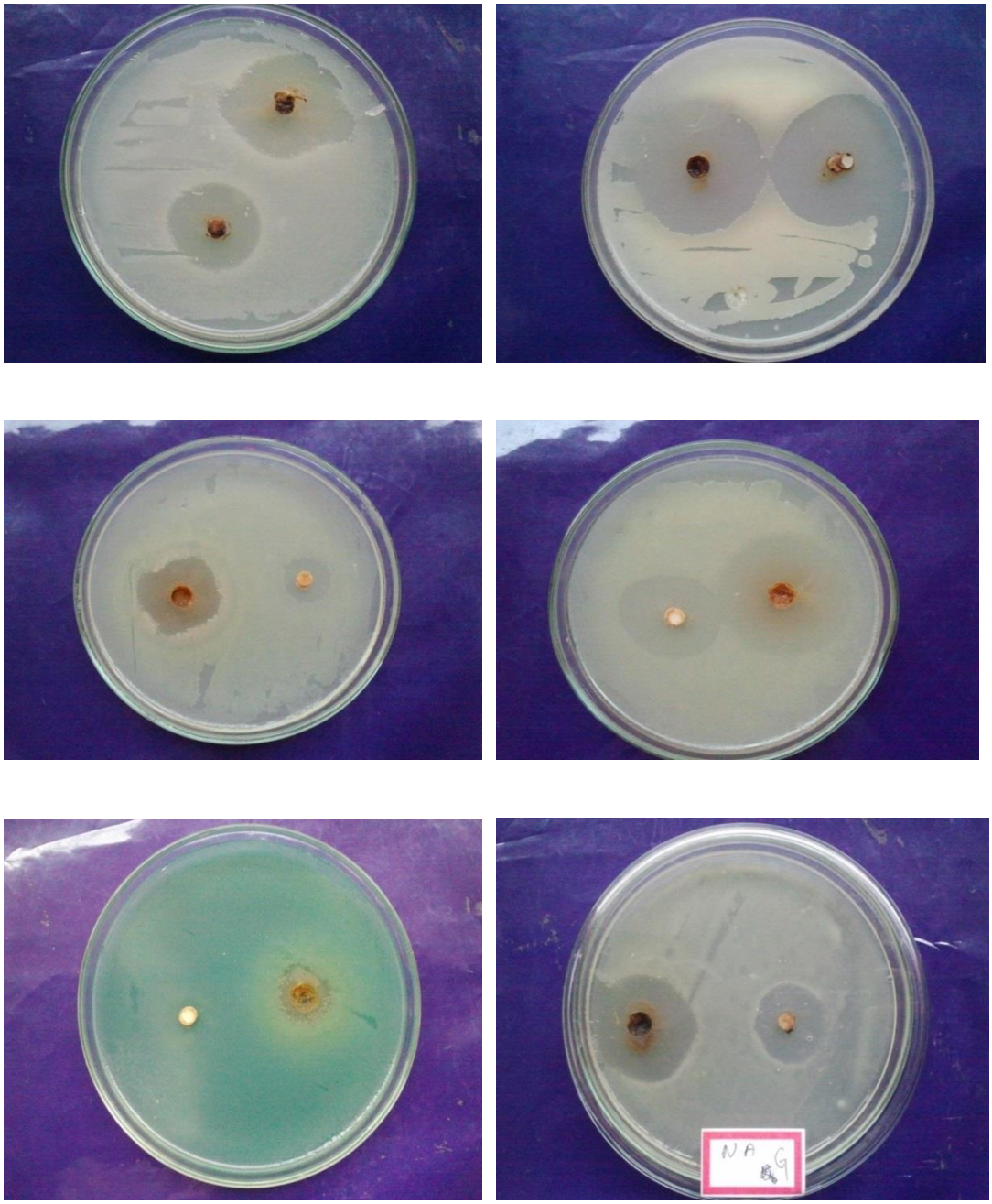


Plate 17: Plates showing antimicrobial activity by different mushroom extracts

The methanolic extracts of the Basdiocarps of 3 different wild mushrooms were tested against three Gram Positive Bacteria (*B.subtilis, S.aureus, M.flavus*), six Gram Negative Bacteria (*P.vulgaris, E.coli, P.aeriginosa, S.abony, K.pneumonia, p.fluroscence)* and two different fungi *(Cryptococcus and C.albicans*). All the extracts showed different degree of antimicrobial activity at 100µg/ml as mentioned

in the table 01 and figure 01, and three representative photos of antimicrobial activity by Agar diffusion assay showing zone of inhibition are shown in plate 11.

Out of three mushrooms, the methanolic extract of *G.applanatum* was found to be more active followed by *T.giganteum* and *B.vitellinus*. *G.applanatum* showed activity against most of the Bacteria, how ever activity was found to be highest for Gram Positive Bacteria, *B.subtilis*, followed by *S.aureus* and *M.flavus*. It even showed the activity against Gram negative bacteria, amoung Gram negative bacteria the extract showed maximum activity against, *E.coli* and *K.pnuemonia* and very less activity against *P.fluroscence* and no activity against *P.aeriginosa*. The extract of *G.applanatum* even was found to be active against *Cryptococcus sps.* and *C.albicans* but the activity was very less when compared to that of standard antibiotics. Smania *et al.,* (1999), Barranco *et al.,* (2010) had reported the similar activity against Gram negative bacteria by *G.applanatum*. Mothana *et al.,* (2000) also reported the similar activity by *G. pfeifferi.* Smania *et al., (2007),* Klaus and Miomiur (2007), Yoon *et al.,* (1994), Sheena *et al.,* (2003), Keypur (2008) reported the smiliar activity of *G.lucidum* however they had reported that the activity against Gram positive bacteria were more than Gram negative bacteria. Lyndal (2004), Beltran-Garcia *et al*, 1997, Ishikawa *et al* (2001) had documented greater resistance to Gram negative bacteria by *Ganoderma* species. This may be due to different extraction procedures followed. 5,8-epidioxy-5a,8a-ergosta-6,22-dien-3b-ol reported by Lindequiest (1998), isolated from *Ganoderma applanatum* (Pers.) Pat., proved to be active against a number of gram positive and gram-negative microorganisms Smania *et al.,* (1999).

T.giganteum showed a good zone of inhibition on gram positive bacteria *B.subtilis, S.aureus, M.flavus* with a zone of inhibition 51mm, 32mm, 35mm of diameter respectively with respect to the standard tetracycline which has a zone of inhibiton of

17mm, 20mm, 20mm. The activity against six gram negative bacteria also showed good activity with respect to tetracycline taken as standard. *(P.vulgaris, E.coli, P.aeriginosa, S.abony, K.pneumonia, P.fluroscence).* The zone of inhibition for *P.vulgaris* is 41mm and wid the standard tetracycline it is 16mm. the zone of inhibitons for *E.coli, P.aeriginosa, S.abony, K.pneumonia, P.fluroscence* was found to be 25mm, 35mm, 30mm, 36mm, and 15mm respectively.*Tricholoma crassum, Tricholoma lobayense* and other *Tricholoma sp.* Are reported to have zero or no inhibition for *S.aureus, E.coli, B.subtilis* by*Krishnendu Acharyaet al., (2012),* where as *T.giganteum* was found to have 32mm, 25mm, and 51mm zone of inhibition.The activity against fungi is different, there is a positive result but the zone of inhibition is very less its about 7mm for *C.albicans* and 0mm for *Cryptococcus*.

The antimicrobial activity of was screened on different bacterial and fungal cultures. The extract of *Bolbitius vitellinus* basidiocarp (100µg/ml) was tested against both gram positive and gram negative bacteria. *Bolbitius vitellinus* showed zone of inhibition on gram positive bacteria *B.subtilis, S.aureus, M.flavus* with a zone of inhibition 14mm, 9mm, 14mm of diameter respectively with respect to the standard tetracycline which has a zone of inhibiton of 17mm, 20mm, 20mm. The activity against six gram negative bacteria also showed good activity with respect to tetracycline taken as standard. *(P.vulgaris, E.coli, P.aeriginosa, S.abony, K.pneumonia, P.fluroscence).* The zone of inhibition for *P.vulgaris* is 25mm and wid the standard tetracycline it is 16mm. the zone of inhibitons for *E.coli, P.aeriginosa, S.abony, K.pneumonia, P.fluroscence* was found to be 32mm, 16mm, 14mm, 19mm, and 0mm respectively. The activity against fungi is very low 0mm for *Cryptococcus* and 12mm for *C.albicans*.

3.5 Screening of Antioxidant Activity of wild mushrooms

3.5.1 2,2-diphenyl-1-picrylhydrazyl (DPPH) Assay

The methanolic extract obtained from the basidiocarp of *Ganoderma applanatum* proved to have antioxidant activity and had the ability to scavage DPPH to 62.24% at 1000 µg/ml. Hence the Ec50 of DPPH was 650µg/ml.(Table 2 and Figure 2). The DPPH was scavenged to 50% by the methanolic extract of the mycelium of *Ganoderma applanatum* at a concentration of 2000µg/ml. (Table 3 and Figure 3). Table 4 and Figure 4, shows that the methanolic extract of *Tricholoma giganteum basidiocarp* had a scavenging activity against DPPH at 700µg/ml. The mycelial methanolic extract of *Tricholoma giganteum* scavenged the DPPH to 50% at a concentration of 2000µg/ml. (Table 5 and Figure 5). At a concentration of 850µg/ml of the methanolic extract from the basidiocarp of *Bolbitus vitellinus* showed 50% scavenging activity of DPPH as in Table 6 and Figure 6. The antioxidant activity was shown by the methanolic extract from the mycelia of *Bolbitus vitellinus* as in the Table 7 and Figure 7, where the Ec50 value was found to be 2100µg/ml.

Concentrations	Scavanging percentage on DPPH
0	0
100 µg/ml	18.7
200 µg/ml	20.6
300 µg/ml	23.4
400 µg/ml	30.2
500 µg/ml	37.4
600 µg/ml	43.1
700 µg/ml	52.2
800 µg/ml	58.4
900 µg/ml	63.3
1000 µg/ml	69.2

Table 2: Antioxidant activity- DPPH assay of *Ganoderma applanatum* basidiocarp

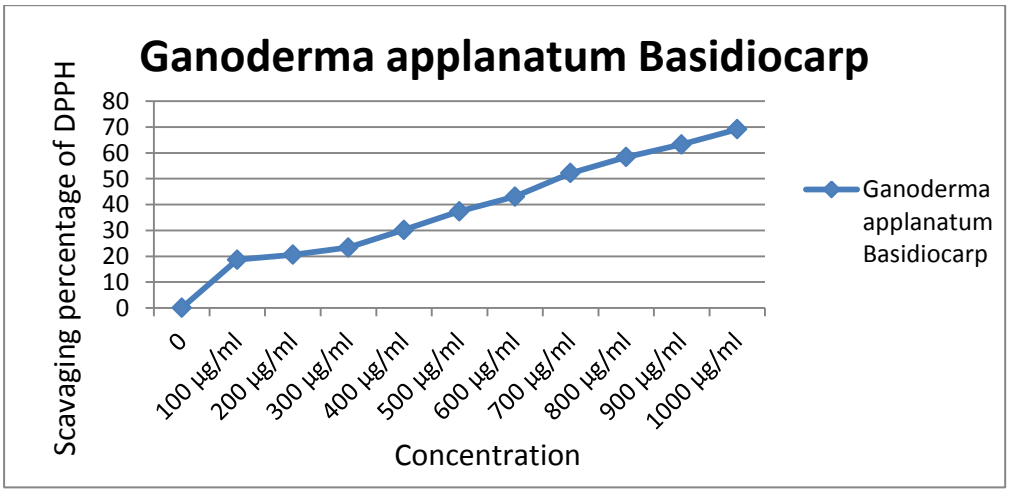


Figure 2: Antioxidant activity - DPPH assay of *Ganoderma applanatum* basidiocarp

Concentration	Scavanging percentage on DPPH
0	0
100 µg/ml	2.7
200 µg/ml	6.9
300 µg/ml	14.2
400 µg/ml	20.4
500 µg/ml	22.2
600 µg/ml	24.8
700 µg/ml	30.4
800 µg/ml	34.1
900 µg/ml	36.2
1000 µg/ml	40.2
1500 µg/ml	47.3
2000 µg/ml	50.6

Table 3: Antioxidant activity - DPPH assay of *Ganoderma applanatum* mycelium

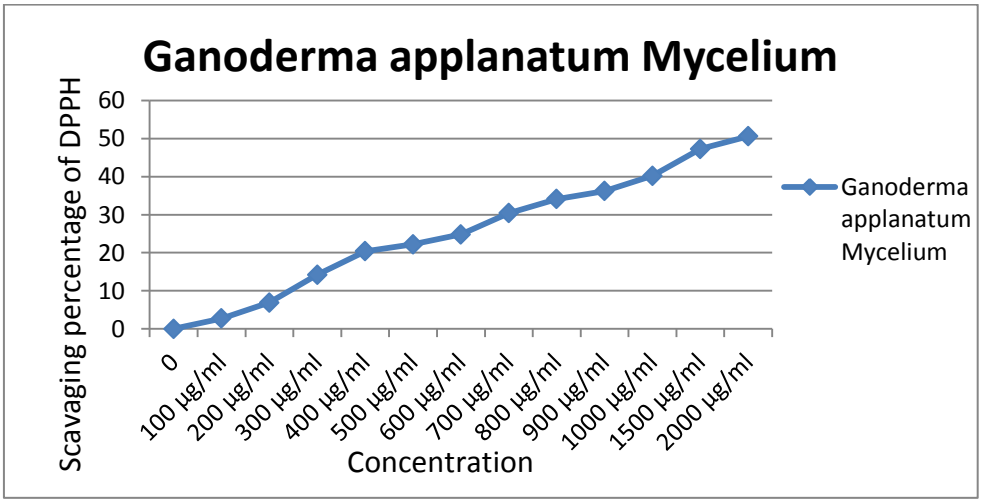


Figure 3: Antioxidant activity - DPPH assay of *Ganoderma applanatum* mycelium.

