

EXPLORING THE APPLICATIONS OF THE EDIBLE MUSHROOM - *PLEUROTUS OSTREATUS*.

Submitted in partial fulfillment of the requirements for the
award of Bachelor of Science degree in
BSc. Microbiology

by

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DEPARTMENT OF BIOTECHNOLOGY

BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **V. POOJA (Reg No. 40300075)** carried out the project entitled **EXPLORING THE APPLICATIONS OF THE EDIBLE MUSHROOM -**

PLEUROTUS OSTREATUS. under the supervision of **Dr. INDUMATHI. SM** and **Dr. JAYASINDU MATHIAZHAGAN** from **SEPTEMBER 2022** to **MARCH 2023**


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ABSTRACT

Mushrooms are generally used as a food all over the world. The genus "*pleurotus*" consists of 40 species. *Pleurotus ostreatus* known as oyster mushroom, is widely consumed all over the world for its flavor, taste, nutritional value and medicinal properties. *Pleurotus ostreatus* shows anti-cancer, anti-tumor, anti-oxidant, antiviral and antibacterial properties. Breast cancer is a disease in which cells in the breast grow out of control. Globally, Breast cancer is the second most common cancer. Previous studies report showed that *P. ostreatus* exhibited the inhibition of breast cancer cell lines (MCF-7). Also, *P.Ostreatus* is known to inhibit the growth of *E.coli*, *S.aureus*, *streptococcus*, *enterococcus*. Different extracts from oyster mushrooms possessed high antioxidant activity due to the presence of polysaccharide plueran (beta glucan). In the present project, the ethanolic extract of *P. Ostreatus* has shown antibacterial, antioxidant and anti-cancer activities. The anti-cancer activity showed inhibition of the cell proliferation in human breast cancer cell lines (MCF-7). The ethanolic extracts of *P.ostreatus* enhanced the activity of antioxidant enzymes. Also, the extract showed the inhibition in growth of *bacillus*, *staphylococcus*, *klebsiella*, *candida* species. Overall, the ethanolic extract of the *P. Ostreatus* showed antibacterial, anti-fungal, antioxidant and anticancer activities.

Keywords: *P. Ostreatus*, antibacterial, antioxidant, anticancer.

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LIST OF ABBREVIATIONS:

- GCMS - Gas Chromatography Mass Spectrum
- TLC - Thin Layer Chromatography
- DPPH - 2,2-diphenyl-1-picrylhydrazyl.
- IC50 - Half-maximal inhibitory concentration
- RSA% - Radical SCavenging Activity
- DMEM- Dulbecco's Modified Eagle Medium
- UV - Ultra Violet
- μ microliter
- nm - nanometer
- OM- Oyster mushroom

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CHAPTER 1

INTRODUCTION

Pleurotus ostreatus is commonly known as “oyster mushroom.” It was first harvested in Germany, and it is cooked and consumed as a food (44). In addition to plants, many fungal species are known to have medicinal benefits, and some are even currently used for that reason. Since ancient times, people all over the globe have used mushrooms as "the ultimate health food" in folk medicine (46). Oyster mushroom is also used industrially for mycoremediation purposes.

Scientific classification of the Oyster Mushroom

Kingdom : *Fungi*

Division : *Basidiomycota*.

Class : *Agaricomycetes*

Order : *Agaricales*

Family : *Pleurotaceae*

Genus : *Pleurotus*

Species : *P.ostreatus*

Mushrooms need a good substrate to grow. Substrate is the medium required for the growth of an organism. The good substrate contains water content, as it is an important factor for mushroom growth. The most common substrate used for mushroom growth is straw substrate and sawdust substrate (66). Mycelia—also known as roots—grow below and inside the substrate during the vegetative phase, when they are essential to the process of nutrition synthesis from the substrate material (71). They discharge enzymes that break down the decaying organic matter into smaller particles that are then taken up as nutrients (61). Mushroom growth cycle starts with a mycelium growth, mycelium is a mesh like structure formed by hyphae. After making a straw substrate with 90% moisture content, spawns should be seeded onto the substrate. The mycelium growth occurs within 10 days after spawning. Mycelium further develops a primordial formation, and it develops into a fruiting body. The mature fruiting bodies are harvested (57).

Oyster mushrooms have various applications. Oyster mushrooms are rich in fiber, protein, and a variety of vitamins and minerals. Oyster mushroom acts as an antioxidant that helps in reducing cell damage (19). They also promote heart health. It reduces the risk of high blood pressure and cholesterol. Oyster mushrooms also regulate blood sugar levels. It supports a healthy immune system and in addition to their excellent nutritional content, researchers have shown that they offer healing powers. The potential of *Pleurotus spp.* as nutritive food ingredients is further demonstrated by reports that they possess nutraceutical and therapeutic qualities, including antihypertensive, antioxidant, antiviral, antibacterial, anti-inflammatory, and immunomodulation activities (15). Treatment with combined pleuran, vitamin C and zinc supplement for HSV-1 known to improve the reduction of HSV- 1 symptoms and severity of respiratory symptoms (67). Oyster mushrooms have antitumor, anti-cancer and anti-inflammatory properties (1). According to preliminary reports, when compared to a regular diet, rabbits and rats' arterial pressure and blood cholesterol levels are reduced more when fed a diet that contains 4-10% dried fruiting bodies of *Pleurotus* species. (13, 14)

1.1. BIO ACTIVE COMPOUNDS OF *P. OSTREATUS*:

Similar to other fungus, *Pleurotus ostreatus* fresh fruiting bodies have a water content of roughly 80–90%. From 100 g of mushrooms, 10 g of dried fruits are produced, with each gram containing 2.5 g of proteins and around 5 g of polysaccharides, mostly starch but also mannitol and trehalose. Due to the high levels of amino acids (arginine, alanine, glutamine, and glutamic acid) present, *P. ostreatus* mycelium has a high nutritional value. The amount of vitamin C in 100 g of fresh mycelia equals 15% of the daily allowance for humans. Additionally, this species has 0.1–0.2 g of fat, which includes oleic acid (40%), linolenic acid (55%), and other substances with hypercholesterolaemia effects. There are just about 10% of saturated fatty acids present (8). Due to their low-calorie count, low fat content, and high quantities of proteins, minerals, and certain polysaccharides, fungi exhibit beneficial dietetic qualities (34).

A major factor in why fungi are employed as food additives and in pharmacology is because of the Glucans b-1, b-14 and b-16; they have also demonstrated positive benefits when used in the treatment of various disorders (63). B-glucans can be found in mushrooms in either their water-soluble or water-insoluble forms. However, studies on humans and animals have demonstrated that the water-soluble form's biological activity is far higher and has a more noticeable impact on their immune systems. (64). The most well-known glucan obtained from oyster mushrooms is pleuran. D-glucose molecules are joined together by bonds of the types 13 and 16. The typical method of obtaining pleuran from fruiting bodies is extraction with hot water (56) A *pleurotus ostreatus* protein that inhibits HIV-1 reverse transcriptase is related to ubiquitin. Low-molecular weight bioactive substances are present in oyster mushrooms and include mono- and sesquiterpenoids, ergosterol, and fatty acid esters (77). (table 1.1)

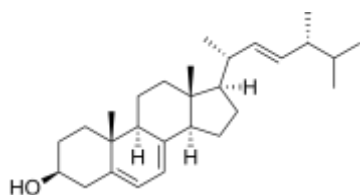


Fig 1.1Ergosterol

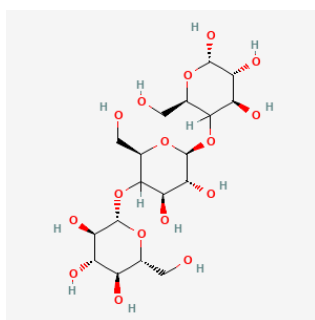


fig 1.2 Beta- glucan

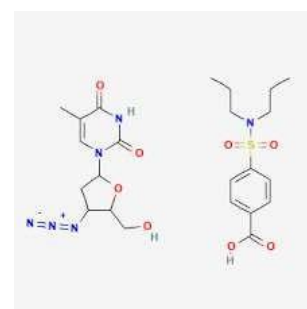


fig 1.3 Concanavalin-A

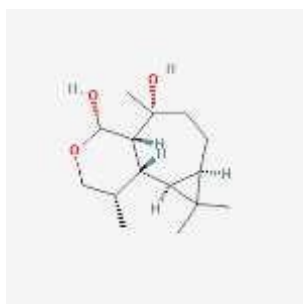


Fig 1.4 Sesquiterpenoid



fig1.5 Linoleic acid

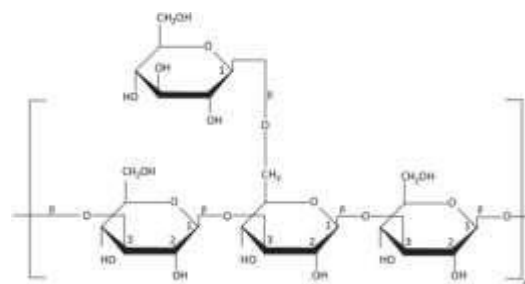


fig 1.6 Beta-D glucan

Table 1.1 BIOACTIVE COMPOUNDS

S.No	BIO COMPOUNDS	ACTIVITY	REFERENCE
1	Phenol Lectin Polysaccharide	Anti-oxidative	Wu et al (2011) De Silva DD et al (2012)
2	β -glucans	beta-glucan on human immune and cancer cells.	Chan GC, Chan WK, Sze DM (2009)
3	β -D Glucan (pleuran)	Antibacterial	Mirunalini S et al (2012) Vamanu E et al (2012)
4	Concanavalin A.	Anti-inflammatory activity	Jedinak A, Dudhgaonkar S, et al (2011)
5	Linoleic Acid, Lovastatin, Ergosterol	Hypocholesterolemic Effect	Schneidera I, Kressel G, et al (2011)

6	1,3- α -glucan	Prebiotic activity	Synytsya A, Míčková K, et al (2009)
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1.2. CANCER:

Cancer is a disease in which some of the body's cells grow uncontrollably and spread to other parts of the body. Normally, human cells grow, multiply, die and the new ones grow. Sometimes this order breaks down and abnormal or damaged cells grow. These cells can form tumours and tumours can be cancerous (76). Tumors can be cancerous or not cancerous (benign). Cancerous tumours may also be named malignant tumours. Many cancers create solid tumours, while malignancies of the blood, such as leukaemias, often do not. Benign tumours do not spread into, or invade, surrounding tissues. When removed, benign tumours normally don't come back, although malignant ones sometimes may. Benign tumours can occasionally be extremely big, though. Some can produce significant symptoms or be life threatening, such as benign tumours in the brain (32)

Few differences between normal cells and cancer cells are; cancer cells grow in the absence of growth signals; normal cells only grow when they receive signals. Cancer cells invade into neighbouring places and spread to other sections of the body. Normal cells stop growing when they encounter other cells, and most normal cells do not travel about the body. Cancer cells tell the blood vessels to expand towards malignancies. These blood arteries deliver oxygen and nutrients to tumours while also removing waste (32)

Cancerous tumours can spread from one organ to another organ through blood, this is known as metastasis (84). The most common cancers are breast, lung, colon and rectum and prostate cancer. Metastatic cancer has the same name as primary cancer and the same sort of cancer cells. Breast cancer that has spread to the lungs, for example, is called metastatic breast cancer rather than lung cancer. Under a microscope, metastatic cancer cells resemble cells from the initial tumour. Moreover, metastatic cancer cells and initial cancer cells frequently share some biological features, such as the presence of particular chromosomal alterations. Treatment may help patients with metastatic cancer survive longer in some cases. In other circumstances, the primary objective of treatment for metastatic cancer is to limit the

disease's spread or to alleviate the symptoms it causes. Metastatic tumours can severely impair how the body works, and metastatic cancer kills the majority of cancer patients (32).

