5. DISCUSSION

The practice of medicine—both in the past and present, often involves the prescription of specific foods or their potent derivatives, to treat a wide spectrum of illnesses (Rigas et al., 2008). Asians have a long history of using mushrooms as medicinal purposes, in which some of them have been reported for their usefulness in pharmaceuticals. Besides, traditional Asian diet contains less animal fats and higher protein contents and they are achieved this by consuming protein rich foods such as mushrooms. The tropical climate in Asian countries allows the growth of wider species of mushrooms and consequently the results leads to higher consumption of mushrooms (Runnie et al., 2004). Mushrooms have been regarded as gourmet cuisine across the globe since antiquity for their unique taste and delicate flavour. Recently, it has been discovered that many mushroom species are used in miniature pharmaceutical factories, producing hundreds of novel constituents with miraculous biological properties.

Currently a large and ever-expanding global population that prefers the use of natural products in treating and preventing medical complications. The worldwide upsurge in the use of traditional medicine preparations and active ingredients isolated from medicinal mushrooms have provided the pharmaceutical industry with one of its most important sources of bioactive compounds, up to 40% of modern drugs are derived from natural sources using either the natural substance or a synthesized version. Furthermore, over a 100 new products derived from fungi are in clinical development, particularly as anti-cancer and anti-infective agents (Gautam et al., 2007; Harvey, 2008).

From time immemorial, mushrooms have been valued by humankind as a culinary wonder and folk medicine in oriental practice. The last decade has witnessed the overwhelming interest of western research fraternity in pharmaceutical potential of mushrooms. The chief medicinal uses of mushrooms discovered so far are as antioxidant, antidiabetic, hypcholesterolemic, anticancer, immunomodulatory, antiallergic, nephroprotective and anti-microbial agents (Sushila et al., 2012).
In contrast to the biological potential of reactive oxygen species formation, the cells have developed a complex defense system, which acts through the enzymatic activities and protection by the low molecular weight antioxidants. Another form of protection is the use of synthetic and natural compounds that show antioxidant effect on the cell. Antioxidants may act as reducing agents (free radical terminators), metal chelating and singlet oxygen quenchers (Tan et al., 2012).

Many mushrooms contain antioxidant compounds and they protect cells against the damaging effects of reactive oxygen species such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite (Ajith and Janardhanan, 2007). Endogenous antioxidants in mushrooms may play an important role in the antioxidative defense against oxidative damage, possibly protecting the biological functions of cells (Jayakumar et al., 2011).

Many medicinal mushrooms are used to treat tumours in the Indian traditional system of medicine (Patel and Goyal, 2012). Hence, enormous scope exists for identifying potent anticancer mushrooms. Many mushrooms belonging to the family, Pleurotaceae are known to exhibit potent antioxidant and anticancer properties.

One such medicinal mushroom was P. florida, which was commonly cultivated in Tamil Nadu, India. It was also called as ‘Dhingri’ (Oyster mushroom). Some of the research reports presents the antimicrobial and antioxidant potential of the crude extract of P. florida mushroom (Rana et al., 2012; Thillaimaharani et al., 2013). However, there is a lack of scientific report regarding the efficacy of the isolated compounds from P. florida against oxidative damage and anticancer activities and also the adsorption of environmental contaminants using spent mushroom substrate of P. florida. With this backdrop, the present study was undertaken to analyze the antioxidant and anticancer ability of the compound and the Fe2+ adsorption by spent mushroom substrate of P. florida. The observations made and the results obtained are discussed in this chapter with reference to the relevant published literature.

5.1. Cultivation, phytochemical and biochemical properties of P. florida

In the initial aspect of the study, P. florida mushroom was cultivated on paddy straw substrate using groundnut husk, maize powder, horse gram powder and coconut oilcake
powder as nutrient supplements from September to November 2010. Among the nutrient supplements, horse gram showed as the best nutrient supplement for cultivation of P. florida mushroom. Hence, the P. florida mushroom was cultivated in huge amount using horse gram as nutrient supplement for further study. Phytochemical and biochemical analysis were determined for methanol, ethanol, aqueous, ethyl acetate and hexane extracts of P. florida, in which methanol was found to be the excellent solvent for extracting the bioactive compounds from P. florida mushroom. Substrate supplementation with organic and inorganic substances often boosts mushroom production and this study aggresses with the previous literatures available (Adejoye and Mesewonrun, 2008; Renganathan et al., 2008).

In Pleurotus spp. the primordial initiation was generally observed on the 24 to 30 days (Markson et al., 2012). Raganathan et al. (1996) reported the day of primordial initiation was from 22 to 27 days. According to Dlamini et al. (2013), in Swaziland the number of days taken by the Pleurotus spp. to colonize the substrates like sugarcane tops, maize stover, maize stover, maize cobs and banana leaves were ranging from 58 to 83 days. In the present study, ramification was observed on the 19th day in horse gram supplemented P. florida mushroom. It was due to the induction of cellulose, which acts as one of the critical factor responsible for increasing yield (Renganathan et al., 2008). Amongst the different substrates like rice hulls, fresh coco coir and banana leaves, rice hull substrate was found as the best for oyster mushroom cultivation (Lasalita, 2010).

Elisashvili et al. (2008) reported that during substrate colonization in P. ostreatus mushroom, the uniformity in the initiation of mycelial growth in all the substrates may be due to low hydrolase activity. The implication is that the level of breakdown of substrate materials for the release of low nutrients; hence, nutrients in the additives may not have been released and consequently not available to the mycelia and hence no influence on the initial growth of the mycelium.