Concentration	Scavanging percentage on DPPH
0	0
100 µg/ml	7.6
200 µg/ml	13.2
300 µg/ml	19.42
400 µg/ml	23.56
500 µg/ml	30.42
600 µg/ml	39.67
700 µg/ml	50.34
800 µg/ml	54.24
900 µg/ml	59.14
1000 µg/ml	62.24

Table 4: Antioxidant activity - DPPH assay of *Tricholoma giganteum* basidiocarp

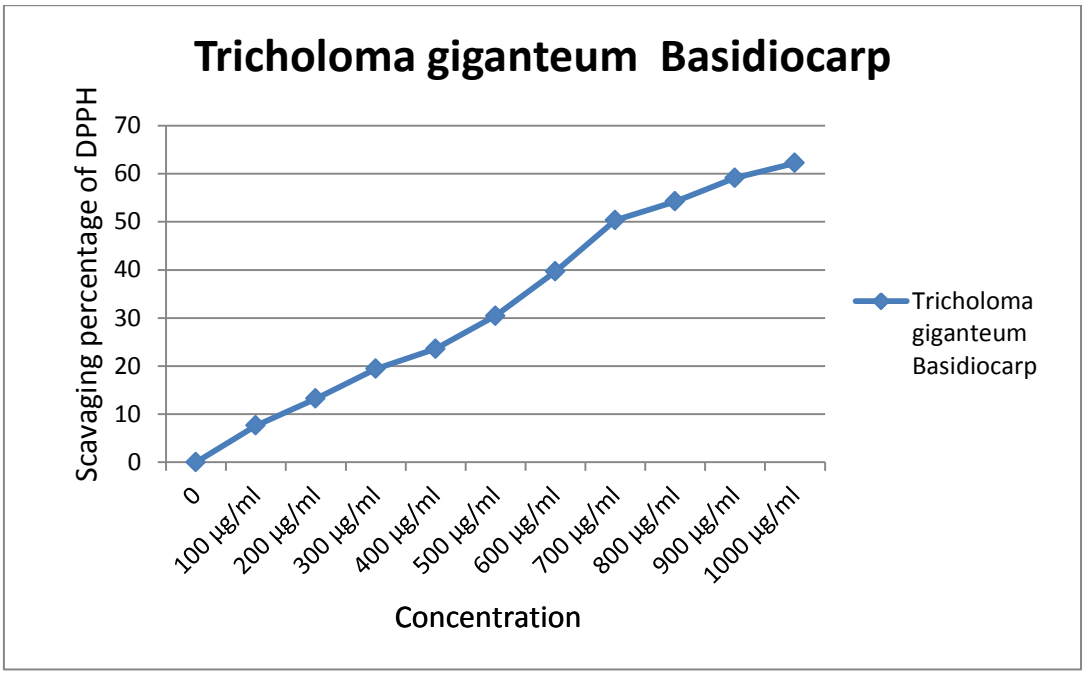


Figure 4: Antioxidant activity - DPPH assay of *Tricholoma giganteum* basidiocarp

Concentration	Scavanging percentage on DPPH
0	0
100 µg/ml	4.69
200 µg/ml	7.32
300 µg/ml	12.05
400 µg/ml	16
500 µg/ml	23.02
600 µg/ml	29.1
700 µg/ml	35
800 µg/ml	38.69
900 µg/ml	42.74
1000 µg/ml	44.69
1500 µg/ml	47.92
2000 µg/ml	50.32

Table 5: Antioxidant activity - DPPH assay of *Tricholoma giganteum* mycelium

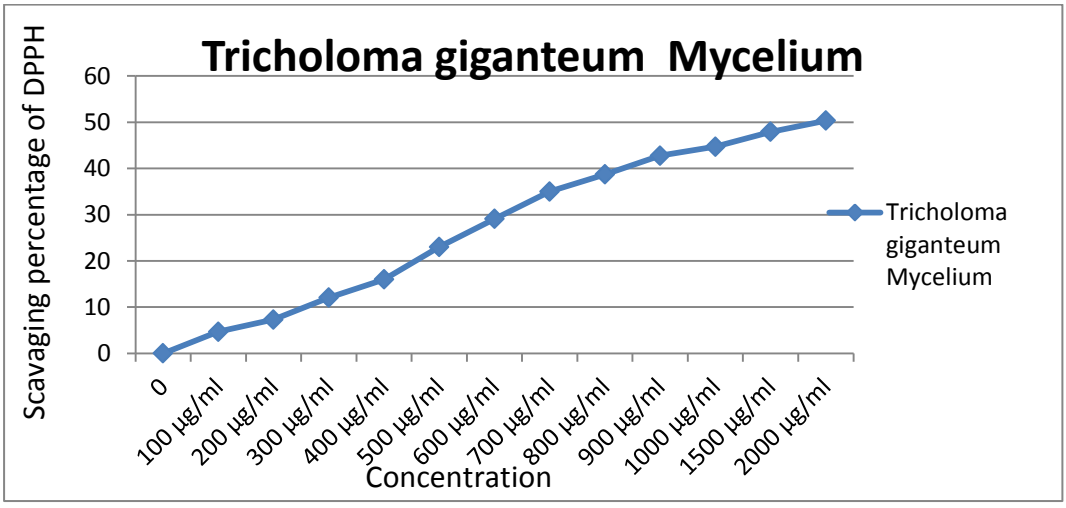


Figure 5: Antioxidant assay- DPPH assay of *Tricholoma giganteum* mycelium

Concentration	Scavanging percentage on DPPH
0	0
100 µg/ml	9.64
200 µg/ml	14.32
300 µg/ml	19.79
400 µg/ml	26.34
500 µg/ml	32.51
600 µg/ml	39.09
700 µg/ml	42.41
800 µg/ml	47.32
900 µg/ml	53.64
1000 µg/ml	57.26

Table 6: Antioxidant activity- DPPH assay of *Bolbitius vitellinus* basidiocarp

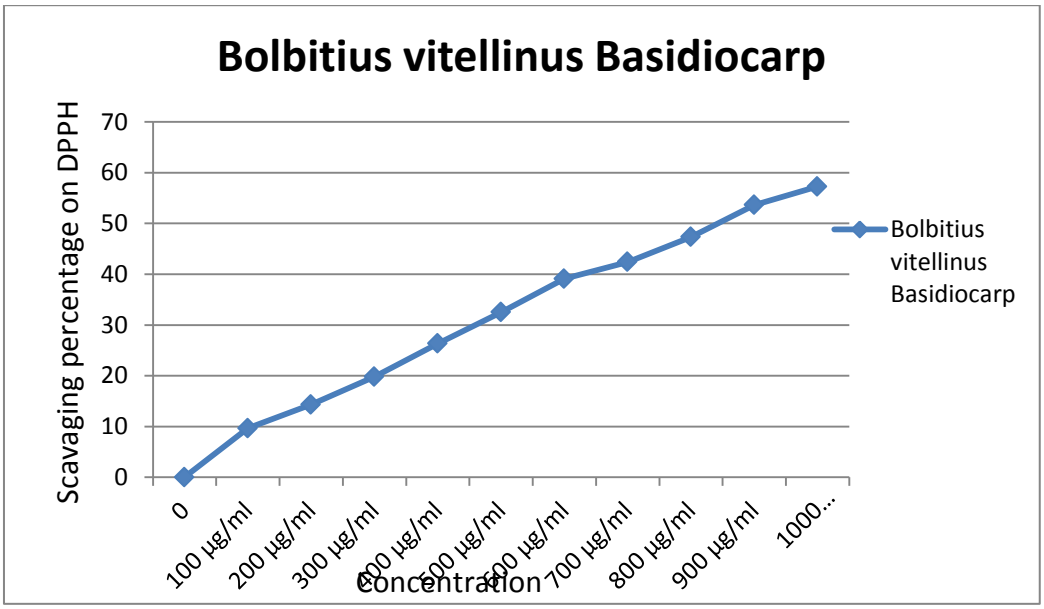


Figure 6: Antioxidant activity - DPPH assay of *Bolbitius vitellinus* basidiocarp

Concentration	Scavanging percentage on DPPH
0	0
100 µg/ml	4.69
200 µg/ml	9.28
300 µg/ml	14.71
400 µg/ml	18.27
500 µg/ml	20.02
600 µg/ml	23.02
700 µg/ml	28.74
800 µg/ml	33.14
900 µg/ml	38.27
1000 µg/ml	42.41
1500 µg/ml	44.69
2000 µg/ml	52.14

Table 7: Antioxidant activity- DPPH assay of *Bolbitius vitellinus* mycelium

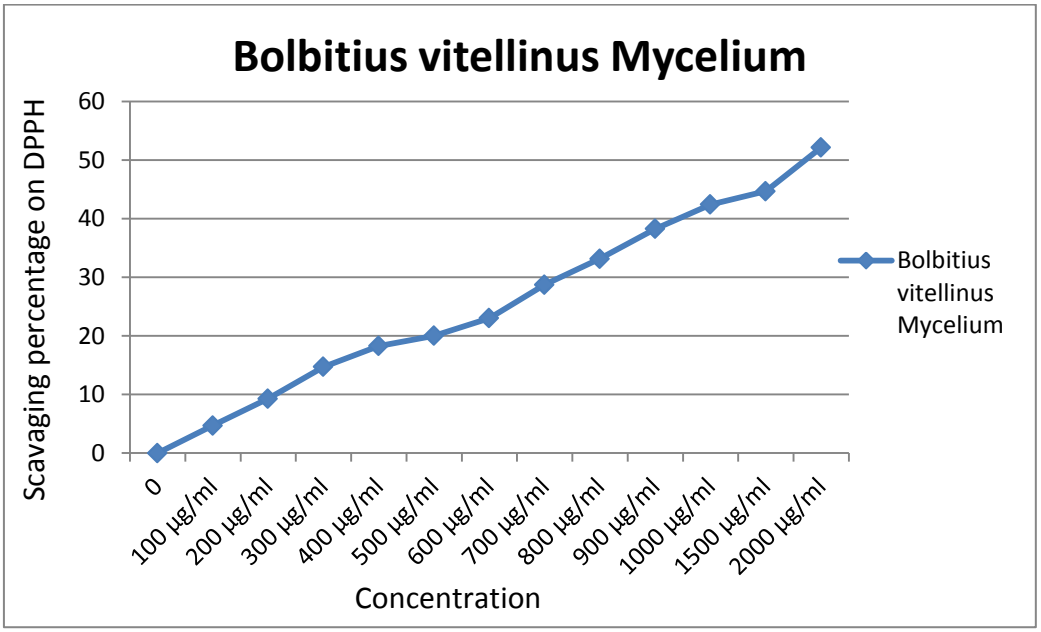


Figure 7: Antioxidant activity - DPPH assay of *Bolbitius vitellinus* mycelium

EC_{50} of Antioxidant activity- (DPPH) for mushroom samples

Ganoderma applanatum Basidiocarp	650 µg/ml
Ganoderma applanatum Mycelium	2000 µg/ml
Tricholoma giganteum Basidiocarp	700 µg/ml
Tricholoma giganteum Mycelium	2000 µg/ml
Bolbitius vitellinus Basidiocarp	850 µg/ml
Bolbitius vitellinus Mycelium	2100 µg/ml

Table 8: EC_{50} of Antioxidant activity - DPPH assay

The wild mushrooms samples showed a promising antioxidant activity by scavenging the activity of DPPH molecule. The basidiocarp extract proved to have scavenged the DPPH at lower concentration of the samples when compared to the mycelial extract of the same mushrooms.

The highest scavenging activity of DPPH was seen in *Ganoderma applanatum* basidiocarp (650 µg/ml) followed by *Tricholoma giganteum* basidiocarp (750 µg/ml) and finally *Bolbitius vitellinus* basidiocarp *(800* µg/ml). The mycelia of all the wild mushrooms did not vary much and showed a scavenging activity at 2000 µg/ml.

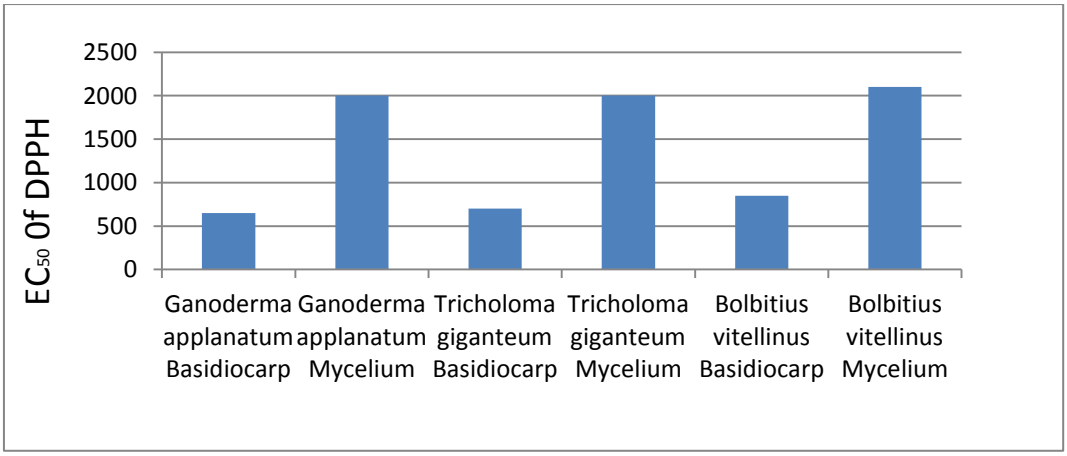


Figure 8: EC_{50} of Antioxidant activity - DPPH assay

Ganoderma applanatum basidiocarp showed 18.7% of scavenging activity at 100 µg/ml and 69.2% scavenging activity at 1000µg/ml. The **EC_{50}** value for *Ganoderma applanatum* basidiocarp has found to be at 650µg/ml. *Ganoderma applanatum mycelium* at 100µg/ml showed 2.7% of scavenging activity and 40.2% of scavenging activity at 1000µg/ml and 50.6% at 2000µg/ml concentration. **EC_{50}** value for *Ganoderma applanatum* mycelium has found to be at 2000µg/ml concentration of the sample.