Cancer is the most leading cause of death worldwide. Each year approximately 40,000 children develop cancer. Cervical cancer is the most common cancer in many cities (40). Breast cancer, lung and bronchus cancer, prostate cancer, colon and rectum cancer, melanoma of the skin, bladder cancer, non-Hodgkin lymphoma, kidney and renal pelvis cancer, endometrial cancer, leukemia, pancreatic cancer, thyroid cancer, and liver cancer are the most common cancers. Prostate, lung, and colorectal cancers are expected to account for 43% of all malignancies diagnosed in males in 2020. Breast, lung, and colorectal cancers are the most frequent malignancies in women. The annual rate of new cancer cases (cancer incidence) is 442.4 per 100,000 men and women (based on 2013–2017 cases). Men have a greater cancer death rate than women (189.5 per 100,000 men and 135.7 per 100,000 women). When comparing groups based on race/ethnicity and gender, African American men have the highest cancer death rate (227.3 per 100,000) while Asian/Pacific Islander women have the lowest (85.6 per 100,000). Cancer is one of the top causes of mortality in the globe. There were 18.1 million new cancer diagnoses and 9.5 million cancer-related deaths globally in 2018. By 2040, the number of new cancer cases per year is predicted to reach 29.5 million, with 16.4 million cancer-related deaths. Cancer rates are often higher in nations with the highest life expectancy, education level, and style of living. Nevertheless, for other cancers, such as cervical cancer, the opposite is true, and the incidence rate is greater in nations with low population density (<https://gco.iarc.fr>)

In most cases, it is impossible to pinpoint why one individual gets cancer while another does not. Yet studies have revealed that some risk factors could raise a person's likelihood of getting cancer. Exposure to chemicals or other substances as well as particular habits are risk factors for cancer. They also comprise factors like age and family history that people cannot change. A potential hereditary cancer syndrome may be indicated by a family history of certain malignancies (32). Cancer is usually caused by physical carcinogens such as radiations, biological carcinogens such as infections from certain viruses, bacteria or parasites and chemical carcinogens such as components of tobacco smoke, alcohol and arsenic. Cancer can be reduced by reducing the use of tobacco, maintaining a healthy immune system. Avoiding and

reducing the consumption of alcohol. Vaccination against HPV and HBV may help. Avoiding exposure to UV rays and pollution may also help in prevention of cancer.

3. BREAST CANCER:

Breast cancer is a disease in which cells in the breast grow out of control. Breast cancer can begin in the ducts and lobules of the breast. Breast cancer can spread to other organs through blood vessels and causes cancer (11) Isolation by size of epithelial tumour cells: A new method for the immunomorphological and molecular characterization of circulating tumour cells (35)

The most common kinds of cancer are; invasive ductal carcinoma and Invasive lobular carcinoma.

- Invasive ductal carcinoma: the cancer cells begin in the ducts and then grow outside the ducts into the other parts of the breast tissues.
- Invasive lobular carcinoma; the cancer cells begin in the lobules and then spread from the lobules to the breast tissue (31)

Breast cancer can be treated in several ways:

1. Surgery/operation by cutting out the cancerous tissue.
2. Chemotherapy; this therapy includes the usage of special medicines or drugs to kill the cancer cells.
3. Hormonal therapy; this therapy blocks cancer cells from getting the hormones for their growth.
4. Biological therapy; it works within our immune system to help it fight against the cancer cells.
5. Radiation therapy; using high energy rays to kill cancer cells.

4. BIO ACTIVITY OF *PLEUROTUS OSTREATUS*

1.4.1.OYSTER MUSHROOM AGAINST BREAST CANCER:

A morphological transition from flattened and star-shaped cells to elongated ones occurred when the breast cancer cell lines MDA-MB-231 cells and MCF-7 cells were exposed to a ethanol extract prepared from the fruiting body of *Pleurotus ostreatus* (0-1.0 mg/ml) for 24 to 48 hours. The cell cycle was arrested at the G0/G1 phase, according to flow cytometry data. Cell proliferation and growth were inhibited

by the upregulation of the genes p21, p53, p27, and p19 caused by methanol extract, and by the downregulation of E2f transcription factor 1, PCNA, CDK4, CDK6, and Transcription factor DP-1 (7) . After 48 hours of exposure to the ethanol extract of *Pleurotus ostreatus*, MCF-7 and normal Vero cells showed signs of oxidative stress, internucleosomal DNA breakage, and loss of mitochondrial membrane potential.

Oral administration of ethanol extract to DMBA induced cancer carrying rats led to significant decrease in tumour volume and an increased body weight however there was no alteration in food and water intake and other behavioural patterns of animals. From this it was concluded that ethanol extract induced cytotoxicity via apoptotic pathway and is devoid of any kind of toxic manifestation. Ethanolic extract also down regulated oestrogen and progesterone receptors which may have been due to presence of ergosterol which probably acted as anti-oestrogen block in receptor mediated pathways. COX2 expression was also inhibited resulting in prostaglandin cascade and hence tumour suppression (48). Lovastatin present in methanolic extract inhibited angiogenesis and metastasis through inhibition of MMP-2 and MMP-9 expression in 4T1 metastatic breast cancer cell line (49).

β -Glucan derived from Oyster mushroom when supplemented to rats carrying DMBA induced breast cancer, resulted in lower tumour incidence, tumour volume and reduction in total number of tumour nodules (38). Pleuran, an active glucan, has been detected in *P. ostreatus* extracts. This is described as an immunomodulatory drug with potential uses for treating cancer, infections, and immune system issues (20).

1.4.2. ANTIBACTERIAL ACTIVITY OF OYSTER MUSHROOM:

It's easy to cultivate *Pleurotus ostreatus*(oyster mushroom) at 15-30°C and use them as food, feed, vitamins, enzymes and several pharmaceuticals, as well as for waste degradation and detoxification. They're one of the most popular edible mushrooms because they're easy to grow at a broad range of temperatures. (28, 43) In tests by Vamanu7, *P. ostreatus* extracts showed narrow antibacterial activity against Gram-negative and Gram-positive bacteria including *Escherichia coli* and *Bacillus cereus*. Aqueous or alcohol extracts of medicinal mushrooms have the most antibacterial properties (33,10).Ethanol extracts from *P. ostreatus* are the most effective in inhibiting and lysing *E.coli* and *K. pneumonia*. Cell wall glucans are well known for their immunomodulatory effects, and many of the externalised secondary metabolites fight bacteria, fungi, and viruses (60).

1.4.3. ANTIOXIDANT ACTIVITY OF OYSTER MUSHROOM:

All living organisms require oxidation to generate energy to power their biological functions. Free radicals with an oxygen center and other reactive oxygen species, which are constantly created in vivo, may cause cell death and tissue loss. These free radicals' oxidative damage may be linked to aging and diseases like atherosclerosis, diabetes, cancer, and cirrhosis (12). With the help of oxidative enzymes like superoxide dismutase (SOD) and catalase (CAT) as well as chemical components like tocopherol, ascorbic acid, carotenoids, polyphenolic compounds, and glutathione, almost all organisms are well-equipped with a variety of defense mechanisms against free radical damage [21, 41]. These systems can't completely prevent damage (58). Nevertheless, antioxidant pills or foods that contain antioxidants to prevent or to reduce oxidative damage.

Natural antioxidants are being examined extensively for their ability to protect organisms and cells against oxidative stress, which is thought to be a cause of aging and degenerative diseases. The phenolic components, including phenolic acids and flavonoids, carotenoids, tocopherol, and ascorbic acid, which are abundant in diets, particularly vegetables, are significant protective agents for human health. These chemicals are abundant in mushrooms (29) and spectrophotometric methods (53). are used to assess their activity. Electrochemical approaches have gradually been developed and tested as an alternative tool for the evaluation of various food extracts, defined in terms of 'antioxidant power,' due to their speed, simplicity, and low cost (36,2,3).

Nutraceuticals are substances that have the potential to be consumed as food or as a component of food and offer advantages to one's health or wellbeing, such as the treatment and prevention of diseases (59, 22)

Mushrooms have gained popularity as a functional food and as a source for the production of pharmaceuticals and nutraceuticals (51) due to their antioxidant, anticancer (47) and antibacterial capabilities. The secondary metabolites that mushrooms accumulate include phenolic compounds, polyketides, terpenes, ubiquinone, and steroids (52). The majority of their antioxidative potential is due to phenolic compounds' redox characteristics, which allow them to operate as reducing agents, hydrogen donors, and singlet oxygen quenchers (17). According to the tests, *P.ostreatus* extract showed higher DPPH and higher phenolic content (60).

CHAPTER 2

LITERATURE SURVEY

Pleurotus ostreatus is the second most cultivated mushroom (68). Because of its digestible proteins, minerals and vitamins and bioactive compounds with pharmacological activities, *P.Ostreatus* is an important mushroom species of dietary and medical significance (6). In extracts of *pleurotus ostreatus*, researchers have found an active beta glucan, named pleuran. This acts as an immunomodulatory agent with applications in treatment of cancer and immune system disorders (20).

Pleurotus ostreatus contains 85%-95% moisture (Khan 2010). Fungal cell walls are rich in non-starch polysaccharides, beta glucan and phenolic compounds have many medical applications (78). *Pleurotus ostreatus* and its fruiting bodies are not often attacked by pests and diseases, they can be cultivated in simple and economic ways (45). Mushroom cultivation not only produces medicinal and nutritious food but also increases the straw quality, making it ideal for animal feed.

The main function of rice straw substrate is to provide cellulose, hemicellulose and lignin required for the growth and fructification (88). Good harvest of *P.Ostreatus* was 3-4 weeks after incubation (Quimio et al.1999). Primordial formation of oyster mushrooms appears in 27-34 days of inoculation (70). Water soluble polysaccharides from oyster mushrooms show anticancer properties.