The variation in the spawn run rate between the treated substrate samples may have resulted from the differences in the chemical composition of both the substrates and additives and the rate at which these materials were hydrolyzed. Variations in the speed of colonization
(spawn run) by mycelia of Pleurotus spp. cultivated on different substrates supplemented with various additives have been reported (Pathmashini et al., 2008; Renganathan et al., 2008; Stanley and Awi-Waadu, 2010). Suitable C: N ratio might be responsible for the higher mycelial growth (Renganathan et al., 2008). The white rot fungus produces a set of lignocellulolytic enzymes, which allows it to grow on lignin rich lignocellulosic substrates. Several lignocellulolytic enzymes are released which play a major role in the biodegradation process. Production of these enzymes is mostly influenced by various organic and inorganic additives (Ramkumar et al., 2011).

According to supplementation, horse gram had a greater potential to improve the accumulation of protein in P. florida. Chopped straw yield better results, because it ruptured the cell walls of the straw to a greater level and potentially making the nutrients in the straw more accessible for mushroom growth. The addition of protein-rich supplements is common practice for nitrogen-deficient composites in the cultivation of mushrooms. Various researchers have used supplements from animal and plant origins, including protein, carbohydrate or oil-rich substances, Agaricus bisporus and Pleurotus spp. (Gurjar and Doshi, 1995; Raymond et al., 2013).

Earlier studies showed that horse gram is a good source of protein (17.9 - 25.3%), carbohydrates (51.9 - 60.9%), essential amino acids, energy and a low content of lipid (0.58 - 2.06%) and is an excellent source of iron and molybdenum (Bravo et al., 1999). The P. florida resulted in 23% higher mushroom yield than the control.

Khan and Tania (2012) reported that Pleurotus spp. generally contain 85 - 94% moisture content. Ahmed et al. (2013) obtained 86.20 to 90% for oyster mushroom species. Alam et al. (2007) found similar data (87 - 87.5%) while evaluating P. florida and P. sajor-caju. In this study, freshly cultivated P. florida mushroom contain high moisture content about 87.3% which was a good agreement with the above reports. Moisture percentage in mushroom depends on the species, maturity of fruiting bodies and storage conditions during processing or packaging (Guillamon et al., 2010).
Variation in water contents among the mushroom samples could be caused by the nature of the mushrooms and the different environmental growth factors such as temperature and relative humidity of the metabolic water (Mattila et al., 2002). The elimination of water content of the sample to dry state will increase the concentration of nutrient relatively. Thus, drying mushrooms is one method that would extend the shelf life of mushrooms by reducing unnecessary biochemical reaction such as enzymatic browning and lipid oxidation that may lead to quality deterioration.

Pleurotus spp. is considered as a good source of superior quality protein, with well distributed essential amino acids as compared to legumes (Patil et al., 2010). Six different Pleurotus spp. was investigated for their protein content and carbohydrate which ranging from 11 - 42%, 36 - 60% (Khan and Tania, 2012) respectively whereas; in the present study it was 50.7 and 26.6%. The protein contents were reported to vary according to genetic structure of species, physical and chemical differences in growing medium, composition of the substrate and harvest time (Akyuz and Kirbag, 2010). Mushroom crude protein rank below animal meats but well above most other foods including milk. On a dry weight basis, mushrooms normally contain 19 to 35% protein as compared to 7.3% in rice, 12.7% in wheat, 38.1% in soybean and 9.4% in corn (Wani et al., 2010). The variations of the protein content among edible mushrooms are affected by a number of factors, namely the type of mushrooms, the stage of development, level of nitrogen available and the location (Okoro and Achuba, 2012).

The preliminary phytochemical tests indicated the presence of flavonoids, phenolics, tannins, saponins and terpenoids in five different P. florida mushroom extracts. Similar results have been observed in petroleum ether and acetone extracts of P. ostreatus (Iwalokun et al., 2007). Several such compounds are known to possess strong antitumor properties (Kintzios, 2006). They are also known for their antioxidant and hepatoprotective properties (Scartezzini and Speroni, 2000).

Phenolic acids were reported to be the main phenolic compounds in mushrooms (Ferreira et al., 2009). According to Puttaraju et al. (2006) gallic acid, tannic acid,
protocatechuic acid and gentisic acids were some of the major phenolics detected in water extracts of several indigenous edible mushrooms from India. Besides, several authors have reported the correlation between the polarity of extraction solvent and phenolic content of resulting extracts (Putteraju et al., 2006; Ferreira et al., 2009).

The total phenolic content in methanol extract of P. florida was higher than that of ethanol, aqueous, hexane and ethyl acetate extract. The total phenolic content per 1 g of dry extract was higher than that reported for garlic extract (0.98 mg GAE/g of extract) (Bozin et al., 2008). For instance, the phenolic content of P. florida mushroom extracts was determined from 10 ± 2.25 to 62.82 ± 1.5 mg catechol equivalent/g of extract, which was supported (6.19 to 63.51 mg GAE/g extract with Ganoderma lucidum) by the findings of Abdullah et al. (2012). Whereas it is differed from that obtained by Wong et al. (2009) who have reported that it was higher than that of Hericium erinaceus methanolic extract of fresh (0.26 mg GAE/g extract), oven-dried (2.37 mg GAE/g extract) and freeze-dried fruiting bodies (0.78 mg GAE/g extract).

Yim et al. (2009) have studied the phytochemical analysis like total phenols, total flavonoids and condensed tannins in crude extracts of Lentinus ciliates, Schizophyllum commune, Hygrocybe conica and P. ostreatus. In all mushroom species the aqueous extract showed the higher results. Total phenols and condensed tannins are present in high amount whereas the total flavonoids contents (5.35 - 184.80 mg catechol equivalent/ 100 g) were present in low amount in all the extracts.