At 100µg/ml concentration of *Tricholoma giganteum* basidiocarp showed 7.6% of scavenging activity and 62.24% scavenging activity at 1000µg/ml. The **EC_{50}** value for *Tricholoma giganteum* basidiocarp has found to be at 700µg/ml. *Tricholoma giganteum* mycelium at 100µg/ml showed 4.69% of scavenging activity and 44.69% of scavenging activity at 1000µg/ml and 50.32% at 2000µg/ml concentration. **EC_{50}** value for *Tricholoma giganteum* mycelium has found to be at 2000µg/ml concentration of the sample.

It has been reported that *Tricholoma matsutake* exhibit several biological activities, such as lower cholesterol, anti- oxidant and immuno-modulating effects in humans (Hoshi *et al.,* 2005; Mau *et al.,* 2002). A recent study examined the free radical-scavenging ability and inhibition of nitric oxide (NO) production by various solvent extracts obtained from TM (Lim *et al.,* 2007).

3.5.2 Reducing Power

The alcoholic extract obtained from the basidiocarp of *Ganoderma applanatum* proved to have reducing power activity with the increased concentration of the extract (Table 9 and Figure 9). The mycelia extract of *Ganoderma applanatum* showed a promising reducing power activity (Table 10 and Figure 10). The reducing power activity was shown by the methanolic extract from the basidiocarp of *Tricholoma giganteum* as in the Table 11 and Figure 11. Table 12 and Figure 12, proves that the basidiocarp extract of *Tricholoma giganteum* has the ability to reduce ferrous ion with the increase in concentration. The basidiocarp extract of *Bolbitus vitellinus* shows an increased reducing power activity and reaches a steady state with high concentration of extract (Table 13 and Figure 13). The ferrous ion were reduced by the methanolic extract of the *Bolbitus vitellinus* mycelia. Which is seen in Table 14 and Figure 14.

Concentration	Optical density at 700nm
0	0
100 µg/ml	0.04
200 µg/ml	0.05
300 µg/ml	0.06
400 µg/ml	0.06
500 µg/ml	0.07
600 µg/ml	0.08
700 µg/ml	0.09
800 µg/ml	0.09
900 µg/ml	0.11
1000 µg/ml	0.16

Table 9: Reducing power of *Ganoderma applanatum* basidiocarp

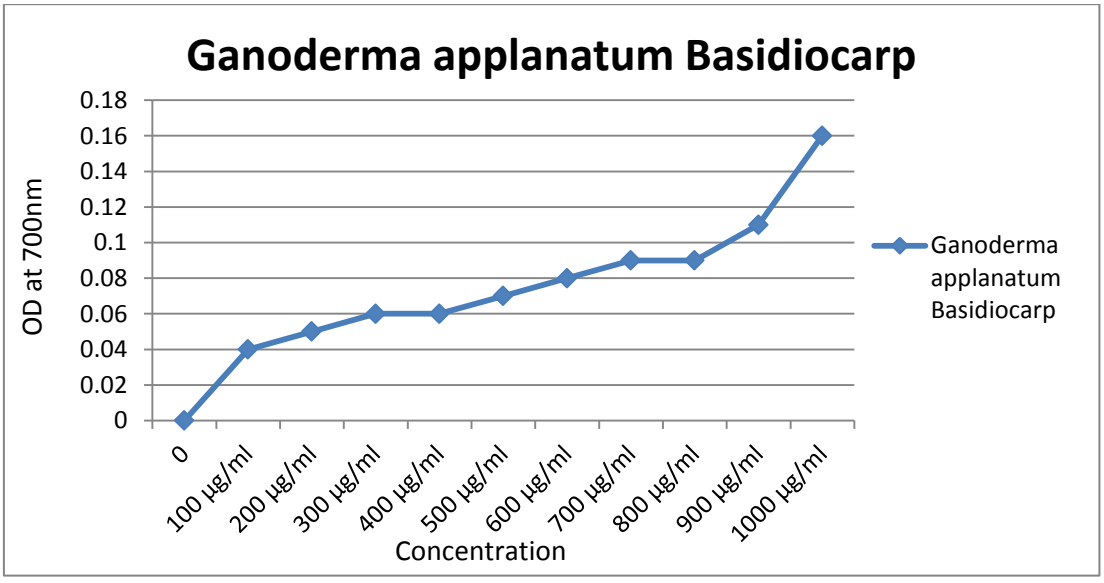


Figure 9: Reducing power of *Ganoderma applanatum basidiocarp*

Concentration	*Ganoderma applanatum* mycelium
0	0
100 µg/ml	0.11
200 µg/ml	0.12
300 µg/ml	0.13
400 µg/ml	0.14
500 µg/ml	0.15
600 µg/ml	0.16
700 µg/ml	0.17
800 µg/ml	0.17
900 µg/ml	0.18
1000 µg/ml	0.19

Table 10: Reducing power of *Ganoderma applanatum* mycelium

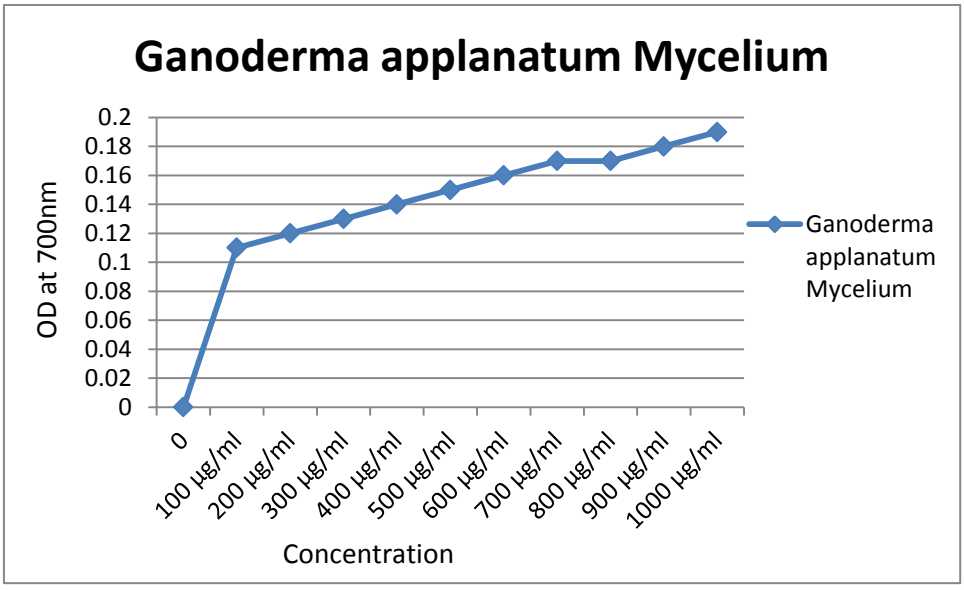


Figure 10: Reducing power of *Ganoderma applanatum* mycelium

Concentration	Optical density at 700nm
0	0
100 µg/ml	0.15
200 µg/ml	0.16
300 µg/ml	0.17
400 µg/ml	0.17
500 µg/ml	0.18
600 µg/ml	0.19
700 µg/ml	0.2
800 µg/ml	0.2
900 µg/ml	0.22
1000 µg/ml	0.26

Table 11: Reducing power of *Tricholoma giganteum* basidiocarp

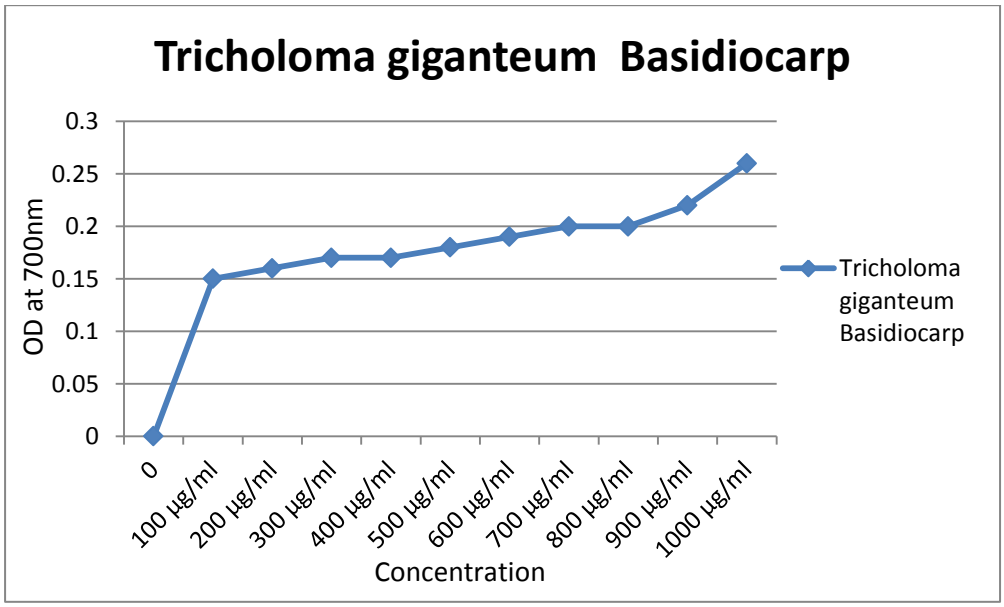


Figure 11: Reducing power of *Tricholoma giganteum* basidiocarp

Concentration	Optical density at 700nm
0	0
100 µg/ml	0.14
200 µg/ml	0.15
300 µg/ml	0.16
400 µg/ml	0.17
500 µg/ml	0.17
600 µg/ml	0.18
700 µg/ml	0.18
800 µg/ml	0.19
900 µg/ml	0.19
1000 µg/ml	0.24

Table 12: Reducing power of *Tricholoma giganteum* mycelium

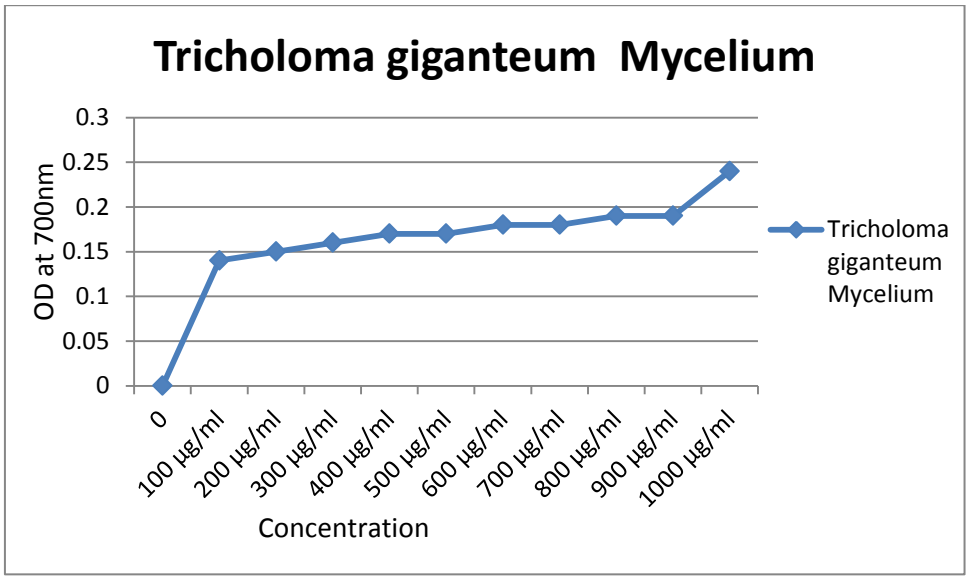


Figure 12: Reducing power of *Tricholoma giganteum* mycelium

Concentration	Optical density at 700nm
0	0
100 µg/ml	0.12
200 µg/ml	0.13
300 µg/ml	0.15
400 µg/ml	0.17
500 µg/ml	0.17
600 µg/ml	0.18
700 µg/ml	0.19
800 µg/ml	0.19
900 µg/ml	0.19
1000 µg/ml	0.19

Table 13: Reducing power of *Bolbitius vitellinus* basidiocarp

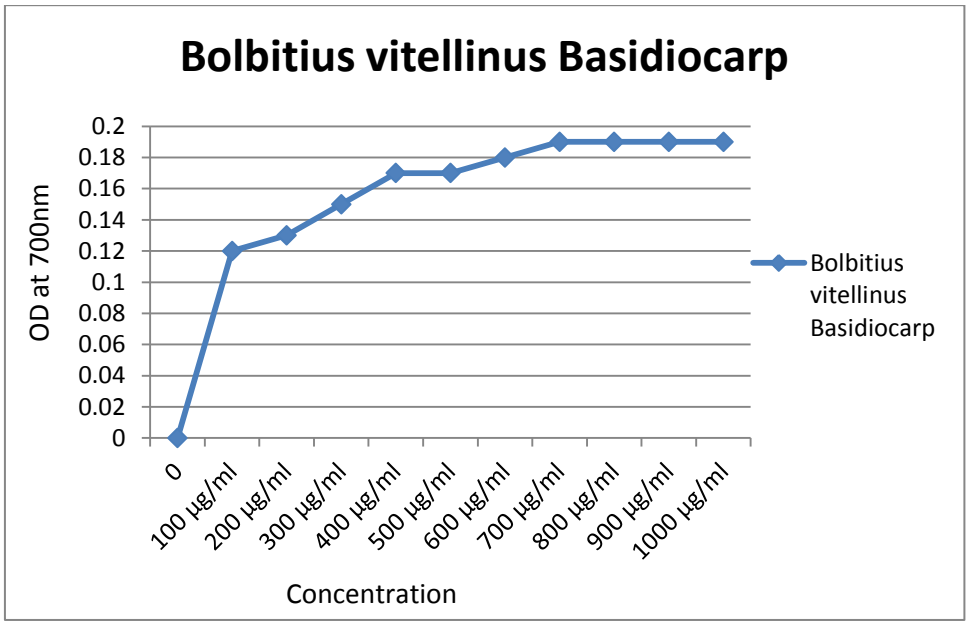


Figure 13: Reducing power of *Bolbitius vitellinus* basidiocarp

Concentration	Optical density at 700nm
0	0
100 µg/ml	0.17
200 µg/ml	0.18
300 µg/ml	0.19
400 µg/ml	0.2
500 µg/ml	0.21
600 µg/ml	0.22
700 µg/ml	0.24
800 µg/ml	0.249
900 µg/ml	0.25
1000 µg/ml	0.25