2.1. BIO ACTIVE COMPOUNDS:

β -glucans acquired from mushrooms differ in their structure, water solubility, size of the molecule and molecular weight. They show a very wide spectrum of health-promoting effects (89,27). The effectiveness of β -glucans depends on their solubility .

Pleuran is the best-known β -glucan extracted from oyster mushrooms. It is composed of D-glucose molecules linked with bonds of the type 1 $\rightarrow$ 3 β and 1 $\rightarrow$ 6 β . Extraction with hot water is the common method of acquiring pleuran from fruiting bodies. It can also be obtained from liquid cultures wasser. (56).

The compound shows anti-neoplastic properties against colorectal cancer cells HT-29 (50), prostate cancer cells PC-3 (36) and breast cancer cells MCF-7 (55). It has anti-oxidative and antiviral properties (69).

A protein from *P. ostreatus* is similar to ubiquitin and inhibits HIV-1 reverse transcriptase (79). A protein extract from *P. ostreatus* showed therapeutic effect towards the colorectal cancer cell line SW 480 and monocytic leukemia THP-1 by inducing their apoptosis (80). Mono- and sesquiterpenoids, ergosterol and fatty acid esters are low-molecular weight bioactive compounds found in oyster mushrooms

2.2. BREAST CANCER:

Cancer is a disease in which the cells grow out of control. Usually cells grow, multiply, and die, this cycle is continued. But when this cycle breaks and the cells start to grow continuously it forms tumors. This condition leads to cancer (www.cdc.gov) Breast cancer is a disease in which the cells in the breast grow out of control. Breast cancer can spread to other parts of the body through blood vessels and lymph vessels, this is known as metastasis. The most common types of breast cancer are invasive ductal carcinoma and invasive lobular carcinoma. Invasive ductal carcinoma; the cancer cells begin in the ducts of the breast and then grow outside the ducts and spread to other parts. Invasive lobular carcinoma; the cancer cells begin in the lobules of the breast and then spread from the lobules to the other breast tissues (www.cdc.gov). Most deaths from breast cancer are not due to the primary tumor itself, it's the result of metastasis in other parts of the body.

Metastasis starts with the invasion of tumors of surrounding host tissue and continues until the tumor cells invade and intravasate into blood and lymphatic vessels (72). E- cadherin (Epithelial cadherin) is a calcium – regulated adhesion molecule present in most normal epithelial tissues (73,11). E-cadherin maintains cell – cell junctions, E- cadherin is known as a determinant of growth of metastatic breast cancer cells (83). According to WORLD HEALTH ORGANIZATION (WHO), the percentage of developing breast cancer is 12.38% in an American female. The mortality rate due to breast cancer has declined in the USA from 1975 to 2000. (Berry D et al 2005). Breast cancer is most common in south Asian developing countries, due to overpopulation (26).

Risk factors of breast cancer include; Menarche and the menstrual cycle, Childbearing, Breastfeeding, Menopause, Endogenous hormones, Oral contraceptives, Hormonal therapy for the menopause, Alcohol and smoking. Alcohol consumption is associated with a moderate increase in the risk of breast cancer. Women who have had at least one full-term pregnancy helps in reduction of breast-cancer risk (64). Because of increased cases of breast cancer, the death rate of the affected women patients has increased. About 48000 death cases were recorded from 1987 to 2006. Most deaths of breast cancer patients are caused due to infection in the pulmonary part of the body. Some die due to organ failure. The treatment of breast cancer metastasis can be divided into standard chemotherapy and targeted therapy. Cytotoxic drugs used in chemotherapy for metastatic breast cancer include anthracyclines, taxemes and 5 – fluorouracil (5). Tamoxifen is an agent that blocks the estrogen receptor, used as a hormonal therapy, it results in tumor regression (Alvarez RH ,2010).

Other ways to prevent breast cancer is to make changes in lifestyle by following good habits like avoiding alcoholic beverages. Accumulation of more body fats could also lead to cancer. (World cancer research fund international) Exercising could reduce the chances of cancer. Leading a healthy lifestyle and following a balanced and healthy diet could prevent this disease naturally without any other preventive methods (65).

Most of the tumor when tested will show biological expression of some receptors. Neoadjuvant treatment is commonly given for breast cancer. This therapy is based on the presence of oestrogen receptor (ER), progesterone receptor (PR) and Human Epidermal Growth Factor Receptor-2 (HER2) receptor in tumor (37). 2ER

expression is said to be a strong predictor of response to endocrine therapy (23). ETR plays a major role in mediating the effects of endogenous hormones and therapeutic agents. Jensen and Jacobson were the first to observe the retention of radiolabelled oestrogen in hormone response targeted tissue (39).

2.3. ANTI-TUMOR PROPERTIES:

Till date, mushrooms are the untouched and unused sources of powerful and new pharmaceutical products (85). About fifty species of mushrooms have potential immune ceuticals that exhibit antitumor activities. About six polysaccharides have been found to have antitumor activities. They are – Lentinan, Schizophyllan, Polysaccharide-K (PSK), Polysaccharide-P (PSP), Active Hexose Correlated Compounds (AHCC), Maitake D fraction (20).

Polysaccharides extracted from *P. ostreatus* show anti-tumor properties in breast cancer cell lines which have the ability to arrest the growth and multiplication of cancer cells. (25). Polysaccharide (POPS-1) from *p.ostreatus* showed anti tumor activity against Hela tumor cells and human embryo kidney 293T cells in vitro (74). Different polysaccharide extract from mycelium of *P.ostreatus* inhibits the development of Ehrlich Tumor (ET) and Sarcoma 180 (S180) (82).

Antitumor properties of *P. ostreatus* are related to the production of heteroglucans (81). Gemmotherapy extract of young parts of *P.ostreatus* shows anti tumor activity on the HTC –116 cell line (Colorectal Carcinoma cell line) . Polysaccharides extracted from *p.ostreatus* mycelium and a compound *Pleurotus ostreatus* mycelium polysaccharide- (POMP-2) tested in male BALB/c (nu/nu) nude mice showed decrease in tumor weight, compared to in vivo (18). Anti-cancer activity of *P.ostreatus* against human androgen – independent prostate cancer PC-3 cells was screened by GU and YH (30). An aqueous polysaccharide extract from the *P.ostreatus* shows anti –proliferative and pro – apoptotic effects on HT-29 colon cancer cells (50). Hot water extract of *P.ostreatus* showed suppression in proliferation of MCF –7 human breast cancer cells (54).

Methanol extract from *P.ostreatus* suppressed the proliferation of breast cancer (MCF –7, MDA-MB-231) and colon cancer (HT-29, HCT-116) cells. *P.ostreatus* showed the expression of the tumor suppressor p53 in MCF-7 and HT-29 cells (42). Protein extract from oyster mushrooms showed effects against human colorectal adenocarcinoma cell line (SW480 cells) and human monocytic leukaemia cell line

(THP-1 cells), (86). The protein extract from oyster mushrooms is considered an important source of a new anti-cancer drug.

2.4. ANTIOXIDANT PROPERTIES:

P.ostreatus are rich in vitamin and selenium content that are natural antioxidants (Chang and Miles 1989) *40. The ethanolic extract of *P.ostreatus* shows antioxidant activity in both in-vitro and in-vivo. It also shows good in-vivo antioxidant activity by enhancing the activities of enzymatic and non-enzymatic antioxidants (75). *P.ostreatus* showed antioxidant properties due to the presence of carbohydrate components (BETA-GLUCANS) that are responsible for antioxidants activity. *P.ostreatus* serves as a good source of the antioxidant (Payel mitra et al 2013).

5. ANTI BACTERIAL:

With a minor modification, the agar-well diffusion technique was used to investigate the antibacterial activity of *P.ostreatus* extracts (4). Using nutrient broth, an overnight culture of each microbial isolate was emulsified to a turbidity of around (10*8) McFarland cfu/ml, or 0.5. Then, to achieve a confluent growth, 100 l of each standard inoculum was streaked on nutritional agar and PDA at 10 6 cfu/ml per spot (9). Using a sterile cork borer, wells were drilled into the agar and filled with 100 L of *P.ostreatus* organic extract. According to the previous description, the plates were incubated. The control organism for bacterial testing was the reference strain of *E. coli* ATCC 25922. With a minor modification, the agar-well diffusion technique was used to investigate the antibacterial activity of *P.ostreatus* extracts (4). Bacterial and fungal control wells contained 100 l of ciprofloxacin and fluconazole, respectively, at 5 g per well. Each microbial isolate overnight culture was emulsified with nutrients. In every test, a well containing 100 l of Tween-20-PBS solution (pH 7.2) was typically utilized as a positive control well. Inhibitory zone diameters to the closest 0.1 mm were used to measure growth inhibition.

6. GAS CHROMATOGRAPHY MASS SPECTROSCOPY:

GC-MS can be used to study liquid, gaseous or solid samples. Analysis begins with the gas chromatograph, where the sample is effectively vaporized into the gas

phase and separated into its various components using a capillary column coated with a stationary (liquid or solid) phase.

According to Egra et al. (2019) (24), *P.ostreatus* mycelium water extract exhibited the greatest ability for cytotoxicity via apoptosis induction in human cancer cells. More so than other commercialized mushrooms, *P.ostreatus* fruiting bodies' methanol extract was found to exhibit stronger antioxidants, reducing power, radical scavenging, and iron chelating properties (87). Okafor et al. (2017) claim that dangerous bacteria like *Bacillus subtilis* and *Pseudomonas aeruginosa*, as well as pathogenic fungi like *Aspergillus niger* and *Fusarium oxysporum*, are prevented by p-anisaldehyde found in hexane-dichloromethane extracts of *P.ostreatus* (62).

CHAPTER 3

AIM:

The aim of the research is to explore the applications of the edible mushroom – *Pleurotus ostreatus* .

OBJECTIVES:

(i)To study the antibacterial activity, anti cancer and antioxidant activity of the oyster mushroom (*Pleurotus ostreatus*).

(ii)Evaluation of antimicrobial activity of the *Pleurotus ostreatus* by Agar Disc Diffusion Method and Well Diffusion Method.

(iii)Chemical screening of crude active compounds to identify the functional groups of *Pleurotus ostreatus*.

(iv)Identification of bioactive compounds from *Pleurotus ostreatus* by Thin Layer Chromatography, GCMS, FTIR

CHAPTER 4

MATERIALS AND METHODOLOGY

1. MUSHROOM CULTIVATION:

The materials used are:

- Paddy straw
- Mushroom spawns
- Coir pith
- Coffee grounds
- Mushroom growth bags
- Spray bottles
- Knife

4.1.1. SUBSTRATE PREPARATION:

Mushrooms need a good substrate for growth. Good substrate includes moisture, humid temperature, and dark places. The substrate, paddy straw, coir pith

and coffee grounds should be autoclaved, and shade dried. The paddy straw should have moisture in it.