In the present study, the flavonoid content was present in range between 7 - 17.7 mg catechol equivalent/100 g. Flavonoids are important for human health because of their high pharmacological activities as radical scavengers. Flavonoids are a group of more than 4000 polyphenolic compounds that occur naturally in foods of plant origin. Flavonoids are a large class of phytochemicals which are present in human diets, which possesses a number of beneficial effects on human health, such as antioxidant, anti-inflammatory, anti-allergic, antiviral and anti-carcinogenic activities (Yao et al., 2004).
Of the five solvent tested, methanol was determined to be the best solvent for extraction, isolation of bioactive secondary metabolites from dried P. florida mushroom followed by ethanol and aqueous extract. However, hexane and ethyl acetate extract of P. florida showed less activity and contain low phenolic compound content. This may be due to the high lipophilic nature of bioactive metabolites. These results indicates that the extraction method had definite effects on the isolation of bioactive principles.

5.2. Antibacterial and antioxidant property of P. florida mushroom extracts

In this aspect, the antibacterial activity of the five different extracts of P. florida mushroom was assessed against Gram positive and negative bacterium. Further the methanolic extract was subjected to six different radical scavenging assays. The methanolic extract showed a significant result in both the antibacterial and antioxidant activities.

In vitro systems are easier, faster and more cost-effective compared to traditional bioassays in vivo. The testing of the antioxidant activity of the compounds in vitro is useful, because if a substrate is poorly effective in vitro, will not be better under in vivo conditions (Aruoma, 2003).

5.2.1. Antibacterial activity

The methanol and ethanol extract of P. florida exhibited considerable antibacterial activity, while the aqueous, ethyl acetate and hexane extract showed the least activity. Jonathan (2007) investigated that methanol extract of P. florida showed activity in E. coli, Klebsiella sp. and no activity against Bacillus sp., Pseudomonas and Proteus sp. In the current study revealed that zone formation in Pseudomonas sp., Salmonella sp. and Klebsiella pneumonia whereas mycelial ethanol extract showed zone formation in Staphylococcus aureus, Streptococcus mutans, Escherichia coli, Micrococcus luteus, Bacillus subtilis and no zone formation against Pseudomonas aeruginosa, Salmonella abony, Klebsiella pneumoniae, Proteus vulgaris and Candida albicans (Gordana et al., 2007).

The methanol and ethanol extracts of the P. florida mushroom inhibited the growth of majority of the bacterium. This result was in agreement with Nwachukwu and Uzoeto (2010) who reported that ethanol extract of P. squarrosolus was better than water extract. Similar
antimicrobial activities were also reported by Iwalokun et al. (2007). This possibly indicated that the extracts possessed substances that can inhibit the growth of some microorganisms (Chika et al., 2007). However, the observed inhibitory activities were more with the methanolic extracts of the mushrooms species. Extracts of P. florida inhibited both Gram positive and negative bacteria suggesting broad-spectrum antimicrobial potentials. However, the inability of the extracts to inhibit the growth of Proteus sp. could be that the organisms possess a mechanism for detoxifying the active components (Chika et al., 2007). But in this study, only methanolic extract of P. florida showed minimal zone of inhibition. The observed antimicrobial properties could be due to the presence of phenolic compounds, tannins, alkaloids and flavonoids which have been shown to possess antimicrobial properties (Draughon, 2004).

The variations in the antimicrobial activities of mushrooms may be due to the differences in their bioactive compositions or concentrations, methods of extraction and mechanism of action of active ingredients in these edible mushrooms (Iwalokun et al., 2007). Based on the results of this study, it can be concluded that the edible mushrooms possessed a broad-spectrum of antimicrobial activities especially in methanolic extract of P. florida.

These results confirmed that bioactive components of mushroom may differ in their solubility depending on the extractive solvents. Antimicrobial activity in natural source of extracts depends not only on the presence of phenolic compounds but also on the presence of various secondary metabolites (Gordana et al., 2007).

The World Health Organization (WHO) estimates globally that about 1500 people die each hour from infectious diseases; half of these are children less than five years of age (Meyler, 2002). The economic worldwide crisis, high cost of industrialized medicines, inefficient public access to medical and pharmaceutical care and side effects caused by synthetic drugs are some of the factors contributing to the central role that medicinal plants have in health care (Johann et al., 2007).

Siddhuraju and Manian (2007) reported that horse gram contained relatively high levels of total phenolics and tannins and it showed higher superoxide anion radical
scavenging and higher radical scavenging activity. These tannins and phenolic compounds are responsible for antibacterial activities. Phytoconstituents such as saponins, phenolic compounds and glycosides have been reported to inhibit bacterial growth and to be protective to plants against bacterial infections (Okwute, 1992). The terpenoids and tannins may elicit the antibacterial properties by cell membrane lysis, inhibition of protein synthesis, proteolytic enzymes and microbial adhesions (Dulger et al., 2002). Saponins are known for their medicinal properties as a natural blood cleanser, expectorant and antibiotics (Kalanithi and Lester, 2001). Among the five solvents tested most of the test pathogens were highly sensitive to methanol and ethanol extract followed by aqueous, ethyl acetate and hexane extracts.

5.2.2. Free radical scavenging assays

Antioxidants are significant in the prevention of human illness and may function as free radical scavengers, complexes of pro-oxidant metals, reducing agents and quencher of singlet oxygen formation (Pawar et al., 2010). Free radical-mediated oxidative stress is believed to be the primary cause of many disease including neurodegenerative diseases, cancer, cardiovascular disease, cataracts and AIDS (Lee et al., 2000; Middleton et al., 2000) and disorders. Hence, therapy using free-radical scavengers (antioxidants) has a potential to prevent, delay or ameliorate many of these disorders. Over the past two decades, an expanding body of evidence from epidemiological and laboratory studies have demonstrated that many mushrooms or their identified ingredients with antioxidant properties have substantial protective effects on human diseases.