Table 14: Reducing power of *Bolbitius vitellinus* mycelium

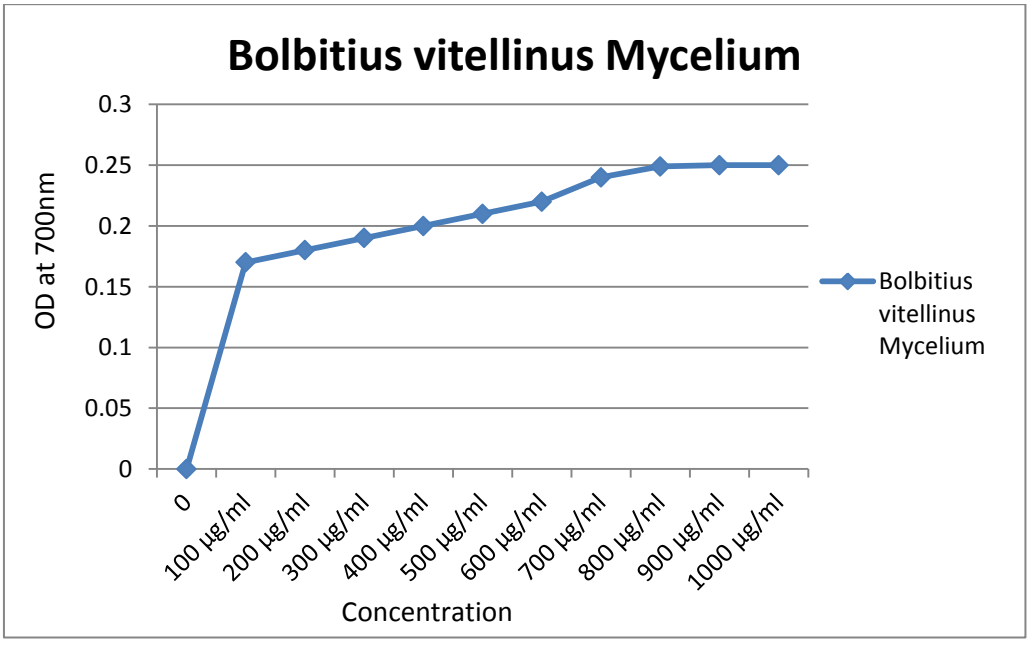


Figure 14: Reducing power of *Bolbitius vitellinus* mycelium

The reducing power increased with concentration, and the values obtained for all the extracts were excellent showing that they had high potential of reducing power.

The lowest yield for Pharmacognostic study was obtained in case of acetone and maximum in methanol for *Tricholoma giganteum*. (krishnanendu acharya *et al.*, 2012). All the extracts of all the mushrooms exhibited positive presence of antioxidant flavonoids and polyphenols indicative their potent medicinal activities. The reducing power increased with concentration, and the values obtained for both basidiocarp and mycelia were excellent showing high potential of reducing power.

3.6 Screening for Anticancer Activity

The anticancer activity was determined for cancer cell lines by MTT assay with different concentrations of samples.

3.6.1 Passaging of Cancer Cell Lines

The cell lines were cultured in T25 flask in the media and the viable cell count was checked with the monolayer formation after 24hrs. once the cell count reaches 5 lakh cells per ml of the media they can be used for further experimentation.



Plate 18: cancer cell lines in t-25 flasks



Plate 19: Inverted Microscope



Plate 20: Carbon dioxide incubator



Plate 21: Passaging of the cancer cell lines

3.6.2 Freezing/Preservation of Cancer Cell Lines

The media containing the cell lines with the cell concentration of 2 lakh cells per ml were freezed in cryovials with DMSO using liquid nitrogen. The preserved cell lines can be retrieved in future for further experimentation.



Plate 22: Cryo vials and liquid nitrogen container

3.6.3 Determination of Cell Viability by Tryphan Blue Staining

The cells suspension mixed with tryphan blue was placed in the haemocytometer and was observed under light microscope to check the viability of the cells. The viable cells appeared colorless whereas the dead cells take up the stain and appear blue in color. If the cell viability was above 2 lakh cells per ml indicated high proliferation of cancer cell lines, this cell suspension can be further used for the MTT assay.

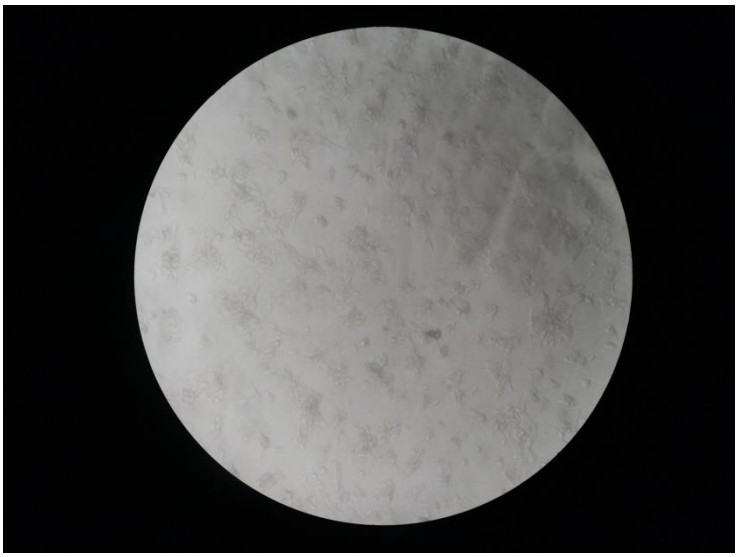


Plate 23: Monolayer of cancer cell lines under inverted microscope

3.6.4 Colorimetric MTT Assay

The alcoholic extract obtained from the basidiocarp of *Ganoderma applanatum* proved to have anticancer activity on HeLa cell lines (Table 15 and Figure 15), ZR-75-30 (Table 22 and Figure 22) and HepG2 (Table 29 and Figure 29). The viability of HeLa, ZR-75-30 and HepG2 cells was decreased to 50% by the mycelial methanolic extract of *Ganoderma applanatum* at a concentration of 9.8µg/ml (Table 16 and Figure 16), 11.9µg/ml (Table 23 and Figure 23) and 13.69µg/ml(Table 30 and Figure 30) respectively. In Table 17, 24, 31 and Figure 17, 24, 31 the anticancer activity by the basidiocarp methanolic extract of *Tricholoma giganteum* is shown for HeLa, ZR-75-30 and HepG2 respectively. The mycelia extract of *Tricholoma giganteum* shows a decreased cell viability for HeLa, ZR-75-30 and HepG2 with the increase in the concentration of the extract (Table 18, 25, 32 and Figure 18, 25, 32). The basidiocarp methanolic extract of *Bolbitus vitellinus* proved to have an effect on HeLa cells, ZR-75-30 and HepG2 at 9.4µg/ml, 11.5 µg/ml and 15.47 µg/ml concentration the cell viability reduced to 50% respectively (Table 19, 26, 33 and Figure 19, 26,33). At 18.5µg/ml, 13 µg/ml and 15.94 µg/ml the cell viability of HeLa, ZR-75-30 and HepG2 respectively reduced to 50% by the mycelia methanolic extract of *Bolbitus vitellinus* (Table 20, 27, 34 and Figure 20, 27, 34).

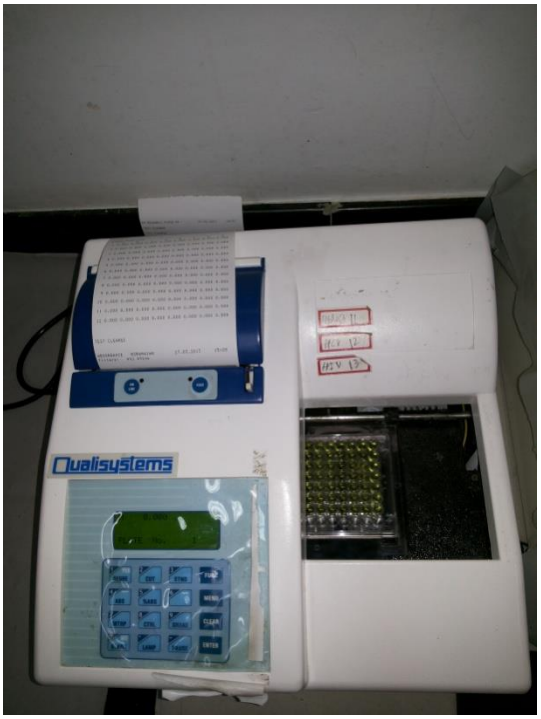


Plate 24: ELISA reader



Plate 25: MTT assay

3.6.4.1 Anticancer activity by the samples on human cervix cancer cell lines (HeLa)

concentration	Viability percentage of cancer cells
0	100
10 µg/ml	50.01
20 µg/ml	45.33
30 µg/ml	42.62
40 µg/ml	42.62
50 µg/ml	41.7
60 µg/ml	40.3
70 µg/ml	37.5
80 µg/ml	36.1
90 µg/ml	32.2
100 µg/ml	26.4

Table 15: Anticancer activity on HeLa by *Ganoderma applanatum* basidiocarp

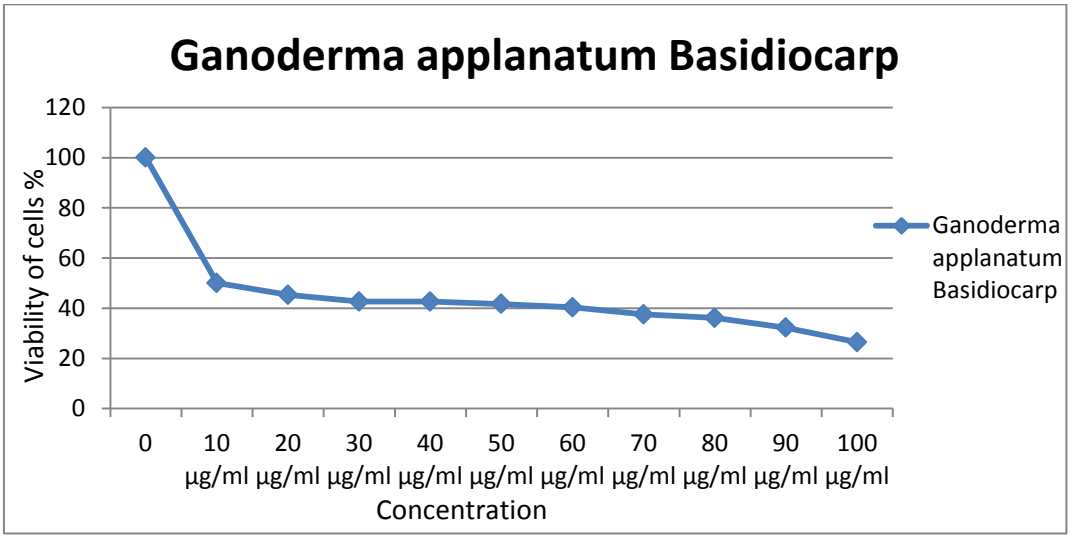


Figure 15: Anticancer activity on HeLa by *Ganoderma applanatum* basidiocarp

concentration	Viability percentage of cancer cells
0	100
10 µg/ml	49.64
20 µg/ml	48.71
30 µg/ml	47.7
40 µg/ml	46.51
50 µg/ml	42.42
60 µg/ml	40.02
70 µg/ml	35.54
80 µg/ml	33.4
90 µg/ml	29.1
100 µg/ml	28.2