2. SPAWNING:

The mushroom growth bag is tied on one end and then, the paddy straw is rolled tightly and inserted into the bag. Four layers should be made. The mushroom spawns are sowed on the sides of the bags up to 3 layers. Make random holes on the bag and hang it down in a dark place.

3. HARVESTING:

Mycelium growth was observed on 7 days of inoculation, primordial formation was observed on 20-21 days of inoculation and mature fruiting bodies were observed on 25-27 days. The mature fruiting bodies should be cut with a sterile knife and stored in a – 20°C freezer.

2. EXTRACT PREPARATION:

The materials used are:

- Oyster mushroom
- Mortar pistol
- Beaker
- Ethanol
- Distilled water

1. MUSHROOM POWDER:

Oyster mushroom was dried in 2 ways. One is by freeze drying, in which the harvested mushroom is kept in – 80°C and then kept for freeze drying for 24 to 48 hrs. Another procedure is by cutting the mushroom into small pieces and keeping it under sunlight for 1 hr for sun drying, then it is kept under the shade for shade drying for 24 to 48 hrs. The dried mushroom is then crushed into fine powder in a mortar pestle.

2. ETHANOL EXTRACT:

The powdered oyster mushroom was mixed with ethanol to a concentration of 1:10 i.eThe mushroom weighed 1 gram and mixed in 10 ml of ethanol. The obtained mushroom powder was 8g, so it was mixed with 80 ml of ethanol. The mixture is then kept in a shaker incubator for 24hrs. Then the mixture is filtered, and the obtained filtrate is the ethanol extract of *Pleurotus ostreatus*.

4.2.3. WATER EXTRACT:

In the water extract of oyster mushrooms the concentrations slightly differ from the ethanol extract. In this about 10 mg of oyster mushroom powder is mixed with 10 ml of distilled water.

3. CELL LINES CULTURE:

The materials used are:

- DMEM- Dulbecco's Modified Eagle Medium
- Complete medium includes DMEM- 89%
- FBS (Fetal Bovine Serum)- 10%, antibiotics-1%
- Plastic flasks (T25, T75 flasks)
- Centrifuge tubes
- Centrifuge
- Microscope
- CO2 incubator and fridge
- Biosafety Cabinets.

4.3.1 METHODOLOGY:

Cell culture is a process of growing animal cells outside the body under controlled conditions. Cell lines are continuous cells. The MCF-7 cell lines were cryopreserved from the sub cultured cell lines. The cell lines are thawed, and should

be warmed rapidly by removing an ampule of the liquid nitrogen and placing it onto a 37°C water bath. Ampules should be washed with 70% ethanol.

Once thawed, the cells should be added to the DMEM in a centrifuge tube. It should be centrifuged for up to 3 minutes. The MCF-7 cells are settled down as pellets. Remove the Supernatant and pipette out the pellets gently. Add the pellet of cells onto the T25 flask with DMEM (complete medium). T25 flask contains 3-5 ml of DMEM and T75 flask contains 15-20 ml of DMEM. The cells should be equally added to the T25 and T75 flask and shake it gently. The flask should be kept in a CO₂ incubator with 5%CO₂ and 35 to 37°C temperature. The flask should be slightly opened before incubation, so that the cell can respire, vented flasks can be kept for incubation without opening the cap. After 48 hrs the cells started growing and should be observed daily.

4. MTT ASSAY:

To perform an MTT assay with oyster mushroom extract, you will need to first extract the bioactive compounds from the mushroom. Here is a general procedure for the MTT assay with oyster mushroom extract

Materials used are:

- Oyster mushroom- ethanol extract.
- Centrifuge tubes
- Pipettes and tips
- MTT solution (5 mg/mL in PBS or DMEM)
- Cell culture medium
- PBS (phosphate-buffered saline)
- DMSO (dimethyl sulfoxide)
- 96-well plate
- Incubator
- Microplate reader

4.4.1.PROCEDURE:

1. Culture cells in appropriate medium until they reach the desired confluence.
2. Aspirate the medium and add the oyster mushroom extract to the cells in triplicate wells.
3. Incubate cells with the extract for the desired length of time (usually 24-72 hours).
4. Remove the medium and rinse cells with PBS.
5. Add MTT solution to each well (enough to cover cells).
6. Incubate the plate for 2-4 hours at 37°C in a humidified atmosphere containing 5% CO₂.
7. Remove the MTT solution and add DMSO to each well (enough to dissolve the formazan crystals).
8. Gently rock the plate to ensure complete dissolution of formazan crystals.
9. Measure the absorbance of each well at 570 nm using a microplate reader.

5. ANTIBACTERIAL ACTIVITY:

The materials used are,

- Muller Hinton Agar
- Petri plates
- Bacterial broth culture swabs
- Discs / well cutter
- Forceps.

4.5.1 METHODS:

Sterilize the conical flasks and petri plates with cotton and ethanol. Weigh 3.8 grams of Muller Hinton agar and put it in the conical flask and mix in 100 ml of distilled water. Sterilize the media and allow it to cool. Wipe and clean the Laminar Air Flow. Sterile swab is dipped into the bacterial inoculum and swabs the surface of the plate completely by rotating the plate. bacterial samples like *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Enterococci* are used for the

antimicrobial activity Allow the places to dry for 5 minutes. This can be done by two methods- disc and well diffusion method .

1. DISC DIFFUSION METHOD:

In the disc method the discs are dipped in the ethanolic extract of oyster mushroom with concentration 25 μ l, 50 μ l, 75 μ l and 100 μ l and negative control of 25 μ l ethanol disc. Allow it to dry. Sterilize the forceps and place all the discs with equal distances on the Petri plate. Finally, incubate the plate upside down for 24 hours at 37°C.

2. WELL DIFFUSION METHOD:

In this method, the agar is poured in a petri plate and allowed to dry for 10 to 15 minutes. Now take desired bacterial sample is swabbed on the agar. Then well is cut using a well cutter or pipette tips . Take the cut agar well part using a inoculum loop. Then add the mushroom extract in the well with concentration of 25 μ l, 50 μ l, 75 μ l, 100 μ l, and pour 25 μ l of ethanol at the center for negative control. Finally incubate the plates for 24 hours at 37°C.

After incubation, zones are observed around the discs or wells. This denotes the antimicrobial activity results are positive. The zones are measured using a scale to see the activity of the added mushroom extract. If there is no zone formation , the result is negative.

6. ANTIOXIDANT TEST:

Materials required are

- 96 well plate
- Pipette
- 0.0024g of DPPH-alpha diphenyl, beta- picrylhydrazyl,
- Ethanol
- Methanol
- DMSO- dimethyl sulfoxide,

- Oyster mushroom extract,
- UV spectrophotometer.

The DPPH assay (2,2-diphenyl-1-picrylhydrazyl) is a widely used method for measuring the antioxidant activity of compounds. This assay involves the use of a stable free radical, DPPH, which changes colour from purple to yellow when reduced by an antioxidant compound. The degree of colour change or reduction in absorbance indicates the scavenging activity of the antioxidant. The higher the degree of colour change, the higher the antioxidant activity.

1. PROCEDURE:

1. The DPPH assay should be performed in a dark room as DPPH is photosensitive.
2. To perform the DPPH assay, a solution of DPPH is prepared in a solvent such as ethanol or methanol. 0.0024 g of DPPH is added to 50 ml of methanol and mixed.
3. Now different concentrations of samples are added in the well from above to below., ie 40 μ l of extract is added in the first well, then 30 μ l of extract is added to the well below to the first well. Add the other concentration 20 μ l, 10 μ l of samples in the wells in same manner. A triplicate method is used for reducing the error while performing the test.
4. The samples are diluted with different concentrations of distilled water by adding it in reverse to the concentration of samples., i.e. 40 μ l of water to the well containing sample concentration of 10 μ l. Now increase the water concentration to the next sample.
5. After diluting the samples, 200 μ l of DPPH is added to the well containing samples.
6. Blank and control wells are prepared on the first two rows of the plate.
7. Then incubate the well plate for 1 hour, at room temperature in a dark room.
8. After incubation, you could observe the colour of the samples change from violet to yellow or colourless.
9. Then run the plate in a UV spectrophotometer at a wavelength of 515 nm.
10. The readings are interpreted in an excel sheet. Then readings are calculated and the results are represented as a graph.

7. PHENOLICS TEST:

Phenolics are a group of organic compounds that are widely distributed in plants and are known for their antioxidant and health-promoting properties. The most common method for testing the phenolic content of a sample is the Folin-Ciocalteu assay, also known as the FC assay.

Materials used for the phenolics test are

- Oyster mushroom extract
- Gallic acid,
- Folin-ciocalteu reagent,
- Sodium carbonate ,
- 96 well plate
- UV spectrophotometer.

1. METHODS:

1. To perform phenolics test or FC assay, FCP reagent is prepared in 1:10 ratio. i.e. 1 ml of Folin-Ciocalteu is mixed in 9 ml of distilled water.
2. Now different concentrations of samples are added in the well from above to below., ie 40 μ l of extract is added in the first well, then 30 μ l of extract is added to the well below to the first well. Add the other concentration 20 μ l, 10 μ l of samples in the wells in same manner. Duplicate method is used for reducing the error while performing the test.
3. The samples are diluted with different concentrations of distilled water by adding it in reverse to the concentration of samples., i.e. 40 μ l of water to the well containing sample concentration of 10 μ l. Now increase the water concentration to the next sample.
4. After diluting the sample, 100 μ l of Folin-Ciocalteu reagent is added to the samples and left aside for 1 minute.
5. Then slowly add 60 μ l of sodium carbonate to the sample .
6. Then add different concentrations of gallic acid, the same as the sample in the first row of the plate and dilute it with distilled water, the same as the samples. Gallic acid is added to find the control for the test.

7. Then add 100µl of Folin-Ciocalteu and add 60µl of sodium carbonate to the gallic acid.
8. Bubbles are observed while adding sodium carbonate to the sample and gallic acid.
9. Then incubate the well plate for 1 hour, at room temperature. After incubation, run the plate in a UV spectrophotometer at a wavelength of 750 nm.
10. The readings are interpreted in an excel sheet. Then readings are calculated and the results are represented as a graph.

4.8. TEST FOR FLAVONOIDS:

Flavonoid test is a series of chemical tests used to detect the presence of flavonoids in a given sample. Flavonoids are a group of plant compounds that are known for their antioxidant and anti-inflammatory properties. The test can be used to identify the presence of flavonoids in the *Pleurotus ostreatus* mushroom extract.