In order to determine if the methanol extract of P. florida was capable of scavenging free radicals, the antioxidant assays was assessed. DPPH is a stable free radical and possesses a characteristic absorbance at 517 nm, which decreases significantly on exposure to radical scavengers by donating a hydrogen atom to become a stable diamagnetic molecule. DPPH radical has certain advantage of being unaffected by side reactions, such as enzyme inhibition and metal chelation (Pal et al., 2010). The principle of the reduction of DPPH free radical is that the antioxidant reacts with the stable free radical DPPH and converts it to 1, 1-diphenyl-2-picryl hydrazine (Sreeyan and Rao, 1996). To determine the reducing power of the extract,
used the method of Berker et al. (2010) to measure Fe$^{3+}$ - Fe$^{2+}$ transformation in the methanol extract. The reducing capacity of extract may be serving as a significant indication of its probable antioxidant capacity and the reducing properties of antioxidants are generally associated with the presence of reductones, e.g., ascorbic acid.

Superoxide radical is a highly toxic species, which is generated by numerous biological and photochemical reactions (Govindarajan et al., 2003). Superoxide radical can further interact with other molecules to generate secondary ROS (e.g., hydroxyl radical, hydrogen peroxide and singlet oxygen), either directly or prevalently through enzyme or metal catalyzed processes (Valko et al., 2007). The hydroxyl radical is the most reactive of the reactive oxygen species and it induces severe damage in adjacent biomolecules (Gutteridge, 1984). The hydroxyl radical can cause oxidative damage to DNA, lipids and proteins (Spencer et al., 1994). Iron can stimulate lipid peroxidation by the Fenton reaction and accelerate peroxidation by decomposing lipid peroxide into peroxy and alcoxy radical that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation (Halliwell and Gutteridge, 1989).

Nitric oxide is an unstable free radical involved in many biological processes which is associated with several diseases. It reacts with oxygen to produce stable product nitrate and nitrite through intermediates. High concentration of nitric oxide can be toxic and inhibition of over production is an important goal (Wang et al., 2005). Nitric oxide plays a role in the modification of the trans-membrane transport of ions and signal transduction pathways that regulate cell functions (Paige and Jaffrey, 2007). Effective inhibition of nitric oxide accumulation represents a beneficial therapeutic strategy (Park et al., 2004). The methanolic extract of P. florida was able to inhibit the nitric oxide generation to a significant extent.

Different concentrations of the methanolic extract of P. florida was used, which showed a dose-dependent percentage of inhibition on the scavenging assays. Therefore, the methanolic extract showed significant antioxidant potential that may reveal its therapeutic potentials for several diseases. Results of the present study revealed that methanol extract of
P. florida mushroom showed relatively high reducing power and DPPH radical scavenging assay (IC$_{50}$ value 110 and 50 µg/ml).

The radical scavenging activity of P. ostreatus mushroom is reported to be higher (6 mg/ml) than those of other mushrooms like Agaricus bisporus, Volvariella volvacea, Calocybe indica and Hybsizus ulmarius (Ramkumar et al., 2010). When compared to P. ostreatus mushroom the methanolic extract of P. florida has higher chelating activity against ferrous ion (Mohamed Imran et al., 2011). The IC$_{50}$ value of ethanolic extract of Agaricus bisporus was 0.37 and 0.38 mg/ml, methanolic extract of Boletus edulis was 0.34 and 0.73 mg/ml in reducing power and DPPH radical scavenging assay respectively (Liu et al., 2013; Vamanu and Nita, 2013). Finimundy et al. (2013) reported that IC$_{50}$ value of DPPH scavenging ability of aqueous extract of P. sajar-caju showed 9.01% and the IC$_{50}$ values of P. abalones in DPPH radicals scavenging ability and reducing power were 8.68 and 4.68 mg/ml respectively (Wang et al., 2012). The IC$_{50}$ value of hot water extract of P. squarrosulus was found to be 340 µg/ml. Therefore, the scavenging activity of P. florida was much effective than those mentioned above.

Apparently, the reducing power of a methanolic extract of P. florida is excellent, when compared to that of other commercial and medicinal mushrooms. A large number of antioxidant components such as phenolic compounds, flavonoids, carotenoids and vitamins C and E have been isolated from the fruit bodies of Pleurotus mushrooms (Jayakumar et al., 2011). The quantity of these antioxidant phytochemicals components vary from strain to strain and also depend on cultivation (Kanagasabapathy et al., 2011).

The present study supports the antibacterial and antioxidant activity may be due to the presence of phenolic compounds present in the mushroom. The mushroom merits further investigation to identify the active principles and the nature of the antitumor activity.

5.3. Isolation, purification and characterization of the active compounds from methanolic extract of P. florida

High number of components isolated from microorganisms and plants have been recorded by asian scientists. Recent research has shown that mushroom derived polyphenolic
compounds and polysaccharides were promising nutraceuticals for control of various disorders such as cardiovascular, neurological and neoplastic disease. The richness of the polysaccharides and polyphenolic contents of mushrooms has made them popular choices for associated antioxidant and anticancer health benefits (Khan et al., 2010).

Some of the most effective anti-tumor agents were subsequently purified and identified as large 1, 6-branched 1, 3-β-glucans. The isolated polysaccharide compounds included lentinan from Lentinus edodes, schizophyllan from Schizophyllum commune, grifolan from Grifola frondosa and SSG from Sclerotinia sclerotiorum (Borchers et al., 2008).