Table 16: Anticancer activity on HeLa by *Ganoderma applanatum* Mycelium

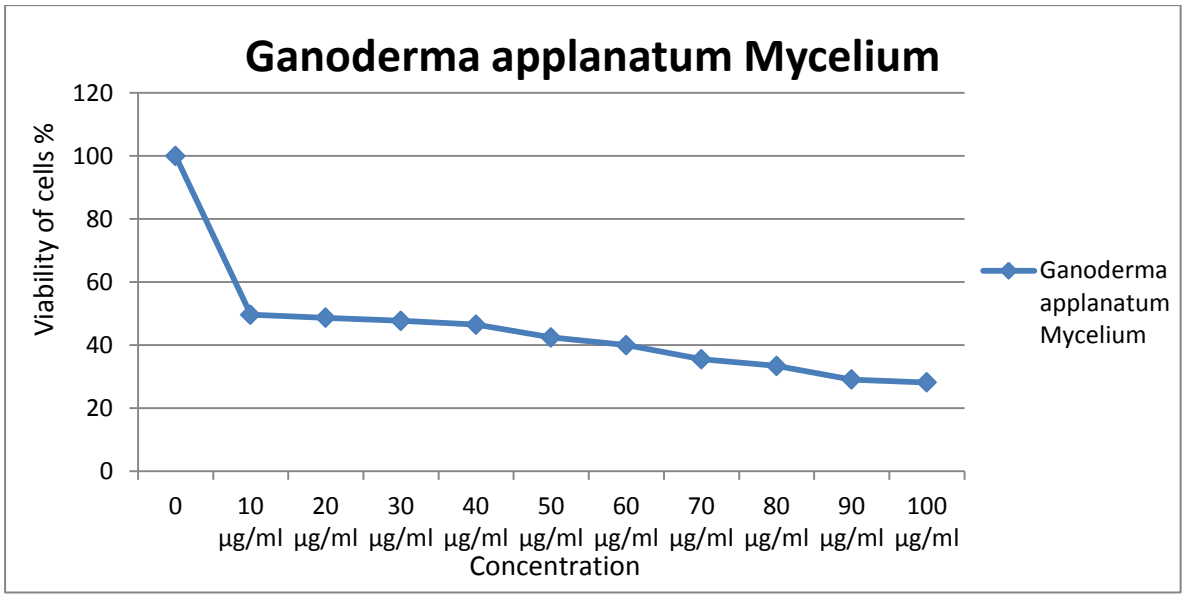


Figure 16: Anticancer activity on HeLa by *Ganoderma applanatum* mycelium

concentration	Viability percentage of cancer cells
0	100
10 µg/ml	49.78
20 µg/ml	49.1
30 µg/ml	48.21
40 µg/ml	46.32
50 µg/ml	43.5
60 µg/ml	40.4
70 µg/ml	39.3
80 µg/ml	38.4
90 µg/ml	38.2
100 µg/ml	35.5

Table 17: Anticancer activity on HeLa by *Tricholoma giganteum* basidiocarp

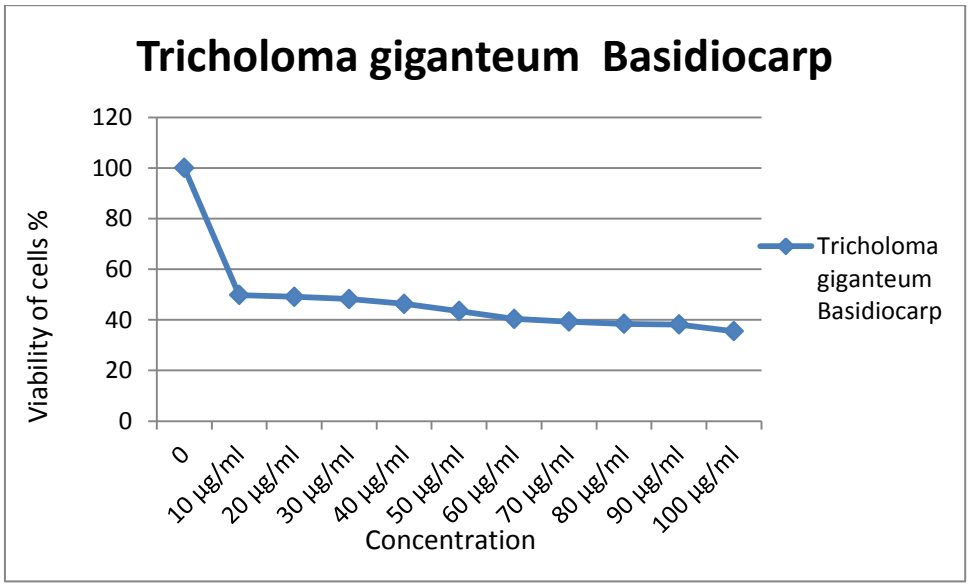


Figure 17: Anticancer activity on HeLa by *Tricholoma giganteum* basidiocarp

concentration	Viability percentage of cancer cells
0	100
10 µg/ml	50.34
20 µg/ml	49.61
30 µg/ml	47.32
40 µg/ml	46.4
50 µg/ml	44.5
60 µg/ml	42.3
70 µg/ml	40.21
80 µg/ml	40.21
90 µg/ml	39.1
100 µg/ml	38.11

Table 18: Anticancer activity on HeLa by *Tricholoma giganteum* Mycelium

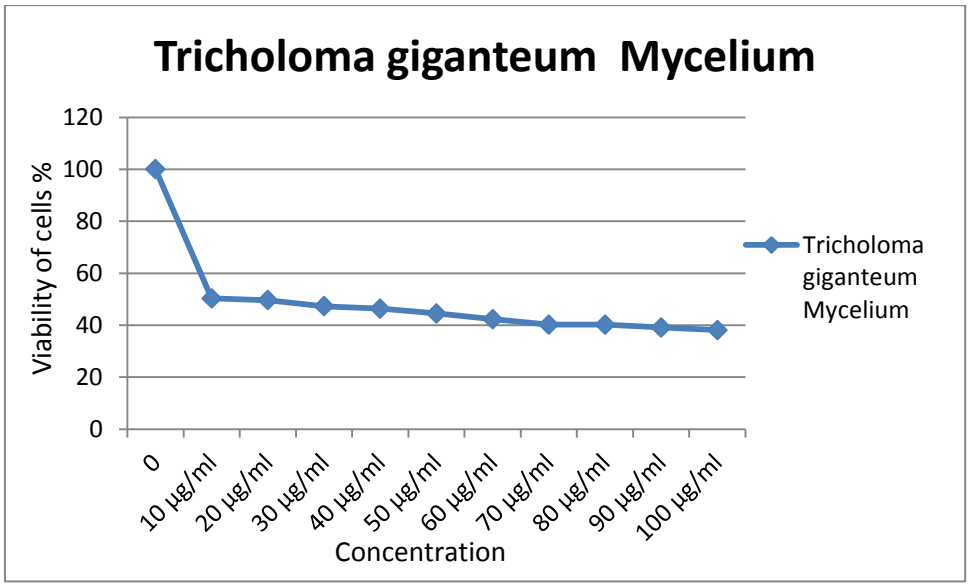


Figure 18: Anticancer activity on HeLa by *Tricholoma giganteum* Mycelium

Concentration	Viability percentage of cancer cells
0	100
10 µg/ml	49.05
20 µg/ml	48.01
30 µg/ml	48.79
40 µg/ml	45.21
50 µg/ml	42.32
60 µg/ml	39.1
70 µg/ml	38.11
80 µg/ml	35.62
90 µg/ml	32.72
100 µg/ml	28.9

Table 19: Anticancer activity on HeLa by *Bolbitius vitellinus* basidiocarp

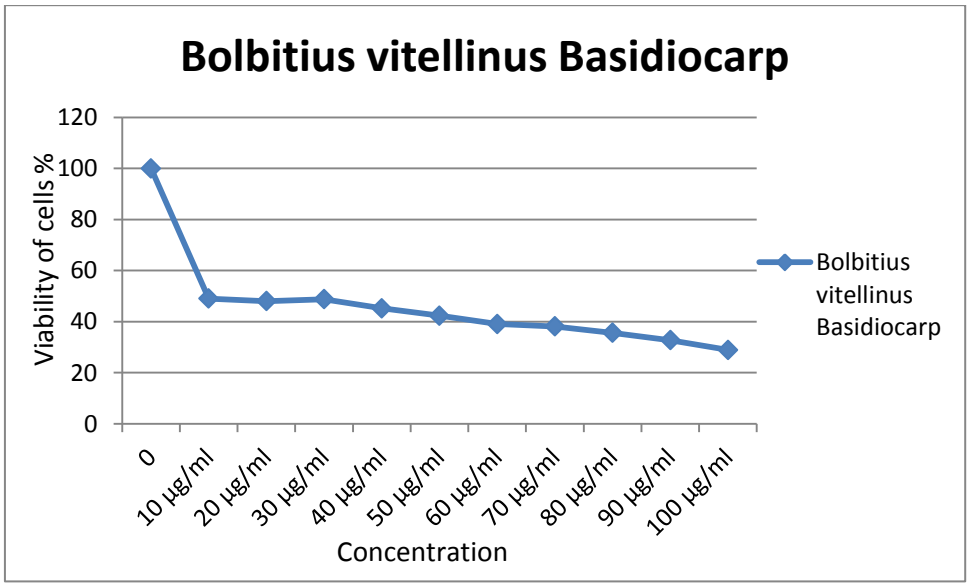


Figure 19: Anticancer activity on HeLa by *Bolbitius vitellinus* basidiocarp

concentration	Viability percentage of cancer cells
0	100
10 µg/ml	54.29
20 µg/ml	48.54
30 µg/ml	45.7
40 µg/ml	42.32
50 µg/ml	40.1
60 µg/ml	38.57
70 µg/ml	35.34
80 µg/ml	34.27
90 µg/ml	32.16
100 µg/ml	31.09

Table 20: Anticancer activity on HeLa by *Bolbitius vitellinus* mycelium

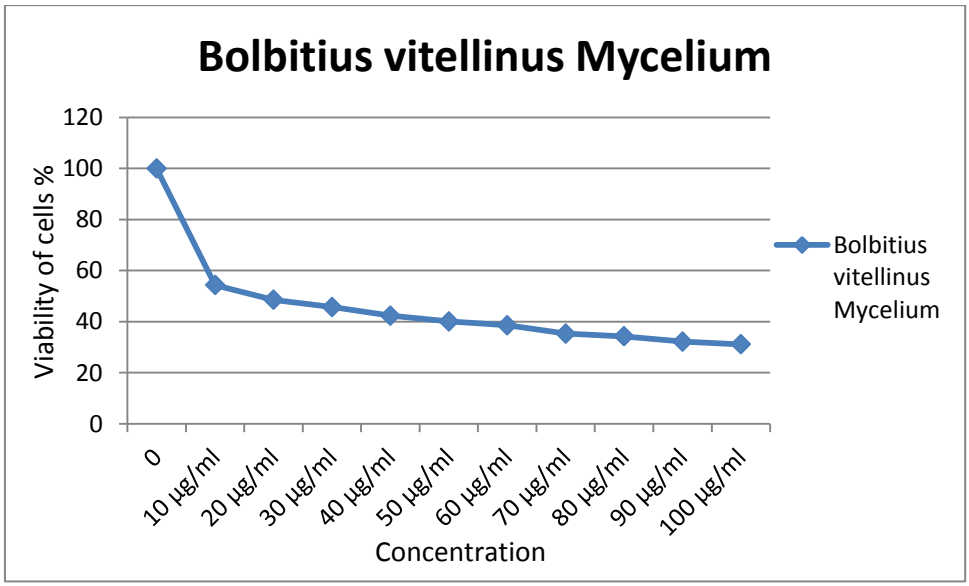


Figure 20: Anticancer activity on HeLa by *Bolbitius vitellinus* mycelium

IC_{50} of mushroom extracts on HeLa

Ganoderma applanatum basidiocarp	*Ganoderma applanatum* mycelium	*Tricholoma giganteum* basidiocarp	*Tricholoma giganteum* mycelium	*Bolbitius vitellinus* basidiocarp	*Bolbitius vitellinus* mycelium
10 µg/ml	9.85 µg/ml	9.8 µg/ml	11.2 µg/ml	9.4 µg/ml	18.5 µg/ml

Table 21: IC_{50} of mushroom extracts on HeLa

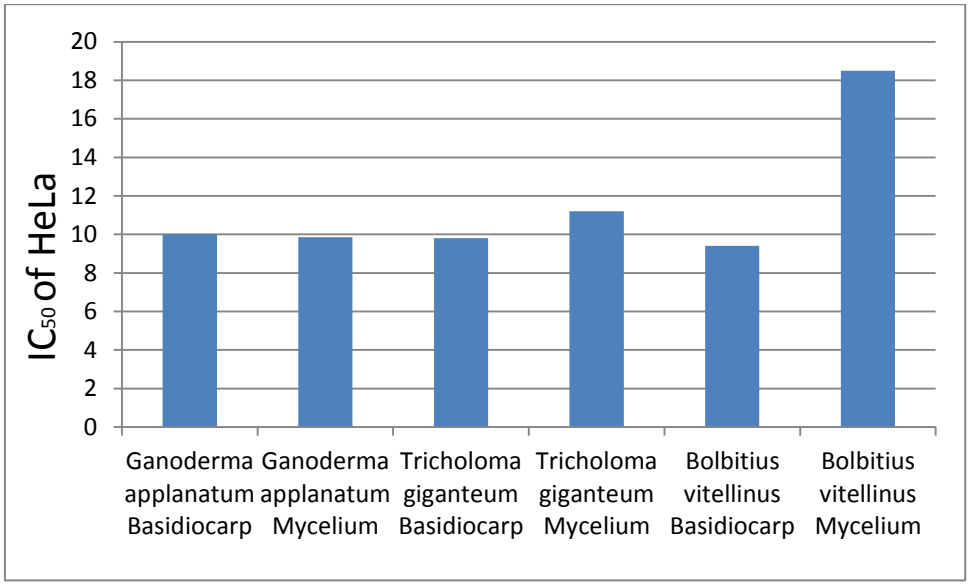


Figure 21: IC_{50} of mushroom extracts on HeLa

The six extracts of 3 wild mushrooms and their mycelium were tested for their anticancer activity on HELA cells. The results summarized clearly indicates that tested extracts exhibited a significant dose-dependent inhibitory effect on cancer cell lines examined. Results indicated that in HELA the methanolic extract of basidiocarp and mycelium of the wild mushrooms equally showed positive result, where in the viability of the cells was reduced to 50% with a concentration ranging from 9 to 19µg/ml. The basidiocarp of *Bolbitius vitellinus* showed the highest anticancer activity at 9.4µg/ml followed by basidicarp of *Tricholoma giganteum* at 9.8µg/ml. the

least anticancer activity was observed in the mycelium of *Bolbitius vitellinus* which showed a activity at 18.5 µg/ml against HeLa cell lines.