Materials used for the test are Oyster mushroom ethanol extract, Sodium nitrate, Aluminium chloride, 1 molar solution of sodium hydroxide, 96 well plate.

1. METHODS:

1. To perform flavonoids test, first the main reagents required for the test are prepared .i.e. 5% sodium nitrate solution, 10% aluminium chloride, 1 molar of sodium hydroxide solution.
2. To prepare 5% of sodium nitrate solution, 0.5 g of sodium nitrate is dissolved in 10 ml of distilled water.
3. For 10% of aluminium chloride solution, 1 g of aluminium chloride is dissolved in 10 ml of distilled water.
4. Then 1 molar of sodium hydroxide solution is prepared by dissolving 0.4 g of NaOH pellets in 10 ml of distilled water.
5. Now different concentrations of samples are added in the well from above to below. 80µl of extract is added in the first well, then 70µl of extract is added to the well below to the first well. Add the other decreasing concentration of

samples in the wells in same manner. Samples are added in a triplicate method to reduce errors.

6. The samples are diluted with different concentrations of distilled water by adding it in reverse to the concentration of samples. 50 μ l of water to the well containing a sample concentration of 13 μ l. Now increase the water concentration to the next sample.
7. After dilution of samples 30 μ l of 5% sodium nitrates solution is added in each well containing the sample and leave it for 5 minutes.
8. Then add 30 μ l of 10% of aluminium chloride solution to the wells and leave it aside for 6 minutes.
9. Add 60 μ l of 1 molar solution of sodium hydroxide to the sample and incubate it for 30 mins.
10. After incubation, run the plate in a UV spectrophotometer at a wavelength of 510 nm.
11. The readings are interpreted in an excel sheet. Then readings are calculated and the results are represented as a graph.

4.9.THIN LAYER CHROMATOGRAPHY(TLC):

Thin layer chromatography (TLC) is a chromatography technique used to separate and analyse different components of a mixture. It is a simple and rapid method that uses a thin layer of adsorbent material, typically silica gel or alumina, coated onto a glass, metal or plastic plate.

1. TLC PLATE PREPARATION:

- Cut the TLC sheet: Use scissors or a cutting tool to cut the TLC sheet or plate to the desired size .i.e. Measure the height of the beaker and cut the plate up to the beaker's height and diameter.
- Typical sizes for TLC sheets are 5 cm x 10 cm or 10 cm x 20 cm. After cutting the TLC sheet, draw two lines on the top and bottom of the TLC sheet leaving 0.5 cm gap from the edge. Then mark the spots with pencil where the samples are to be added.

4.9.2 SAMPLE PREPARATION:

The sample to be used for TLC should be a concentrated solution of sample and solvent. 100mg of oyster mushroom extract powder is mixed in 1 ml of ethanol.

3. MOBILE PHASE PREPARATION :

- Mobile phase is the one that moves and consists of a solvent mixture or a solvent. This phase should be particulate-free. This phase carries the bio components across the TLC plate.
- The solvents used in this TLC procedure are toluene, ethyl acetate, formic acid and methanol. The mobile phase is prepared in a beaker with the ratio of 7T: 2EA: 1FA .i.e. 7 ml of Toluene is mixed with 2 ml of Ethyl Acetate and 1 ml of Formic acid.

4. STAIN PREPARATION:

Staining is a method for the visualisation of organic compounds on a TLC plate. Many stain methods are used in TLC. Here, two staining methods are used for visualising organic compounds.

SULPHURIC ACID STAIN: Weigh 1 gram of ceric sulphate and 2.5 grams of ammonium molybdate and put it in a clean beaker. Measure 90 ml of distilled water and add it to the mixture. Pipette out 10 ml of sulphuric acid and slowly release on the wall of the beaker and mix well.

IODINE VAPOUR STAIN: The TLC plate is stained using granules of Iodine Resublimed. The vapours from the granules stains the TLC plate. Place a TLC sheet in a beaker or glass container and add some granules of Iodine Resublimed near to the bottom of the sheet and close the container tightly with the aluminium foil.

4.9.5 PROCEDURE:

1. Take the TLC plate which is cut with required length and width and place it over a clean surface.
2. Now add the concentrated solution of the sample over the dots marked by a pencil on the TLC plate, and let it dry.
3. A small amount of the sample is then applied to the dots marked by a pencil on the TLC plate, using a micropipette or a capillary tube. Let the sample dry.

(NOTE) The sample should be spotted at a specific distance from the bottom of the plate to ensure that the separation occurs in a defined region.

4. After the spot is dried, again add the sample over the dot. Repeat it for another 10 to 15 times until all the compounds are deposited on the spot. And let it dry.
5. Then, the TLC plate is placed on the mobile phase (solvent) at 45° angle and allows the solvent to transport. Tightly close the lid of the beaker with an aluminium foil.
6. Leave it aside, the solvent starts to move from the bottom to the top on the TLC plate.
7. After the solvent reaches the peak, take it out and air dry for a few minutes.
8. View it under UV radiation for any fluorescent compounds present.
9. For viewing the compounds in the staining method, the plate is soaked in the sulphuric acid stain, and heat the plate over a hot plate, at 100°C for a few seconds and observe for spots (unknown compounds).

10. GAS CHROMATOGRAPHY MASS SPECTRUM- GC MS:

Gas chromatography-mass spectrometry (GCMS) is an analytical technique used to identify and quantify chemical compounds based on their separation and detection by gas chromatography and mass spectrometry.

GCMS is a powerful technique for analysing a wide range of volatile and semi-volatile compounds, including organic molecules, inorganic molecules, and biomolecules. It is used in a variety of fields, including environmental analysis, pharmaceuticals, food and beverage analysis, forensic science, and many others.

1. PROCEDURE:

The GCMS procedure involves several steps:

1. **Sample preparation:** The sample must be prepared for analysis by appropriate extraction, purification, or derivatization methods to ensure that it can be volatilized and separated by gas chromatography. So the mushroom

extract is dissolved in either DMSO or ethanol and the procedure is performed.

2. **Injection:** The prepared sample is injected into the gas chromatography column, which separates the individual components of the mixture based on their differing physical and chemical properties.
3. **Separation:** The separated components are carried through the column by a gas mobile phase and exit the column at different times, resulting in a chromatogram that reflects the composition of the original sample.
4. **Ionization:** The separated components are then ionized in the mass spectrometer, typically by electron ionization or chemical ionization, which generates a mass spectrum for each component.
5. **Detection and analysis:** The mass spectrum is then analyzed to identify the individual components based on their characteristic fragmentation patterns and molecular weight. Quantitative analysis can also be performed by comparing the abundance of each component to an internal or external standard.

Interpretation and reporting: The results are interpreted and reported, typically including the identified compounds, their concentrations, and any other relevant information.

11. FTIR- FOURIER TRANSFORM INFRARED SPECTROSCOPY:

Fourier transform infrared (FTIR) spectroscopy is a technique used to obtain information about the molecular vibrations of a sample. In FTIR spectroscopy, infrared radiation is passed through a sample, and the amount of radiation absorbed by the sample is measured. The resulting spectrum can be used to identify the functional groups and chemical bonds present in the sample.

1. PROCEDURE:

The procedure for FTIR spectroscopy involves several steps:

1. **Sample preparation:** The oyster mushroom extract powder is dissolved in a suitable solvent (ethanol) to allow for transmission of infrared light.
2. **Instrument setup:** The FTIR instrument must be set up with the appropriate sampling accessory, such as a transmission cell, attenuated total reflectance (ATR) attachment, or Fourier transform Raman spectrometer (FTR) attachment. The instrument is then calibrated with a reference standard, such as air or a known standard compound.
3. **Baseline correction:** A baseline correction is performed to eliminate any background interference in the spectra.
4. **Spectrum acquisition:** The infrared light is passed through the sample and the resulting spectra are recorded as a function of wavelength. Multiple spectra are typically collected and averaged to improve the signal-to-noise ratio.
5. **Data analysis:** The acquired spectra are analysed to identify and quantify the various components in the sample. This is typically done using software that matches the spectrum to a database of known compounds or functional groups.
6. **Interpretation and reporting:** The results are interpreted and reported, typically including the identified compounds, their concentrations, and any other relevant information.

NOTE: The FTIR procedure is relatively straightforward, but requires careful attention to sample preparation, instrument setup, and data analysis to ensure accurate and reliable results.

4.12. CHEMICAL SCREENING TESTS:

Chemical screening tests are used to identify the presence of specific chemical constituents in the oyster mushroom extract. These tests can be qualitative or quantitative, depending on the goal of the analysis and used to detect the presence of specific groups of secondary metabolites, such as alkaloids, tannins, terpenoids, saponins, etc. These tests involve the use of specific reagents that produce characteristic color changes or precipitates in the presence of these compounds.

1. ALKALINE REAGENT TEST:

Add 100µl of mushroom extract and add a few drops of 20% of NaOH solution. If the solution changes to yellow or colourless solution then it shows the presence of Alkaloids.

2. TEST FOR TANNINS:

Take a few µl of mushroom extract and add a few drops of Ferric Chloride, yellow colour appears in ethanol extract.

3. TEST FOR ANTHRAQUINONES:

Add 100µl of extract and add 2 ml of Ammonia solution. Separated Aqueous layers were observed. Then colour changes to pale pink colour, which shows the presence of Anthraquinones.

4. TEST FOR TERPENOIDS:

Add 100µl of extract and add 2 ml of Chloroform, 3 ml of conc. H₂SO₄. Reddish brown colour indicates the presence of Terpenoids.

5. TEST FOR STEROLS:

Take some extract, add a few drops of conc. H₂SO₄ and shake well. Reddish brown colour indicates the presence of Sterols.

6. TEST FOR SAPONINS:

0.5 g of oyster mushroom powder extract is taken in a test tube containing water. shake it vigorously. Formation of foams indicates the presence of saponins.

4.12.7. TEST FOR ALKALOIDS:

100µl of extract and 1 ml of Wagner's reagent (Iodine in Potassium Iodine) were added. Presence of brown/reddish colour indicates the presence of Alkaloids.

8. TEST FOR PHENOLS:

Add 100µl of extract, 0.5ml of Ferric Chloride, 2.5ml of Sodium Carbonate. Dark blue colour indicates the presence of Phenols.

9. TEST FOR CARDIAC GLYCOSIDE:

Take 1ml of extract and add 0.5ml of Glacial Acetic Acid and Ferric Chloride followed by 1 ml of Conc. H₂SO₄. Blue colour indicates the presence of Cardiac Glycoside

10. FERRIC CHLORIDE TEST FOR FLAVONOIDS:

Take 2 ml of extract and add a few drops of Ferric Chloride. Green-Blue or violet color indicates the presence of the Phenolic Hydroxyl group.