The polyphenolic compounds found as integral components of the human diet. The ability of phenolic compounds to scavenge free-radicals and block lipid peroxidation raises the possibility that they may act as protective factors against carcinogenesis (Tseng and Lee, 2006). Scalbert et al. (2005) documented that the consumption of phenolic compounds is associated with the prevention and reduced risk of several degenerative diseases including atherosclerosis, cardiovascular complications and cancer.

Ferulic acid is an antioxidant nutrient commonly found in fruits and vegetables such as tomatoes, sweet corn and rice bran (Zhao and Moghadasian, 2008). Ferulic acid accounts for 90% of the total phenolic acids in common flour (Manach et al., 2004). Ferulic acid is a strong membrane antioxidant in humans and is known to protect against cancer, cold, flu, influenza, diabetes, cholesterol, skin aging, degenerative diseases and muscle wasting (Fazary and Ju, 2007).

A white rot fungus isolated from decaying wood has the ability to metabolise ferulic acid, transforming it to 4-vinyl guaiacol, which was further metabolized to give acetovanillone (Mabinya et al., 2006), a product with potential applications in the pharmaceutical industry (Vanden et al., 2001). Xie et al. (2010) suggested that when edible mushroom mycelium grown in wheat bran could have the capacity to release ferulic acid by secreting cellulase and ferulic acid esterase enzyme. Dogan and Akbas (2013) identified phenolic compounds like catechin, ferulic acid, p-coumaric acid and cinnamic acid using gas chromatography.
In this study, the P. florida mushroom was cultivated using paddy straw as substrate with horse gram as nutrient supplement. The composition of phenolic compounds in paddy straw and horse gram reported in the literature and their analysis revealed that ferulic acid was the dominant phenolic acid which possesses significant antioxidant activity (Sun et al., 2001; Srerama et al., 2010; Srerama et al., 2012).

Visualization of certain phenolic monomers can be achieved by a brief exposure to UV light after separation on thin layer chromatography (TLC) plates (absorbance at 254 nm and fluorescence at 366 nm) and by the colour developed after spraying with various spray reagents (Mabinya et al., 2002). The rapid quantitative and qualitative analysis by TLC from crude extracts offers a practical and simple procedure for many applications.

TLC is a most widespread technique used for the separation of natural bioactive compounds. It is the standard technique that separates low molecular weight organic compounds based on their polarity (Anjana and Virendra Kumar, 2009). Using solvent extraction process, isolation of phenolic compound is supposed to be a highly tedious process, because of its magnitude of reactivity with other molecules of the mushrooms such as proteins and carboxylic acids to form esters during extraction in addition to their delicate nature of decomposition in presence of heat, acids, bases and electropositive inorganic metal ions. Prabhu et al. (2011) showed that for the TLC investigation of phenolic compounds, flavonoids and glycosides, the medium and high polar solvents like ethylacetate, methanol and water were used.

In the present study, we observed that n-hexane: dichloromethane: methanol in the ratio of 6: 3.8: 0.2 was the best solvent system for the separation of phenol and flavonoid compounds. The process of drug discovery is a complicated and risky work but it involves the use of various techniques resulting in emergence of innovative drug. In recent years thousands of compounds are tested for pharmacological activities and in that only few of compounds are therapeutically effective. The P. florida mushroom contains a large number of phytoconstituents like polysaccharides, phenols, flavonoids, terpenoids, steroidal, glycosides and carbohydrates (Ganeshpurkar et al., 2012). To our best knowledge, the present study was
the first report to isolate and purify the bioactive compounds ferulic acid and cholest-5-en-3-ol from P. florida mushroom.

5.4. Antioxidant and anticancer activity for the purified active fractions of P. florida mushroom

An angle of this research has been the study of bioactive components and their biological properties like antioxidant and anticancer properties from methanolic extract of P. florida mushroom were studied. The present study has been verified that remedial mushroom could be a good source of antioxidant and anticancer substance. It has been acknowledged that phenolic compounds show significant antioxidant action on human health and fitness. The high potential of phenolics to scavenge free radicals may be due to many phenolic hydroxyl groups they possess (Sawa et al., 1999).

5.4.1. Radical scavenging activity

The DPPH radical is frequently used to determine the radical scavenging activity of natural product (Pukalskas et al., 2005). This compound is characterized as stable free radical by its property of the delocalization of the electron pair over the molecule as whole, thus the molecules do not dimerise as would be the case with most other free radicals (Molyneux, 2004). Many reports in the literature associate the DPPH* scavenging activity of mushrooms and their components with strong antioxidant activity. The fraction 5 and 8 exhibited a better DPPH radical scavenging activity followed by the fraction 4, 6 and 7 from methanolic extract of P. florida.

High radical scavenging activity against DPPH radical scavenging activity has been reported in water soluble heteroglycan isolated from aqueous extract of edible hydrid mushroom P. florida and Calocybe indica var. APK2 (Maity et al., 2011). A heteroglycan (PS) isolated from the mycelia of P. ostreatus has been shown to render protection against hydroxyl and superoxide generation (Patra et al., 2013).

The aqueous extracts of Lentinus edodes and P. sajor-caju exhibited high antioxidant property as reflected by the capacity to scavenge DPPH and SO radical (Finimundy et al., 2013). Liu et al. (2010) proposed that the DPPH scavenging assay results revealed that the
intracellular polysaccharide probably contained substances that were proton donors and could react with free radicals to convert them to stable diamagnetic molecules.