Three terpenoids, lucidenic acid O, lucidenic lactone, and cerevisterol in *G. lucidum* could selectively inhibit eukaryotic DNA polymerase activities. The bioactive components in the spores of *G. lucidum* might be inhibitors of the onset of DNA synthesis, and could also strongly reduce the level of intracellular calcium, which resulted in the inhibition of cell growth when the spores were used to treat human cervix uteri tumor HeLa cells (H.S.Zhu *et al.*, 2010).

3.6.4.2 Anticancer activity by the samples on ductal carcinoma cancer cell lines (ZR-75-30)

Concentration	Viability percentage of cancer cells
0	100
10 µg/ml	54.14
20 µg/ml	42.1
30 µg/ml	40.12
40 µg/ml	40.1
50 µg/ml	38.7
60 µg/ml	36.42
70 µg/ml	35.72
80 µg/ml	32.75
90 µg/ml	30.6
100 µg/ml	28.5

Table 22: Anticancer activity on ZR-75-30 by *Ganoderma applanatum* basidiocarp

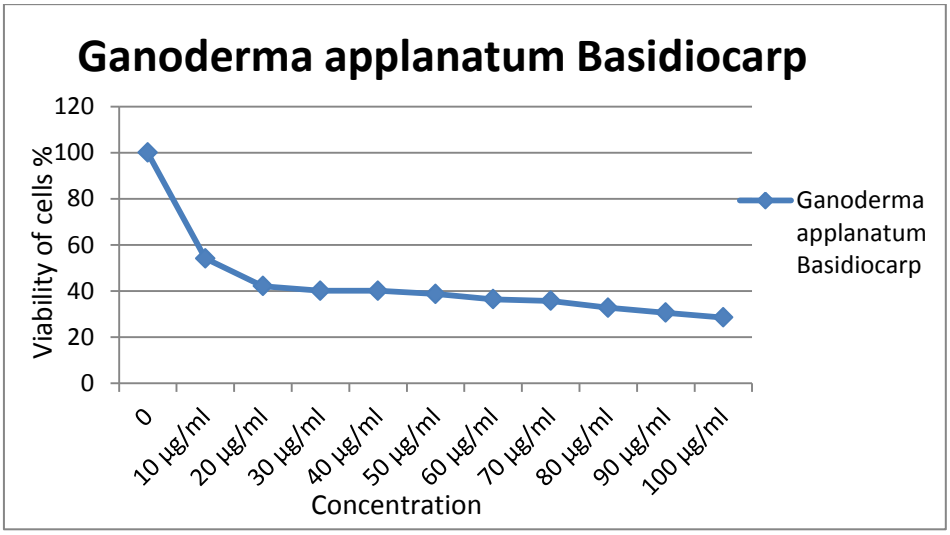


Figure 22: Anticancer activity on ZR-75-30 by *Ganoderma applanatum* basidiocarp

Concentration	Viability percentage of cancer cells
0	100
10 µg/ml	53.32
20 µg/ml	47.12
30 µg/ml	46.12
40 µg/ml	45.75
50 µg/ml	44.32
60 µg/ml	42.45
70 µg/ml	39.3
80 µg/ml	38.54
90 µg/ml	35.56
100 µg/ml	34.32

Table 23: Anticancer activity on ZR-75-30 by *Ganoderma applanatum* mycelium

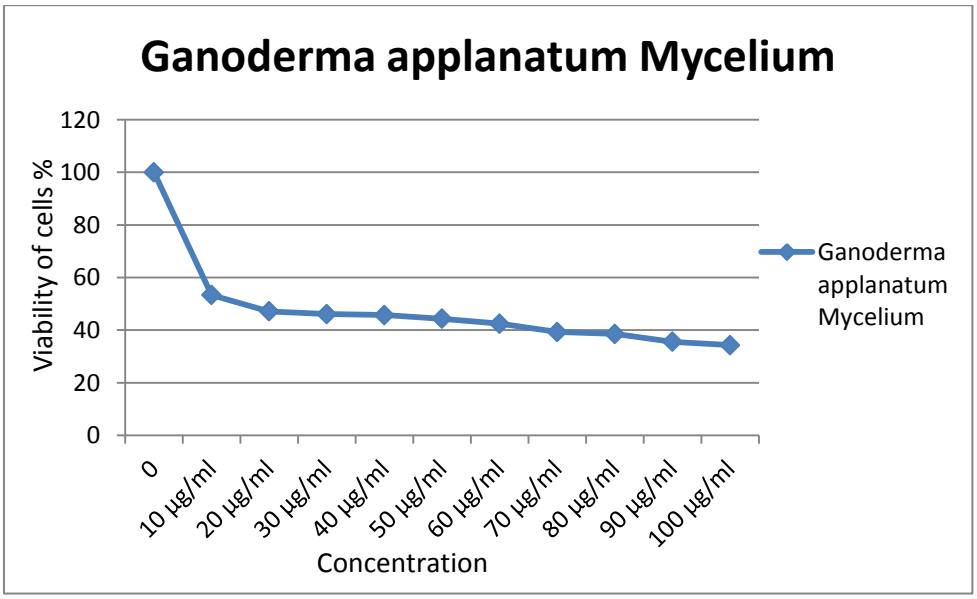


Figure 23: Anticancer activity on ZR-75-30 by *Ganoderma applanatum* mycelium

Concentration	Viability percentage of cancer cells
0	100
10 µg/ml	50.32
20 µg/ml	49.32
30 µg/ml	48.3
40 µg/ml	47.5
50 µg/ml	45.7
60 µg/ml	44.62
70 µg/ml	42.3
80 µg/ml	40.11
90 µg/ml	39.15
100 µg/ml	37.32

Table 24: Anticancer activity on ZR-75-30 by *Tricholoma giganteum* basidiocarp

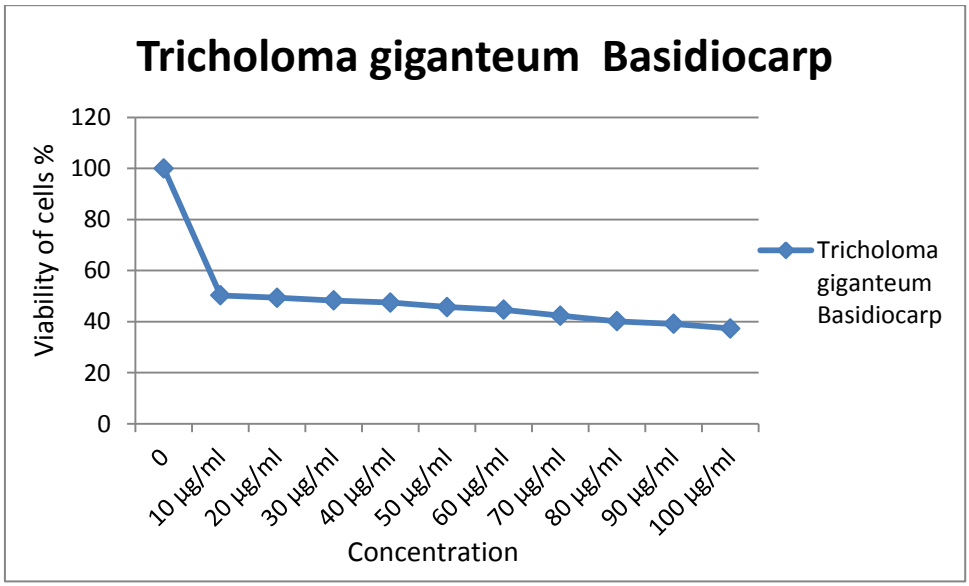


Figure 24: Anticancer activity on ZR-75-30 by *Tricholoma giganteum* basidiocarp

Concentration	Viability percentage of cancer cells
0	100
10 µg/ml	54.23
20 µg/ml	48.14
30 µg/ml	47.56
40 µg/ml	45.72
50 µg/ml	44.3
60 µg/ml	42.7
70 µg/ml	41.2
80 µg/ml	38.19
90 µg/ml	37.47
100 µg/ml	36.1

Table 25: Anticancer activity on ZR-75-30 by *Tricholoma giganteum* mycelium

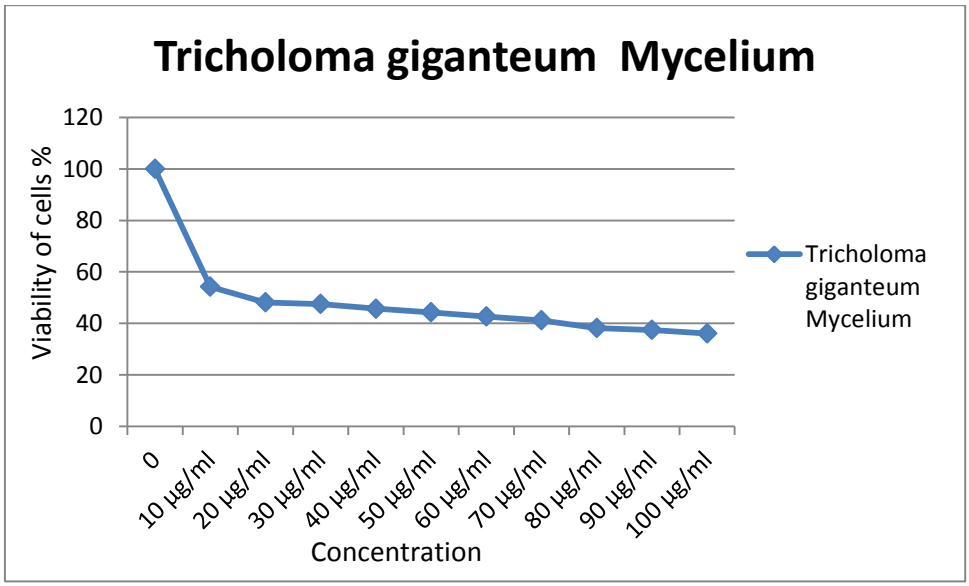


Figure 25: Anticancer activity on ZR-75-30 by *Tricholoma giganteum* mycelium

Concentration	Viability percentage of cancer cells
0	100
10 µg/ml	56.34
20 µg/ml	48.9
30 µg/ml	47.23
40 µg/ml	45.1
50 µg/ml	42.3
60 µg/ml	40.2
70 µg/ml	39.65
80 µg/ml	37.3
90 µg/ml	36.4
100 µg/ml	35.3

Table 26: Anticancer activity on ZR-75-30 by *Bolbitius vitellinus* basidiocarp

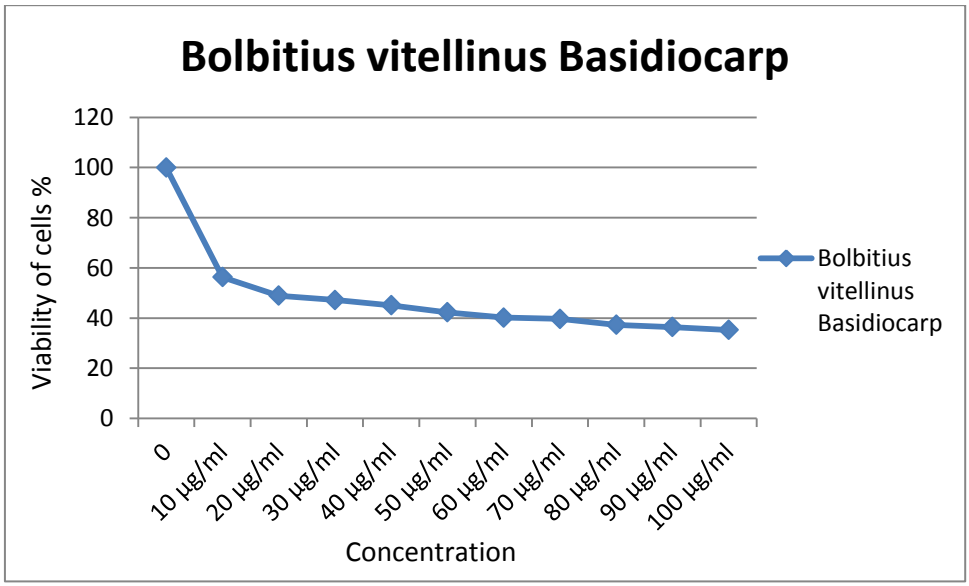


Figure 26: Anticancer activity on ZR-75-30 by *Bolbitius vitellinus* basidiocarp

Concentration	Viability percentage of cancer cells
0	100
10 µg/ml	54.78
20 µg/ml	47.14
30 µg/ml	46.13
40 µg/ml	44.21
50 µg/ml	42.23
60 µg/ml	40.16
70 µg/ml	38.12
80 µg/ml	36.33
90 µg/ml	36.23
100 µg/ml	35.4