11. MOLISCH'S TEST FOR CARBOHYDRATES:

Few drops of Molisch's reagent dissolved in distilled water and slowly added. Add 1 ml of conc. sulphuric acid by the sides of the test tube. Leave it aside for 2 minutes. Then dilute it with 5 ml of distilled water. Formation of red or violet colour at the interface of two layers shows the presence of carbohydrates.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 MUSHROOM CULTIVATION:

Oyster mushrooms are well known for their nutritional and medicinal properties. They were widely used for various purposes such as food, to treat diseases etc. The first bud of mushrooms were harvested 27 days after spawning. After 3-4 days of the cultivation of the first bud, the second bud of the mushroom was harvested.

YIELD:

The yield of the first bud was 350 grams. The yield of the second bud was 150 grams. The yield of one bag is 500 grams (0.5 kg). (fig 5.1, 5.2)



Mature fruiting body(fig 5.1)

Fruiting body formation(fig 5.2)

5.2 EXTRACT PREPARATION:

The oyster mushroom extract preparation process uses the polar solvent, i.e, Ethanol. Ethanolic extract is made in a concentration of 1g per 10 ml. (fig 5.3)



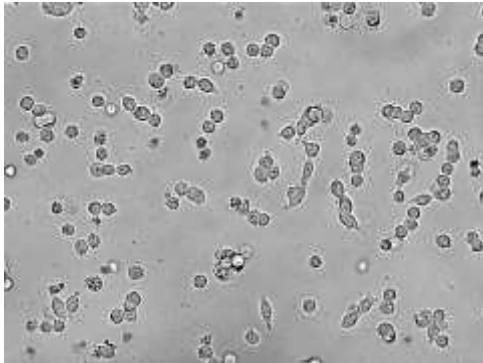
fig 5.3 Ethanol extract

5.3 CELL LINE CULTURE

The cells start to proliferate after 48 hours in the T flask. It should be observed daily for 4to 5 days.The given images show the growth of the cancer cells over the period of time.

In (fig 5.4) the cells start to grow after 48 hours of incubation.

The number of cancer cells increases day by day until it reaches the confluency stage.
(fig 5.5, 5.6)



MCF-7 Cells Fig 5.4

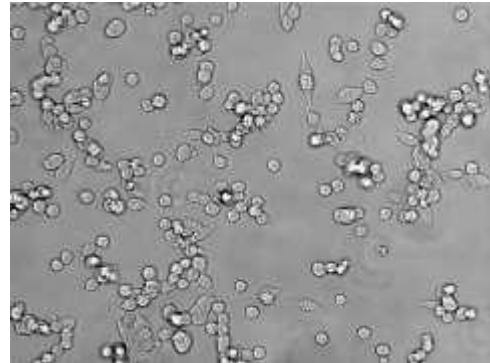


fig 5.5 (after 72 hrs of incubation)

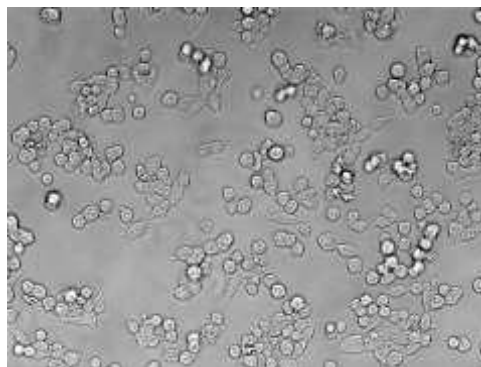


Fig 5.6 (after 3 days of incubation)

5.4 MTT ASSAY:

We could clearly observe the reduction in the cancer cell count after the addition of oyster mushroom extract (fig 5.7, 5.8). This clearly shows that the oyster mushroom has the ability to kill cancer cells.

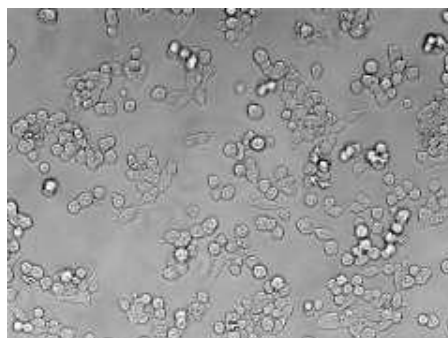


Fig 5.7 (cell culture before adding mushroom extract)

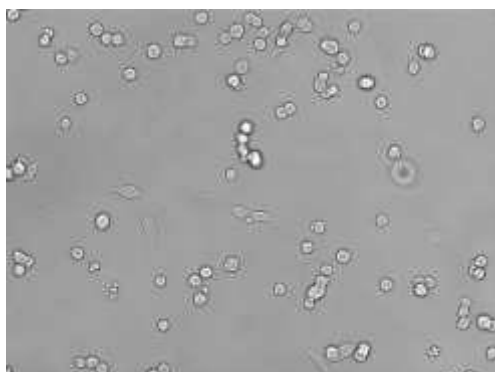


Fig 5.8 (cell culture after adding mushroom extract)

NOTE: The sample was dissolved in 1% DMSO (2 mg/ml conc) as stock. The working was prepared as serial dilution higher conc was 1000 ug/ml (9 conc) were treated with MCF-7 breast cancer cells 24 hrs. Then an MTT assay was performed.

Table 5.1 Readings of MTT assay

Sample conc.(mg/ml)	µg/ml	% Viability
0.004	3.906	80.41
0.008	7.813	74.39
0.016	15.625	71.32
0.031	31.250	67.66
0.016	15.625	64.59
0.125	125.000	42.16
0.250	250.000	35.91
0.500	500.000	33.31
1.000	1000.000	19.98

Table 5.2 - IC₅₀value

IC ₅₀ values µg
33.10 (upto133.8 µg/ml)

The above table 5.1 shows the readings of the MTT assay and the viability of cells. For calculating the IC₅₀ value, subtract the absorbance of the blank wells (no cells) from the absorbance of each well to obtain the corrected absorbance values.

Calculate the percentage of cell viability/proliferation using the following formula: [(mean absorbance of treated wells)/(mean absorbance of control wells)] x 100.

The calculation is done and the values are interpreted on the excel sheet given below. Then the IC₅₀ value is found (table 5.2) and depicted on the graph (fig 5.9)

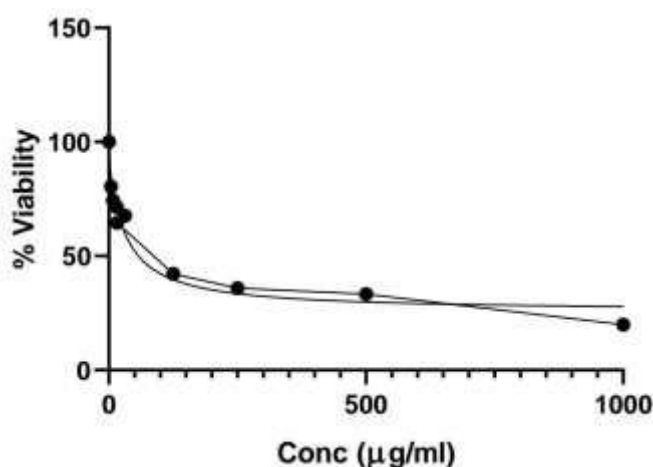


fig 5.9 graph of MTT assay

The IC₅₀ value of the calculated values is 33.10. This confirms that the oyster mushroom has the anti cancer property against breast cancer cell lines.

5.5 ANTIBACTERIAL ACTIVITY:

The *Pleurotus ostreatus* is considered as the most promising source of several antimicrobial drugs. In fact, these mushrooms exhibit significant antibacterial effects against Gram positive and Gram negative bacteria.(table 5.3)

Table 5.3 measurements of the zones

ORGANISMS	CONCENTRATION	ZONE OF INHIBITION
<i>Pseudomonas</i> (fig 5.10)	50µl	0.7 cm
	75µl	2.1 cm
	100µl	3 cm
<i>Enterococci</i> (fig 5.11)	50µl	0.5 cm
	75µl	1 cm
	100µl	2 cm
<i>Salmonella</i> (fig 5.12)	50µl	0.25 cm
	75µl	0.75 cm
	100µl	0.5 cm



fig 5.10 pseudomonas



fig 5.11 enterococci



Fig 5.12 salmonella

5.6 ANTIOXIDANT ACTIVITY:

Oxidative stress caused by free radicals may lead to aging and chronic diseases. Edible Oyster mushrooms contain a wide variety of bioactive compounds such as phenolics, flavonoids, ascorbic acid, glycosides, polysaccharide and carotenoids. The presence of these compounds provide defense against free radicals that reduces oxidative stress. A DPPH radical scavenging activity test is done to prove the antioxidant activity of the edible mushroom, *Pleurotus ostreatus*. This assay is based on the measurement of the scavenging capacity of antioxidants towards it. The 96 well plates with samples (fig 5.13) are run in an UV spectrometer at 515 nm. The readings are interpreted on the excel sheet and it is given in table 5.4.

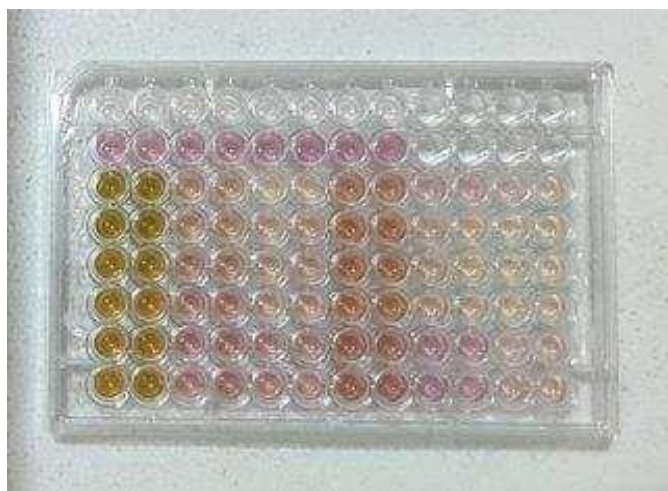


fig 5.13 (DPPH assay)

Table 5.4 Readings of DPPH

Sample 1 reading	Sample 2 reading
0.295	0.26
0.277	0.254
0.183	0.143
0.179	0.132

Table 5.5 Calculation of DPPH

Concentration (μ l)	Sample reading	control	RSA%	IC ₅₀
10	0.2775	0.4125	32.7273	7.09685
20	0.2655	0.4125	35.6364	11.499
30	0.163	0.4125	60.4848	42.3143
40	0.1555	0.4125	62.303	20.3034

The above table 5.5 shows the calculation of DPPH readings. The percentage of the RSA (Radical Scavenging Activity) is calculated using the formula. The B row contains the control.