The reducing power of bioactive compounds was associated with antioxidant activity. Thus, it is necessary to find out the reducing power of phenolic constituents to reveal the relationship between their antioxidant effect and their reducing power (Siddhuraju et al., 2002). Liu et al. (2013) reported that the methanolic extract of Agaricus bisporus showed excellent reducing power activity (IC50 value - 0.37 mg/ml). The antioxidant activity of P. ostreatus mushroom is reported to be higher (6 mg/L) than those of other mushrooms like Agaricus bisporus, Volvariella volvacea, Calocybe indica and Hybsizus ulmarius (Ramkumar et al., 2010). In the present study, the fractions from the methanolic extract showed maximum DPPH radical scavenging and reducing power assay. This observation indicates the strong antioxidant activity of P. florida mushroom.

5.4.2. Cytotoxic activity of 4-hydroxy-3-methoxycinnamic acid from methanolic extract of P. florida

The principle of SRB, which is a bright pink aminoxanthene dye, is that it is ananionic protein stain containing two sulfonic groups which bind to protein basic amino acid residues in TCA-fixed cells under mildly acidic conditions. The protein-bound dye is then solubilized by weak base for spectrophotometry. This colorimetric assay can be used to estimate the cell number indirectly by providing a sensitive index of total cellular protein content which is linearly related to cell density (Skehan et al., 1990). This assay was found to give good results over a wide range of cell density (Freshney, 1994). The MTT assay is based on metabolic reduction of colorless tetrazolium salt, by mitochondrial enzyme activity in viable cells, to formazan salt (blue), which can be quantified spectrophotometrically. It is particularly useful for assaying cell suspensions because of its specificity for living cells (Mosmann, 1983).

The trypan blue exclusion assay is a direct method for measuring cell growth or cytotoxicity, but it involves many steps that may introduce experimental errors. Trypan blue, a diazo dye, is a vital stain used to selectively color dead tissues or cells blue. Live cells or tissues with intact cell membranes are not coloured. Since cells are very selective in the
compounds that pass through the membrane, in a viable cell, trypan blue is not absorbed; however, it traverses the membrane in a dead cell. Hence, dead cells are shown as a distinctive blue color under a light microscope. Since live cells are excluded from this staining, this staining method is also described as a dye exclusion method (Shapiro, 1988; Haldar et al., 2010).

In the present study, the 4-hydroxy-3-methoxycinnamic acid from methanolic extract of P. florida was tested for the effect on the extent of survival of A549 caused a remarkable decrease in the viability of cancer cell line and the addition of compound 4-hydroxy-3-methoxycinnamic acid rendered good protection and destroyed the cancer cells.

The reasons why cancer cells are addicted to aerobic glycolysis and the underlying molecular mechanisms still remain controversial. Mitochondrial dysfunction caused by mitochondrial DNA mutations, oncogenic signals and ROS stress may be an important event that forces cancer cells to be more rely on the glycolytic pathway for energy production and for generation of metabolic intermediates for biogenesis. Glycolysis may be still highly active even in cancer cells with competent mitochondria function and activate oxidative phosphorylation, and thus blocking a single energy metabolic pathway might not be effect in killing cancer cells (Moreno-Sanchez et al., 2010).

The effect of ferulic acid derivatives on cellular ROS and redox states is rather complex, since this compound has been shown to have antioxidant and pro-oxidant functions (Maurya and Devasagayam, 2010). Pleurotus spp. is promising as medicinal mushrooms and exhibiting hematological, antiviral, antitumor, antibiotic, antibacterial, hypocholesterolic and immunomodulation activities (Cohen et al., 2004; Singh et al., 2011a). Ferulic acid derivative was indicated for cancer, not only for the breast and lung but also for the colon, stomach and tongue cancer (Fazary and Ju, 2007). It was observed that administration of ferulic acid possess a variety of pharmacological properties including hepatoprotective, antimalarial, antioxidant and antityrosinase activities (Prabhakar and Doble, 2011).

The anticancer activity of many of natural compounds isolated from different mushroom extracts has been reported that lentinan, krestin, hispolon, lectin, calcaelin, illudin
S, psilocybin, Hericium polysaccharide A and B (HPA and HPB), ganoderic acid, schizophyllan and laccase (Patel and Goyal, 2012).

The hyperbranched beta-glucan, extracted from P. tuber-regium has been shown to inhibit the proliferation of HepG2 human hepatocellular carcinomas (Tao et al., 2006). The polysaccharide from P. ferulae has been reported to have cytotoxic effects on human lung cancer and cervical cancer cell lines (A549, SiHa and HeLa cells) (Choi et al., 2004).

In our study, MTT, SRB and Trypan blue assays were used to test the cytotoxicity of the from methanolic extract of P. florida. The results on the cytotoxicity tests conducted against A549 cell line exhibiting a significant activity against the lung cancer cell line. The results obtained from the present study showed that the 4-hydroxy-3-methoxycinnamic acid is cytotoxic in nature and may possess antitumor activity. It may be used as a supportive supplement to decrease the toxicity of anticancer drugs.

Asia is one of the most promising regions for discovering novel biologically-active substances from its flora. More efforts are needed to explore potent anticancer drug from the mother earth and save humans around the world from cancer. Although cancer is a multifactorial disease, researches have shown that a healthy diet rich in vegetables, mushroom and low in fats is the key to lower the risk of such terrible diseases.

5.5. Fe$^{2+}$ removal from metal contaminated ground water using spent mushroom substrate

In this aspect, the live, dead and chemically treated (sodium hydroxide, formaldehyde and orthophosphoric acid) SMS of P. florida were used for the removal of ferrous iron (Fe$^{2+}$). SMS were used in different dosage levels at different time intervals and the effect of pH and temperature were also studied.