Table 27: Anticancer activity on ZR-75-30 by *Bolbitius vitellinus* mycelium

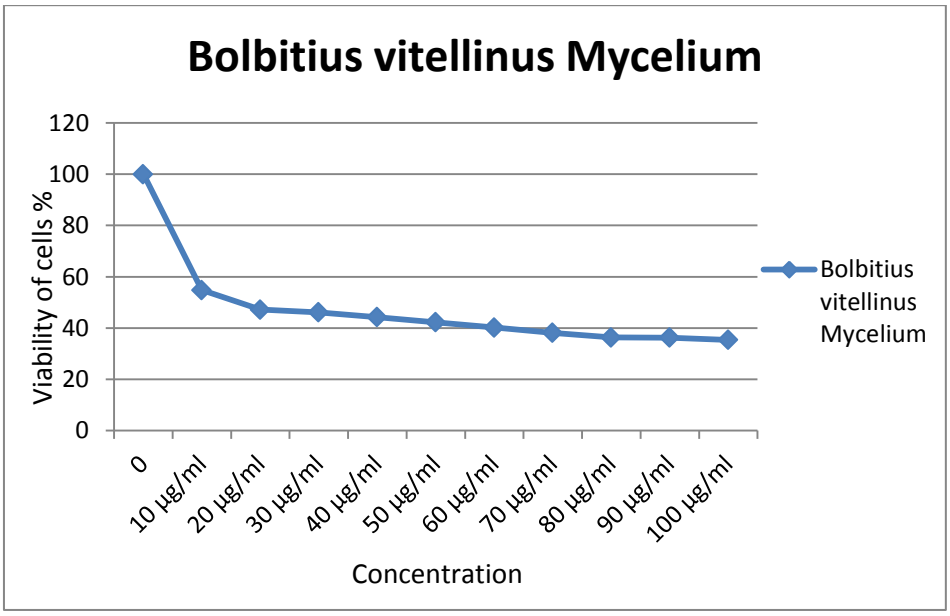


Figure 27: Anticancer activity on ZR-75-30 by *Bolbitius vitellinus* mycelium

IC_{50} of mushroom samples on ZR-75-30

Ganoderma applanatum basidiocarp	*Ganoderma applanatum* mycelium	Tricholoma giganteum basidiocarp	*Tricholoma giganteum* mycelium	*Bolbitius vitellinus* basidiocarp	*Bolbitius vitellinus* mycelium
14 µg/ml	11.9 µg/ml	11 µg/ml	12.2 µg/ml	11.5 µg/ml	13 µg/ml

Table 28: IC_{50} of mushroom samples on ZR-75-30

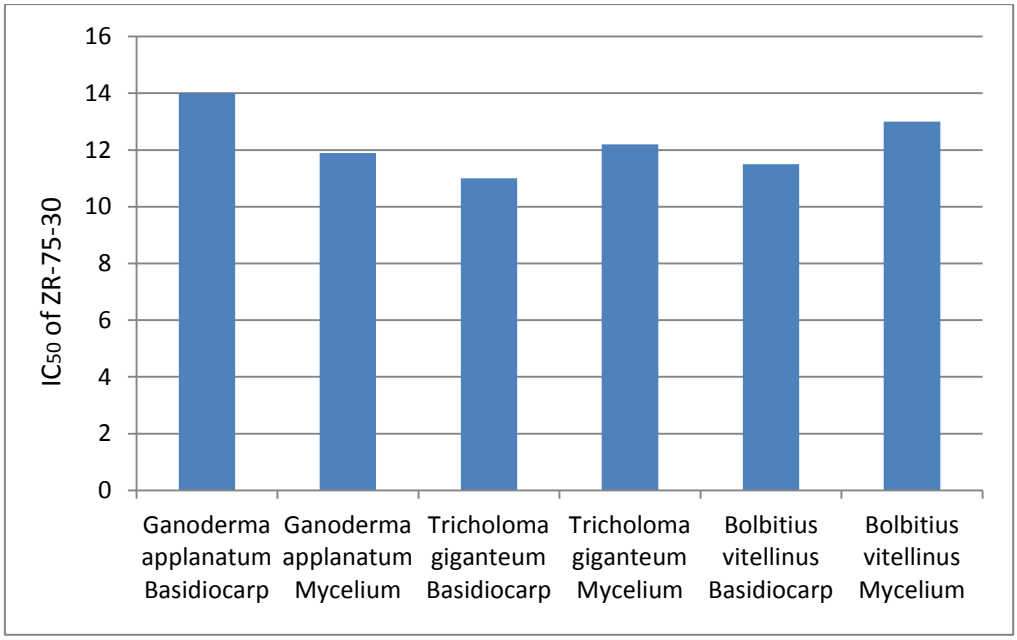


Figure 28: IC_{50} of mushrooms samples on ZR-75-30

The six extracts of 3 wild mushrooms and their mycelium were tested for their anticancer activity on ZH-75-30 cells. The results summarized clearly indicates that tested extracts exhibited a significant dose-dependent inhibitory effect on cancer cell lines examined. Results indicated that in ZH-75-30 the methanolic extract of basidiocarp and mycelium of the wild mushrooms equally showed positive result, where in the viability of the cells was reduced to 50% with a concentration ranging from 10 to 14µg/ml. The basidiocarp of *tricholoma giganteum* showed the highest anticancer activity at 11µg/ml followed by basidicarp of *bolbitius vitellinus* at

11.5µg/ml. the least anticancer activity was observed in the basidiocarp of *Ganoderma applanatum* which showed a activity at 14µg/ml against ZH-75-30 cell lines.

Daniel Sliva, *et al.*, (2002) have demonstrated that phosphatidylinositol 3-kinase (PI 3-kinase) and nuclear factor-j B (NF-jB) regulate motility of highly invasive human breast cancer cells by the secretion of urokinase-type plasminogen activator(uPA). The effect of *G. lucidum* on highly invasive breast and prostate cancer cells.

3.6.4.3 Anticancer activity by the samples on Human liver cancer cell lines (HepG2)

Concentration	Viability percentage of cancer cells
0	100
2 µg/ml	89.07
4 µg/ml	82.41
6 µg/ml	76.23
8 µg/ml	64.9
10 µg/ml	60.1
12 µg/ml	59.12
14 µg/ml	58.34
16 µg/ml	50.04
18 µg/ml	48.16
20 µg/ml	47.18

Table 29: Anticancer activity on HepG2 by *Ganoderma applanatum* basidiocarp

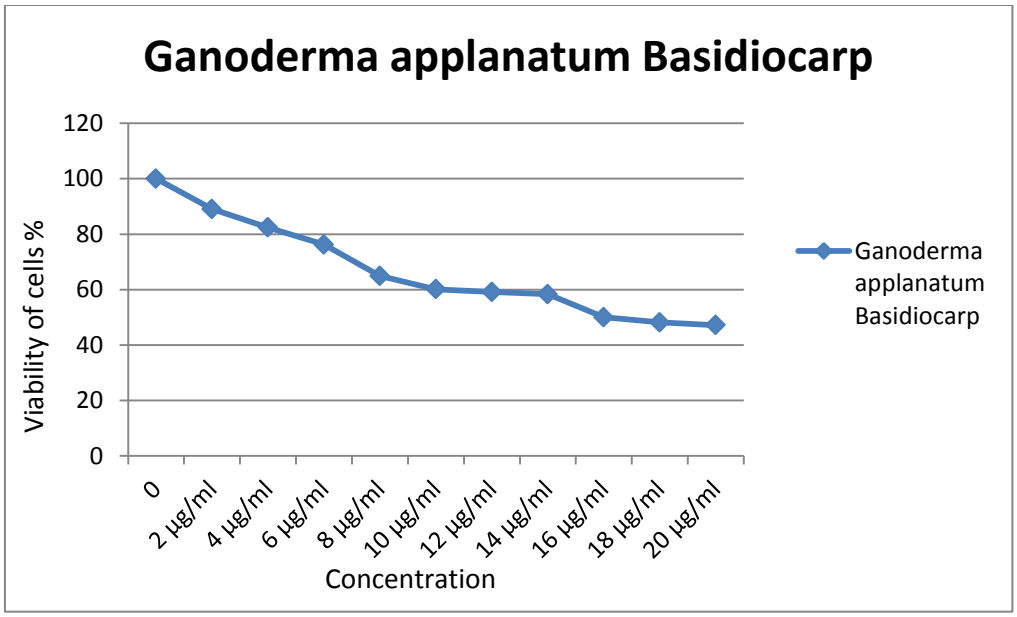


Figure 29: Anticancer activity on HepG2 by *Ganoderma applanatum* basidiocarp

Concentration	Viability percentage of cancer cells
0	100
2 µg/ml	92.04
4 µg/ml	84.03
6 µg/ml	80.01
8 µg/ml	74.09
10 µg/ml	68.04
12 µg/ml	59.12
14 µg/ml	50.49
16 µg/ml	48.96
18 µg/ml	45.32
20 µg/ml	44.17

Table 30: Anticancer activity on HepG2 by *Ganoderma applanatum* mycelium

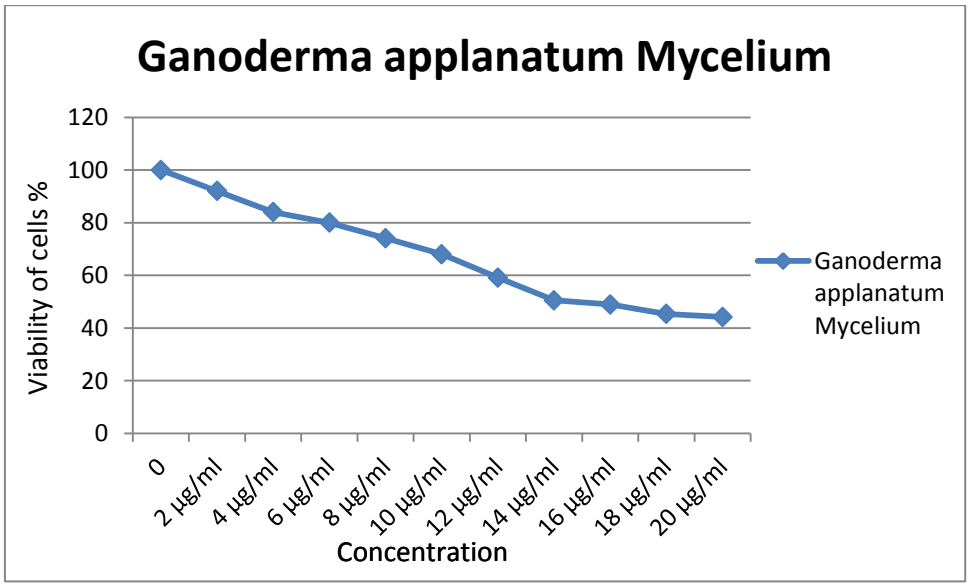


Figure 30: Anticancer activity on HepG2 by *Ganoderma applanatum* mycelium

Concentration	Viability percentage of cancer cells
0	100
2 µg/ml	87.4
4 µg/ml	74.32
6 µg/ml	66.13
8 µg/ml	60.21
10 µg/ml	54.14
12 µg/ml	49.46
14 µg/ml	48.14
16 µg/ml	47.12
18 µg/ml	44.23
20 µg/ml	41

Table 31: Anticancer activity on HepG2 by *Tricholoma giganteum* basidiocarp

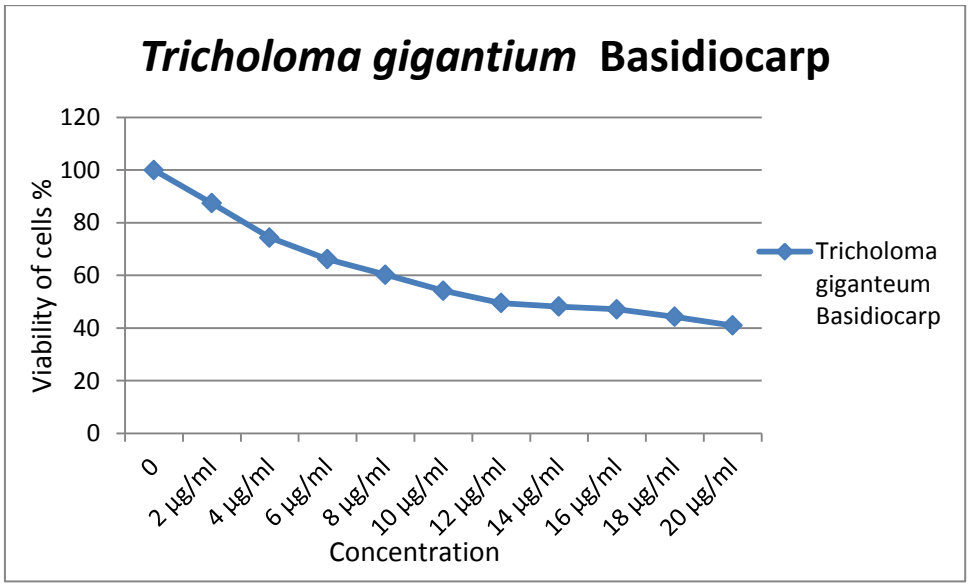


Figure : Anticancer activity on HepG2 by *Tricholoma giganteum* basidiocarp

Concentration	Viability percentage of cancer cells
0	100
2 µg/ml	94
4 µg/ml	87
6 µg/ml	72
8 µg/ml	63.2
10 µg/ml	54.8
12 µg/ml	49.1
14 µg/ml	48.2
16 µg/ml	47.31
18 µg/ml	46.02
20 µg/ml	45.15

Table 32: Anticancer activity on HepG2 by *Tricholoma giganteum* mycelium

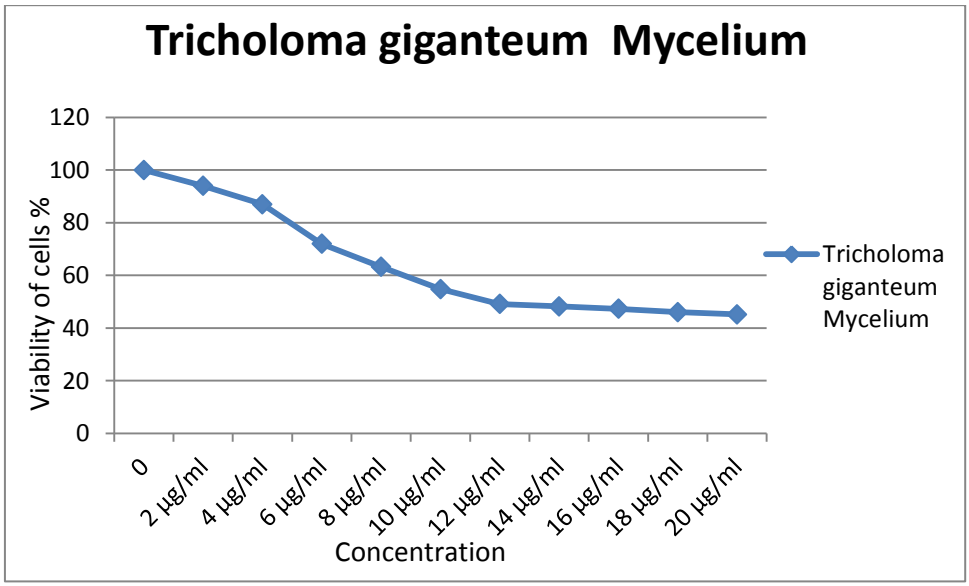


Figure 32: Anticancer activity on HepG2 by *Tricholoma giganteum* mycelium

Concentration	Viability percentage of cancer cells
0	100
2 µg/ml	87
4 µg/ml	80.21
6 µg/ml	74.22
8 µg/ml	68.17
10 µg/ml	62.21
12 µg/ml	60.01
14 µg/ml	54.67
16 µg/ml	49.12
18 µg/ml	49
20 µg/ml	48.09