$$\text{RSA}\% = \frac{[(\text{absorbance of control} - \text{absorbance of sample}) / (\text{absorbance of control})] \times 100}$$

The calculated values are given as graphical representation (fig 5.14)

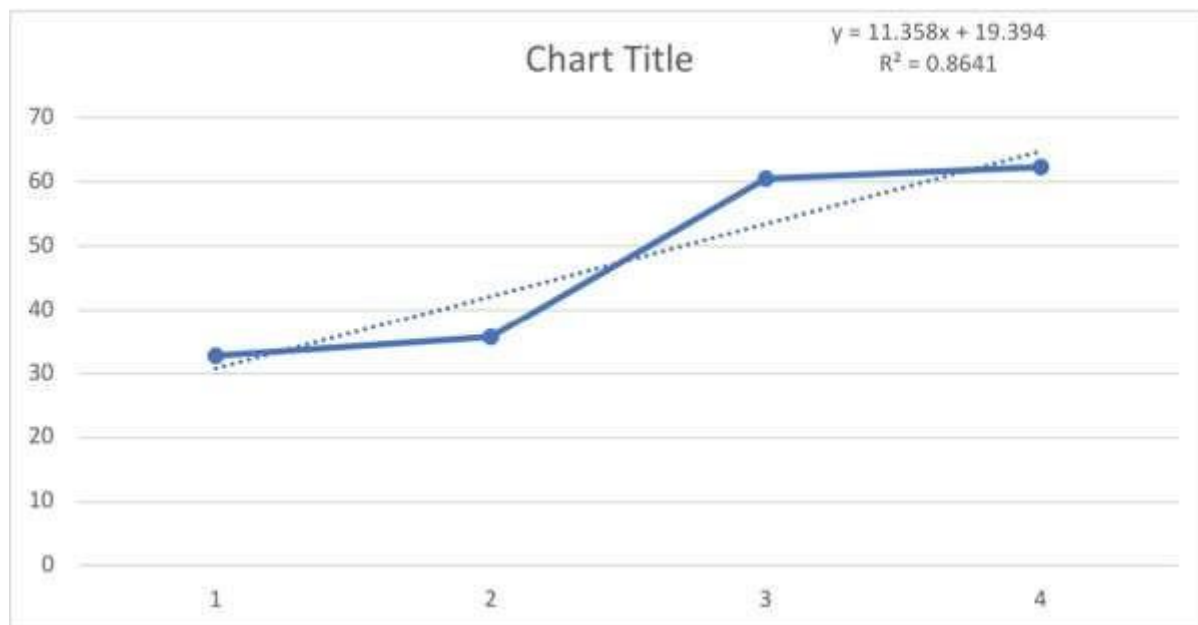


fig 5.14 Graphical representation of RSA%

NOTE: The X axis in the graph denotes the concentration of the sample and the Y axis denotes the RSA percentage.

Then the IC₅₀ value (Half Maximal Inhibitory Concentration) is calculated with the slope from the graph. The slope forms the $y = Mx + b$ equation. The IC₅₀ value is calculated by substituting the values in the equation.

5.7 PHENOLICS TEST :

The phenols test by folin- ciocalteu reagent proves the reduction of folin ciocalteu reagent in the presence of phenols (fig 5.15) that is measured spectrophotometrically at 750 nm. The readings are interpreted in the excel sheet.(fig 5.9)

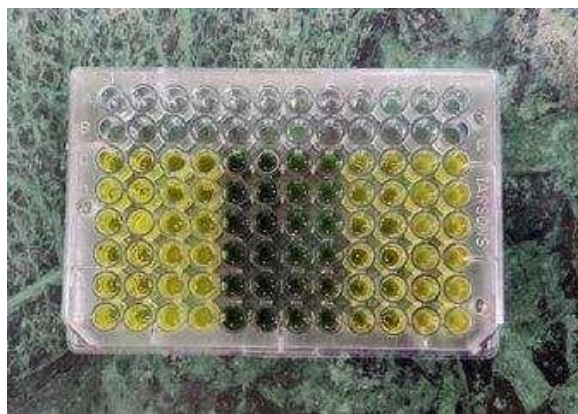


Fig 5.15 Phenolics test

Table 5.6 Readings of phenolics test

Sample 1 reading	Sample 2 reading
0.185	0.203
0.149	0.177
0.044	0.049
0.041	0.04

From the readings given in table 5.6, Calculate the RSA% , we need to find the average values of the samples with different concentration and the control.

Then substitute the values in the formula

$$\text{RSA\%} = \left[\frac{(\text{absorbance of control} - \text{absorbance of sample})}{(\text{absorbance of control})} \right] \times 100$$

After calculating, the RSA%(table 5.7) the values are interpreted in the graph (fig 5.15).

Table 5.7 calculation for phenolics test

Concentration (μ l)	Sample	control	RSA%
10	0.194	0.2005	3.2419
20	0.163	0.2005	18.7032
30	0.0465	0.2005	76.808
40	0.0405	0.2005	79.8005

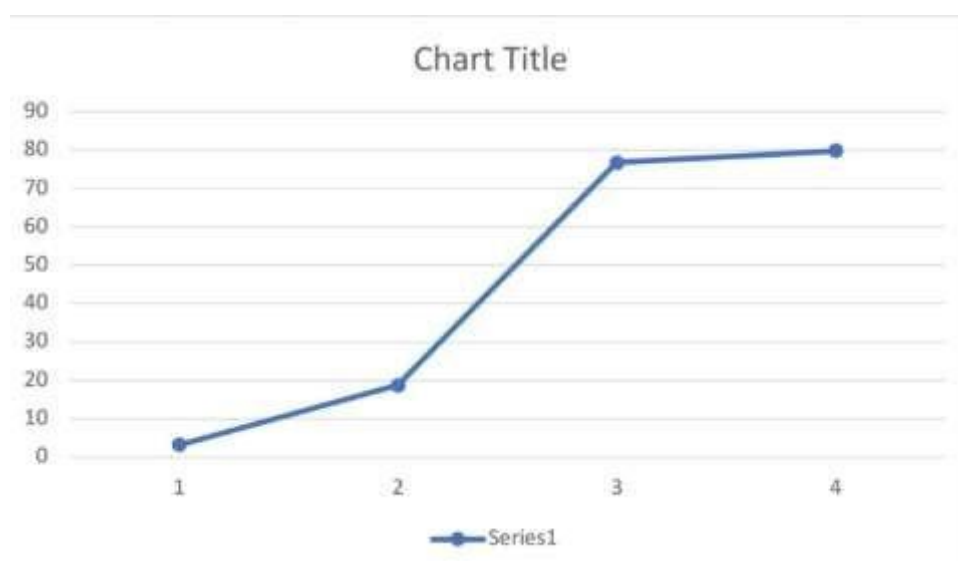


Fig 5.16 Graphical representation of RSA%

NOTE: The X axis in the graph denotes the concentration of the sample and the Y axis denotes the RSA percentage.

5.8 TEST FOR FLAVONOIDS:

The above performed test confirms the presence of flavonoids in the mushroom sample(fig 5.17).The readings are given below table 5.8 after running in a UV spectrophotometer.

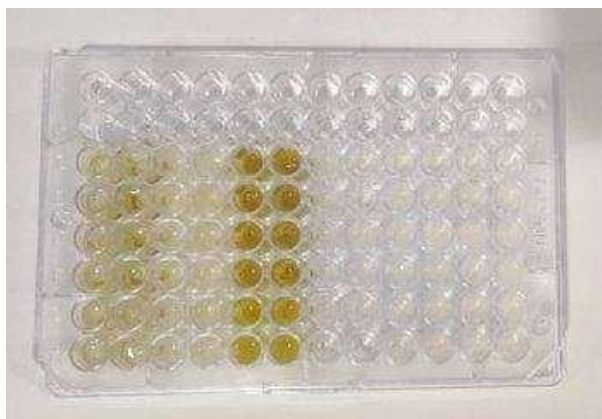


Fig 5.17 test for flavonoids

Table 5.8 Readings of flavonoids test

Sample 1	Sample 2	Sample 3
0.889	0.403	0.222
0.889	0.313	1.108
0.949	0.759	0.872
0.59	1.081	1.018
0.658	1.715	0.757

Calculate the RSA% using the values given in table 5.8 , we need to find the average values of the samples with different concentration and the control.(table 5.9)

Then substitute the values in the formula

$$\text{RSA}\% = \frac{[(\text{absorbance of control} - \text{absorbance of sample}) / (\text{absorbance of control})]}{\times 10}$$

After calculating , the RSA% values are interpreted in the graph(fig 5.18)

Table 5.9 Calculation for flavonoids test

Concentration (µl)	Sample	Control	RSA%
40	0.5046	0.0415	91.7768
50	0.77	0.0415	94.6104
60	0.86	0.0415	95.1744
70	0.896	0.0415	95.37
80	1.043	0.0415	96.0224

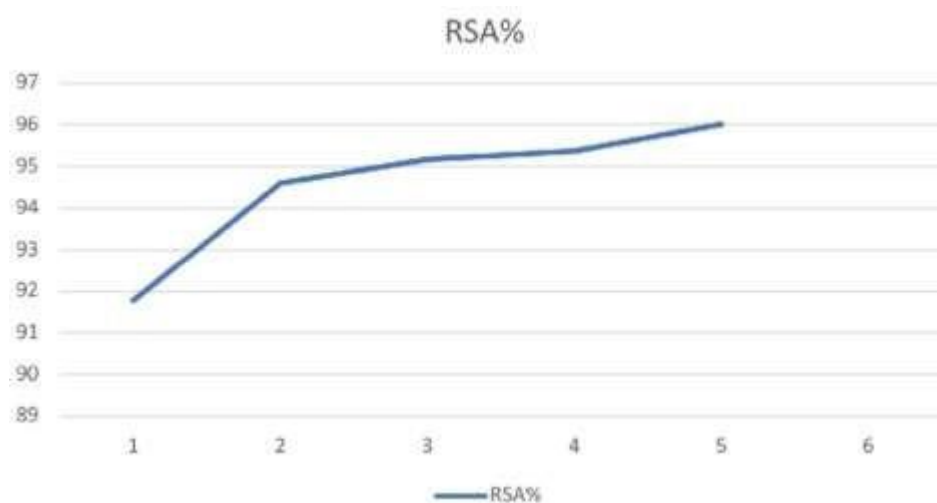


fig 5.18

NOTE: The X axis in the graph denotes the concentration of the sample and the Y axis denotes the RSA percentage.