Water is one of the environmental substances most exposed to pollution. The number of pollutants so far identified in water was larger than in the air. Among those, the main pollutants are heavy metals cations, such as Cadmium, Chromium, Cobalt, Copper, Ferric, Manganese, Nickel, Lead and Zinc. They are not biodegradable and tend to accumulate in living organisms, thus causing different disorders (Al-Deqs et al., 2003).
Presence of iron in water imparts the colour, odour, makes the teeth and nail as black and weaker as well, as leads to roughness of skin and stickiness of hair. According to Environmental Protection Authority (EPA), the acceptable value of iron in drinking water was 0.3 mg/l. Iron overload may lead to debilitating and life-threatening problems such as diabetes, heart failure, poor growth, haemochromatosis, accumulation of iron in the hippocampus of the brain leads to Alzheimers disease and Parkinson disease (Ncibi et al., 2007; Devi Prasad and Abdullah, 2009).

Many physico-chemical methods like ion exchange, membrane separation, coagulation, flocculation and oxidation are available for the treatment of heavy metals. Major drawbacks of these methods are high sludge production, handling and disposal problems, high cost and technical constraints. This necessitates cost effective and environmentally sound techniques for treatment of wastewaters containing metals. Adsorption is now recognized as an effective and economic method for heavy metal removal in wastewater. The major advantages of biosorption are its high effectiveness in reducing the heavy metal and the use of inexpensive biosorbents.

Previously it has been demonstrated that mushroom mycelium and spent mushroom substrate are used as a potential biosorbents for the removal of heavy metals (Gui-qui et al., 2005; Bayramoglu and Arica, 2008; Javaid et al., 2011a) and this fact also has been confirmed in this study. Fungi offer a wide range of chemical groups that can attract and sequester the metals in biomass. Cell walls are composed of structural polysaccharides, proteins and lipids that offer metal-binding functional groups (Tomko et al., 2006).

The SMS of Lentinus edodes mushroom has been successfully used as biosorbent for the removal of cadmium, lead and chromium from aqueous solution owing to the presence of hydroxyl, phosphoryl and phenolic functional groups on the surface of the SMS (Gui-qui et al., 2005). Much number of studies was reported in literature on biosorption of iron onto different fungal mycelium and plant biomass (Ahalya et al., 2006; Wuyep et al., 2007; Abou Zeid et al., 2009; Prabhakaran and Arivoli, 2011). Up to my knowledge this is the novel report for the adsorption of Fe^{2+} using SMS of P. florida.
A novel approach was carried out with the SMS of P. florida on Fe\textsuperscript{2+} removal was carried out using live, dead and chemically treated SMS were used in different dosage levels such as 0.25, 0.50, 1.0 and 1.50 g/50 ml (4, 6 and 8 ppm of Fe\textsuperscript{2+}) at different time intervals till 90 min. The live SMS of P. florida showed potential Fe\textsuperscript{2+} removal (100%) from aqueous solution in all the tested concentrations. The evaluations seemingly are in agreement with observations recorded in the case of different fungal biomasses. Cabuk et al. (2005) and Shokoohi et al. (2009) obtained 95% of iron adsorption with 0.9 g of dried biomass of activated sludge as dosage. The reduction of biosorption capacity in autoclaved P. florida SMS may be attributed to the loss of intracellular uptake.

The maximum removal (91.98%) of Fe\textsuperscript{2+} was observed at 1 h of contact time using the live biomass of P. ostreatus as adsorbent (Arbanah et al., 2012). Mamun et al. (2011) investigated the dead fungal biomass showed no obvious change in the metal adsorption capacity for first 4 h, 56.56% for Cu (II) and 22.30% for Cr (VI).

The constant value at equilibrium stage is due to the vacant space is fully filled by heavy metal ions and after some time, there is no available place for heavy metals ion binding to the cell wall surface. As a result, a repulsive force occurred. Biosorption of heavy metals ions occurred in two stages of process where the initial rapid uptake due to surface absorption to the cell walls and subsequent slow uptake due to membrane transport into the cytoplasm of the cells (Saglam et al., 2002).

Fungal biosorbents can sorb heavy metals which are dependent on fungal species, biosorbent size and concentration, solution pH and ionic composition. Biomass concentration in solution seems to influence the specific uptake and lower values of biomass increases uptake as an increase in biomass leads to interference between binding sites (Tomko et al., 2006).

The value of pH is also an important parameter for adsorption of metal ions from metal contaminated water because it affects the solubility of the metal ions, concentration of the counter ions on the functional groups of the adsorbent and the degree of ionization of the adsorbent during reaction and also the solution chemistry of heavy metals (Dursun, 2006). Solution chemistry includes hydrolysis, complexation by organic/inorganic ligands, redox
reactions, precipitations, speciation and biosorption availability of heavy metals (Ozer and Ozer, 2003). The potential binding sites on the adsorbents might be carbohydrates, amino groups, hydroxide groups and carboxylic groups. These functional groups might dissociate or ionize at different pH values. So, the surface chemistry of the functional groups also plays an important role in the adsorption process (Soundarajan et al., 2013). Several researchers have investigated the effect of pH on biosorption of heavy metals by using different kinds of microbial biomass.

For example, the biosorption of lead and copper using fungal biomasses was pH dependent and maximum biosorption was obtained in the range of pH 4 to 6 (Ozsoy, 2010; Joo et al., 2011; Gazem and Nazareth, 2012). Wuyep et al. (2007) analyzed that fungal biomass has maximum sorption capability for cationic metal ions at pH values of 4 to 6, but in case of pH below 3, uptake of heavy metal ions were negligible, because of cation competition effects with hydronium ion H$_3$O$^+$. In addition, the biosorption capability of heavy metals by fungi is strongly pH dependent, such that biosorption increases with increase in pH.