Table 33: Anticancer activity on HepG2 by *Bolbitius vitellinus* basidiocarp

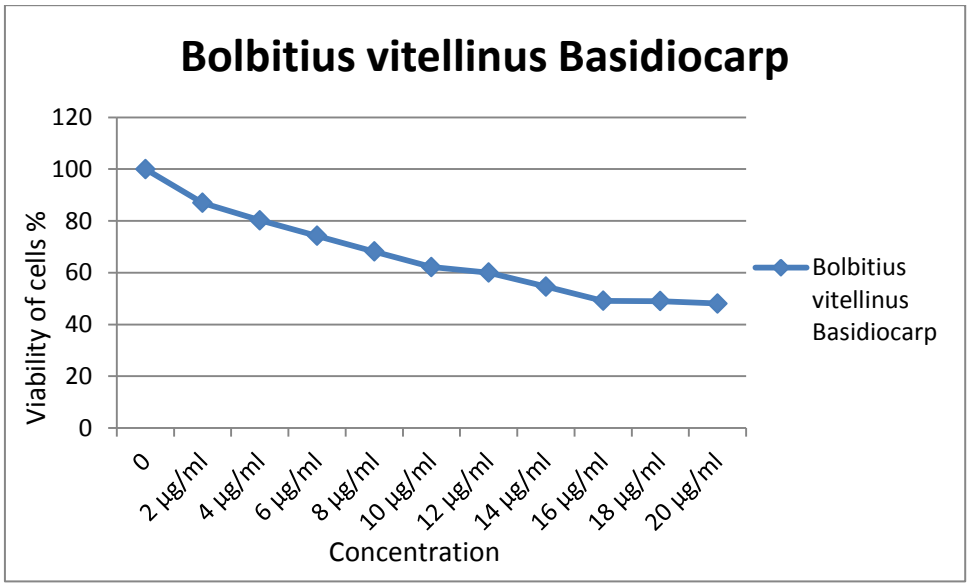


Figure 33: Anticancer activity on HepG2 by *Bolbitius vitellinus* basidiocarp

Concentration	Viability percentage of cancer cells
0	100
2 µg/ml	91
4 µg/ml	84.3
6 µg/ml	76.33
8 µg/ml	70.12
10 µg/ml	60.21
12 µg/ml	57.87
14 µg/ml	54.56
16 µg/ml	49.56
18 µg/ml	48.45
20 µg/ml	47.43

Table 34: Anticancer activity on HepG2 by *Bolbitius vitellinus* mycelium

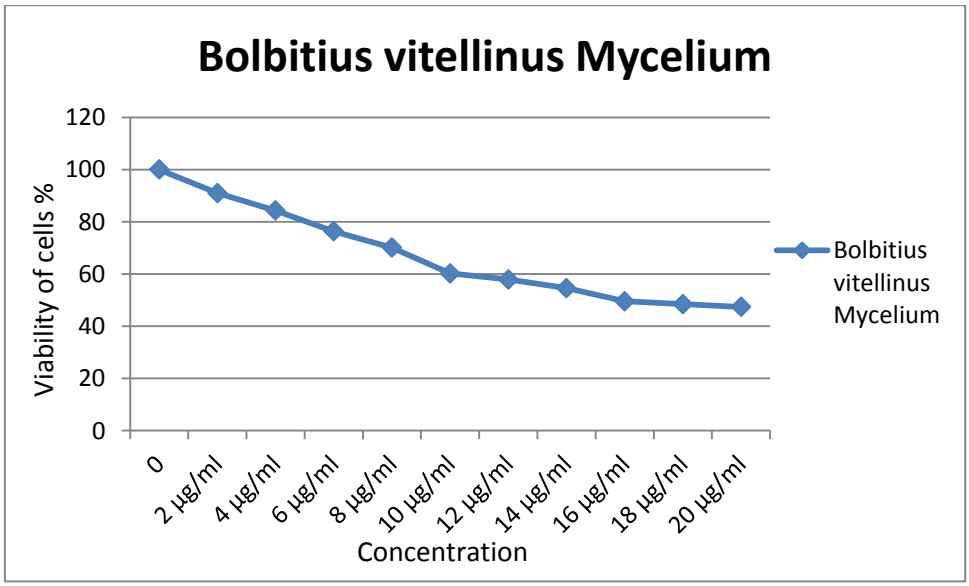


Figure 34:Anticancer activity on HepG2 by *Bolbitius vitellinus* mycelium

IC_{50} of mushroom samples on HepG2

Ganoderma applanatum basidiocarp	*Ganoderma applanatum* mycelium	*Tricholoma giganteum* basidiocarp	*Tricholoma giganteum* mycelium	*Bolbitius vitellinus* basidiocarp	*Bolbitius vitellinus* mycelium
16.02 µg/ml	13.69 µg/ml	11.47 µg/ml	10.64 µg/ml	15.47 µg/ml	15.94 µg/ml

Table 35: IC_{50} of mushroom samples on Hep G2

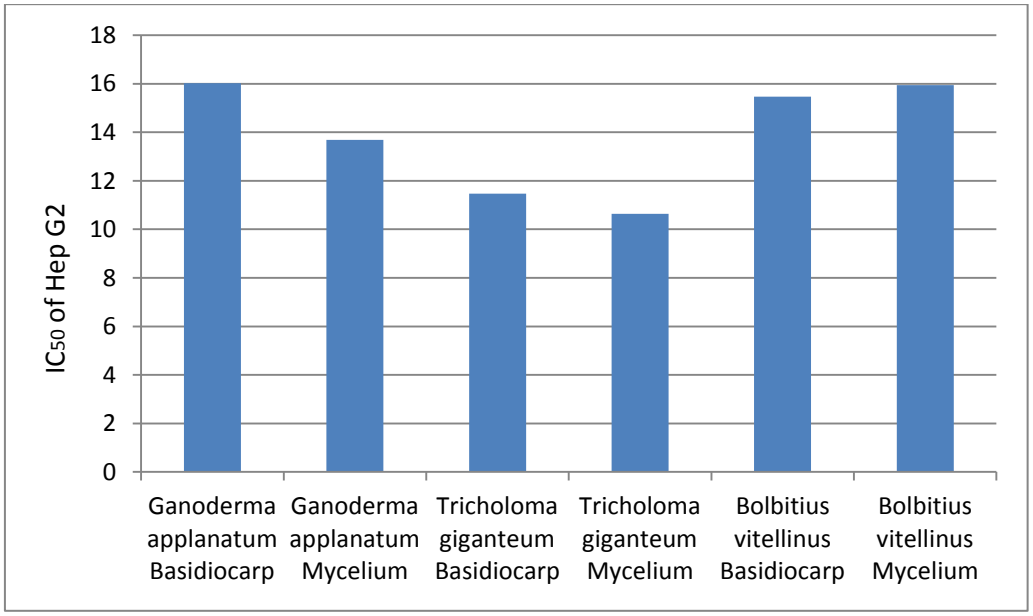


Figure 35: IC_{50} of mushroom samples on HepG2

The six extracts of 3 wild mushrooms and their mycelium were tested for their anticancer activity on HepG2 cells. The results summarized clearly indicates that tested extracts exhibited a significant dose-dependent inhibitory effect on cancer cell lines examined. Results indicated that in HepG2 the methanolic extract of basidiocarp and mycelium of the wild mushrooms equally showed positive result, where in the viability of the cells was reduced to 50% with a concentration ranging from 10 to 16µg/ml. The mycelium of *Tricholoma giganteum* showed the highest anticancer activity at 10.6µg/ml followed by basidicarp of *Tricholoma giganteum* at 11.4µg/ml.

the least anticancer activity was observed in the basidiocarp of *Ganoderma applanatum* which showed a activity at 16µg/ml against HepG2 cell lines.

It has been reported that the mycelial preparation of *Tricholoma matsutake* mushroom prepared in bulk culture was shown to have antitumor activity against the in vivo growth of mouse syngeneic fibrosarcoma (Ebina *et al.,* 2002) as well as preventive activity against the formation of azoxymethane-induced precancerous lesions in the colon (Matsunaga *et al.,* 2003).

The aqueous extracts of *Ganoderma* species have already been found to have potent antibacterial, antiviral, antiparasitic and anticancer properties readily make it a central tool for nanotechnology research in order to enhance its potency and activity along those lines.(Ofodile, 2005, Jonathan, 2010 and Liandris, 2011).

Qun-Hao Zhang(1999) investigated the antitumor activity of GL-B, a purified polysaccharide isolated from *Ganodenna lucidum*. The results showed that GL-B inhibited the growth of implanted Sarcoma 180 significantly and in a dose dependent, combination immunotherapy, GL-B with a chemotherapy such as cyclophosphamide achieved the best effect.

In the investigation carried out by Hongbo HU *et al.,* (2002) showed that the alcohol extract of *Ganoderma lucidum* inhibited the proliferation of MCF-7 cells in a dose- and time-dependent manner. After 48 hr of treatment, at 50µg/ml, this alcohol extract caused nearly a 70% inhibition of cell growth compared to the control. Sliva *et al.,* (2002) have recently investigated and showed that *Ganoderma lucidum* inhibits cell migration of highly invasive human prostate cancer cells PC-3.

CHAPTER 4

CONCLUSION

Wild mushrooms play an important and beneficial role in the environment. They grow in a wide variety of habitats. Most of the mushrooms seen on a walk through a woods are beneficial. Many species are quite specific about their food source. Some are important as decay organisms, aiding in the breakdown of logs, leaves, stems and other organic debris. This important role of mushrooms results in recycling of essential nutrients.

Some mushrooms grow in, and form their fruiting structures on living trees causing decay of the sapwood or of the heartwood. Many woodland mushrooms are essential to good growth, and even survival of trees. They establish a relationship with roots of living trees that is mutually beneficial. These are called mycorrhizal mushrooms and few mushrooms have a beneficial role in curing many diseases like those diseases which are caused by many prokaryotes and also used as a anticancer agents to use against cancer.

Diseases such as Oxidative Stress and Neurodegenerative Diseases (Eales' disease: accumulation of reactive oxygen intermediates and lipid peroxides and decrease of antioxidants causing inflammation, neovascularization and retinal damage) is caused due to accumulation of free oxygen radicals in the body. Many mushrooms are capable of producing antioxidans and help in removal of such highly reactive free radicals.

Our study is about three wild mushrooms *Ganoderma applanatum*, *Tricholoma giganteum*, and *Bolbitus vitellinus* which showed very good results for all three activities i.e., Antimicrobial, Antioxidant and Anticancer activities.

Ganoderma is a genus of polypore mushrooms which grow on wood, and include about 80 species, many from tropical regions, Because of their extensive use in traditional Asian medicines, and their potential in bioremediation, they are a very important genus economically.

Tricholoma is a genus of fungus that contains a large number of fairly fleshy white-spored gilled mushrooms which are found worldwide generally growing in woodlands.

Bolbitius is a genus of small mushrooms in the Bolbitiaceae family.The methanolic extract of basidiocarps and mycelial mat of all the above mentioned mushrooms was analysed. The methanolic extracts were tested against three Gram positive, six Gram negative bacteria and five fungal cultures. Out of three mushrooms *Ganoderma applanatum* was found to be more active against bacterial cultures, followed by *Tricholoma giganteum* and *Bolbitius vitellinus*. *G.applanatum* and *T.giganteum* was found to be more sensitive to Gram Positive bacteria than Gram negative bacteria, how ever *B.vitellinus* was found to very active against Gram negative bacteria especially *E.coli.*

Antioxidant activity of methanolic extract of basidiocarp and mycelium was characterised using 1,1,-diphenyl-2- picrylhydrazyl (DPPH) radical scavenging assay and reducing power assay. The result of present study indicates that all the 3 mushrooms showed activity. The IC_{50} of basidiocarp and mycelial mat of *G.applantum* was found to be 650µg/ml and 2000µg/ml respectively. Similarly

extract of *T.giganteum* showed IC_{50} of 700µg/ml and 2000µg/ml. The IC_{50} of *B.vitellinus* was 850µg/ml and 2100µg/ml.

Anticancer activity of methanolic extracts of mushrooms was determined through MTT (Tetrazolium) assay against Cervix, Liver and Ductal carcinoma cancer cell lines,all the extract showed decrease in viability of the cells to 50% with a concentration below 20µg/ml.

This study reveals that not only basidiocarps of mushroom but also mycelial mat of the pure culture of *G.applantum, T.giganteum* and *B.vitellinus* has potential antimicrobial, anticancer and antioxidant activity, hence these mushrooms can be exploited for their useful metabolites in food and Pharmaceutical industries. Further studies on isolation and identification of the active compounds may provide a better source for developing new therapeutic agents.

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