In this study, it was found from the result that the uptake of Fe depends on pH, where optimal metal removal efficiency occurred at pH 7. The medium pH affects the solubility of metals and the ionization state of the functional groups (carboxylate, phosphate and amino groups) of the fungal cell wall. The carboxylate and phosphate groups carry negative charges that allow the fungal cell wall components to be potent scavengers of cations (Say, 2000). At low pH (pH < 5), desorption of metal ions was occurred when metal contaminated water was applied for the treatment.

Biosorption was also affected with a lesser extent within the temperature ranges from 20 to 35°C. Higher temperatures usually enhance sorption due to the increased surface activity and kinetic energy of the solute (Mudhoo et al., 2012). In the present investigation, maximum biosorption was obtained at temperature of 30°C. Biosorption efficiency was increased due to higher affinity of active sites for heavy metals ion attraction. It was found that the highest biosorption efficiency of Fe$^{2+}$ was 100, 100 and 98.62% in 4, 6 and 8 ppm respectively. In case of temperature 35, 40 and 45°C the adsorption effectiveness was found
to be 95.5, 58.25 and 65.62% at 8 ppm Fe$^{2+}$ concentration. This indicates that 30°C was the best temperature for biosorption process and the results were in agreement in Horsfall and Spiff (2005).

Arbanah et al. (2012) stated lower temperature was suitable for the cell wall components of mushroom mycelium to reorient and also for more binding. Since the biosorption efficiency of heavy metals ion is an exothermic process, the increasing temperature from 30 to 45°C decreased the biosorption efficiency. The results also show that Fe$^{2+}$ biosorption decreased from 98.62 to 65.62%. It was believed that at 35°C, some of the active sites responsible for biosorption on the biosorbents surface were distorted due to high temperature (Ozer and Ozer, 2003). Furthermore, too high temperature is unfavorable for practical application because it can increase operating cost.

In present investigation, P. florida SMS was pretreated with formaldehyde, sodium hydroxide and ortho-phosphoric acid and was evaluated for Fe$^{2+}$ removal from aqueous solution. Assessment revealed that the P. florida SMS exposed to pretreatments induced reduction in uptake efficiency and capacity of the adsorbents as compared to untreated live SMS. Presently the recorded reduction in sorption capacity of tests SMS for the removal of Fe$^{2+}$ was evidenced due to acid pretreatment. The assessments seemingly are in agreement with observations recorded in the case of Mucor rouxii (Yan and Viraraghavan, 2000), Aspergillus fumigatus (Saleh et al., 2009) and A. niger (Javaid et al., 2011b). H$^+$ ions binding to the fungal biomass after pretreatment are responsible for the reduction in adsorption of heavy metals. This indicates that the acids destroyed the adsorbing groups and their positive ions (H$^+$) may covalently bind to the adsorbing surfaces. Thus, the remaining H$^+$ ions on the pretreated biomass may change the biomass electronegativity, resulting in a reduction in biosorption capacity (Javaid et al., 2011b).

Generally mushrooms can act as an effective biosorbents of toxic heavy metals since they are growing in natural habitat having large, tough texture and conductive characteristics required for their development into sorbents, thus obviating for immobilization process which was required for other microbial sorbents (Das, 2005).
The SMS include mostly lignocellulosic materials, decomposed and permeated by the fungal mycelium. High levels of residual nutrients and enzymes are still left in SMS (Semple et al., 2001). The live SMS, without any treatment has the advantage of including viable mycelia and several active enzymes produced by the fungus throughout the growing cycle. Consequently, this fresh SMS may perform more efficiently in remediation activities than autoclaved and chemical treated SMS. Therefore, if P. florida SMS are to be used as an adsorbent, then their use as fresh (without any treatment) products should also be considered. SMS also has the ability to chemically adsorb the organic and inorganic pollutants, while the diverse category of microbes it harbours has the capability of biological breakdown of the organic xenobiotic compounds present in soil and water (Ahlawat et al., 2010).

Column mode adsorption studies are considered to be very important in treatment application point of view because it is economical to treat any wastewater in continuous mode than in batch mode. Column mode adsorption studies were carried out to find the efficiency of the adsorbent for continuous removal of Fe$^{2+}$. Column study was carried out only with live P. florida SMS as a adsorbent, which showed that the maximum adsorption of Fe$^{2+}$ than batch mode studies. The efficiency of a column mode experiment depends on the flow rate at which constant flow rate was maintained. Results obtained that increase in adsorption of Fe$^{2+}$ due to column bed volume. It may be due to the availability of more surface functional groups on bed volumes.

The results revealed that Fe$^{2+}$ was removed effectively by the incorporation of SMS as a bioadsorbent. The current investigation concluded that spent mushroom substrate can be used as a potential biosorbent for the removal of Fe$^{2+}$.

The value of mushroom could be greatly enhanced since mushrooms are described as “Precious Pearls of Cookery” by Hema (2002). Thus, the outcome of the present study highlights the antioxidant and anticancer potential and biosorption capacity of Fe$^{2+}$ using P. florida. The findings of the present study are summarized and the conclusions drawn are elaborated in the next chapter following the future research directions.
Future Research Directions

The outcome of the present study has opened up several promising insights of possible research. Some of them which can be followed up for active research are as follows:

A The anticancer activity of the compounds isolated from P. florida can be further probed using various cancer cell lines.

A The effects of the compounds isolated from P. florida can be investigated against oxidative stress caused by different types of oxidative stress caused by different mechanisms.

A The mechanisms of the compounds isolated from P. florida in protecting biomolecules can be probed further.

A The isolated and purified active components and their effects can be studied further against oxidative stress and different types of cancer cells in vitro and in vivo.

The knowledge accumulated on various uses of mushrooms shows that it is a gift of Mother Nature, which could be exploited in numerous ways for the benefits of humanity.