5.9 THIN LAYER CHROMATOGRAPHY:

After placing the TLC plate in the mobile phase, the mobile phase starts to ascend towards the top of the plate. After running the plate in the mobile phase, the

TLC plate is kept in the UV light to visualize the compounds that are isolated on the plate(fig 5.21). The distance travelled by the compound and the distance travelled by the solvent are measured. The Rf value is calculated by using the formula below;

$R_f = \text{Distance travelled by compound} / \text{Distance travelled by solvent.}$

$R_f = 6.4 / 10.$

$R_f = 0.64.$

TLC is widely used in analysis because of its simplicity, low cost, high sensitivity and speed of separation. The purpose of this has been achieved which is the Rf value was determined for OM sample, the analysis technique by using TLC was successfully performed.



Fig 5.19 TLC plate



fig 5.20 Iodine vapour staining

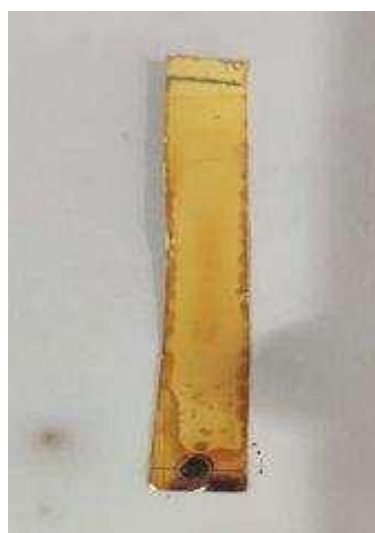


Fig 5.21 TLC in UV light

fig 5.22 compounds visualised on TLC

The compounds on the TLC plate are clearly visualised using iodine vapour staining method.(fig 5.22).

5.10 GC-MS GAS CHROMATOGRAPHY AND MASS SPECTROMETRY

The readings of the oyster mushroom extract for GCMS is given in the graph (fig 5.23). By inferring the values in the graph, the names of the compounds in the sample are given in the (table 5.10)

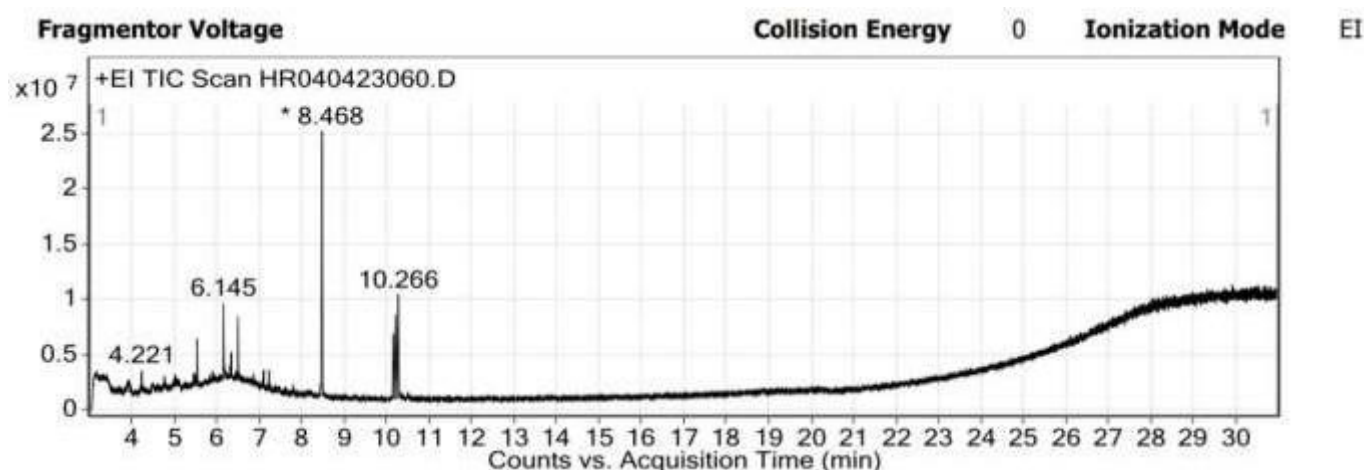


Fig 5.23

Table 5.10 Names of the compounds

S. No	Compound Label	RT	Name	DB Formula	Hits (DB)
1	Cpd1: 1-Ethynyl-3,trans(1,1-dimethylethyl)-4,cis methoxycyclohexan-1-ol	3.907	1-Ethynyl-3,trans(1,1-dimethylethyl)-4,cis methoxycyclohexan-1-ol	C13H22O2	3
2	Cpd 3: 4-(2,5-Dihydro-3-methoxyphenyl)butylamine	4.221	4-(2,5-Dihydro-3-methoxyphenyl)butylamine	C11H19NO	3

3	Cpd 4: 9-Oximino-2,7-diethoxyfluorene	4.752	9-Oximino-2,7-diethoxyfluorene	C17H17NO3	3
4	Cpd 6: 1-(2-Acetoxyethyl)-3,6-diazahomoadamantan-9-one	5.008	1-(2-Acetoxyethyl)-3,6-diazahomoadamantan-9-one	C13H20N2O3	3
5	Cpd 8: Androst-4-en-3-one, 17-methoxy-, 3-methoxime, (17.beta.)-	5.096	Androst-4-en-3-one, 17-methoxy-, 3-methoxime, (17.beta.)-	C21H33NO2	3
6	Cpd 10: 1,1'-Biphenyl, 2,2'-dimethyl-6,6'-dinitro	5.439	1,1'-Biphenyl, 2,2'-dimethyl 6,6'-dinitro	C14H12N2O4	3
7	Cpd 12: Benzaldehyde, 2,4-dimethyl	5.527	Benzaldehyde, 2,4-dimethyl	C9H10O	3
8	Cpd 13: Lycorine, 2-O-methyl(ether)	5.821	Lycorine, 2-O-methyl(ether)	C17H19NO4	3
9	Cpd15: Bacteriochlorophyll-c stearyl	6.145	Bacteriochlorophyll-c-stearyl	C52H72MgN4O4	3
10	Cpd 17: Tetradecanedioic acid, 3-oxo-, dimethyl ester	6.33	Tetradecanedioic acid, 3-oxo-, dimethyl ester	C16H28O5	3
11	Cpd 19: Phenol, 2,4-bis(1,1-dimethylethyl)-	6.49	Phenol, 2,4-bis(1,1-dimethylethyl)-	C14H22O	3
12	Cpd 22: Z-9-Pentadecenol	7.095	Z-9-Pentadecenol	C15H30O	3
13	Cpd 28: Methyl 10-methyl undecanoate	8.468	Methyl 10-methyl undecanoate	C13H26O2	3
14	Cpd 29: 11-Octadecenoic acid, methyl ester	10.152	11-Octadecenoic acid, methyl ester	C19H36O2	3
15	Cpd 30: Methyl 12-methyl tridecanoate	10.208	Methyl 12-methyl tridecanoate	C15H30O2	3
16	Cpd 31: Methyl 11,14-octadecadienoate	10.266	Methyl 11,14-octadecadienoate	C19H34O2	3
17	Cpd 32: Doconexent	10.498	Doconexent	C22H32O2	3

5.11 FTIR- FOURIER TRANSFORM INFRARED SPECTROSCOPY:

After running the oyster mushroom ethanol extract in the FTIR the peak level of each compound present in the sample are displayed in the graph below.(fig 5.24)

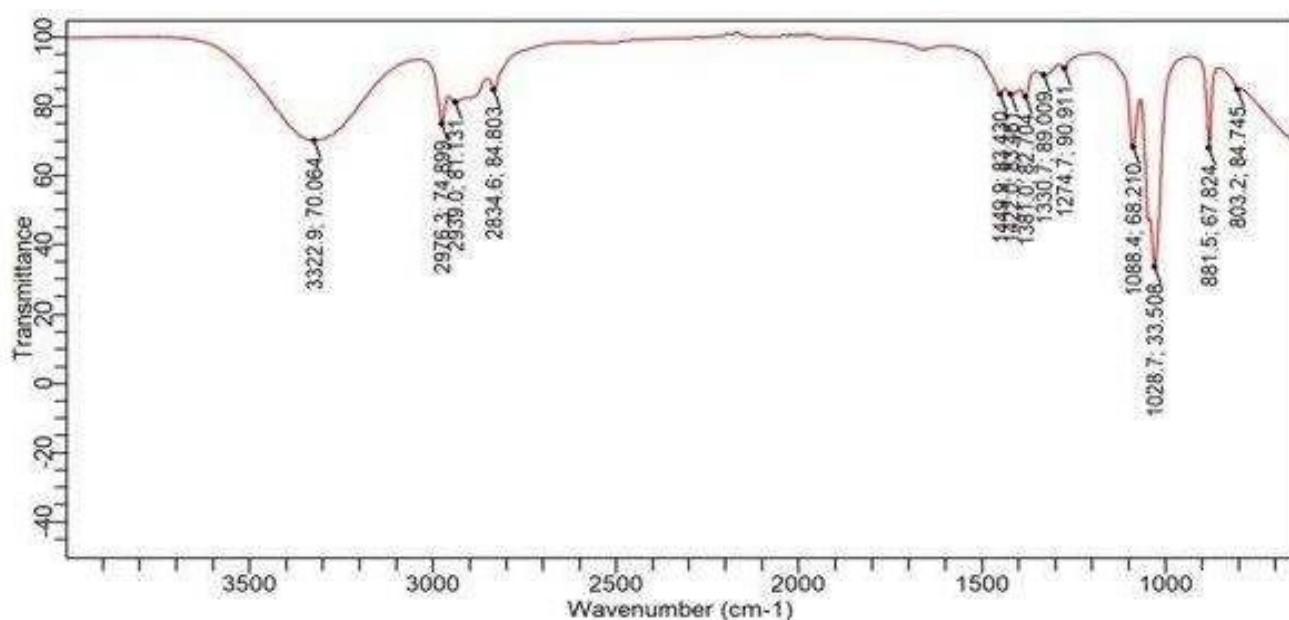


Fig 5.24

With the help of the wavenumbers interpreted on the above graph (fig 5.24), we can find out the functional groups of each compound which is given the (table 5.11)

Table 5.11 wavenumbers and functional groups

S.No	Wavenumber (cm-1)	Functional group present
1	803.24130	Aromatic ring (aryl) C-H 1,4- Distribution (para)
2	881.51540	C-H 1,3 - Distribution (meta)

3	1028.74524	Aliphatic phosphates (P-O-C) stretch
4	1088.38265	Primary amine, CN stretch
5	1274.74954	Tertiary amine, CN stretch
6	1330.65961	Aromatic primary amine, CN stretch
7	1380.97867	Aliphatic nitro compounds
8	1421.97938	Organic sulphates
9	1449.93442	Vinyl C-H in plane bend
10	2834.64043	Methoxy, methyl ether O-CH ₃ , C-H stretch
11	2939.00588	Methylene C-H asym . /sym . stretch
12	2976.27926	Alkanes C-H stretch
13	3322.92168	Aliphatic secondary amine >N-H stretch

5.12. CHEMICAL SCREENING TESTS

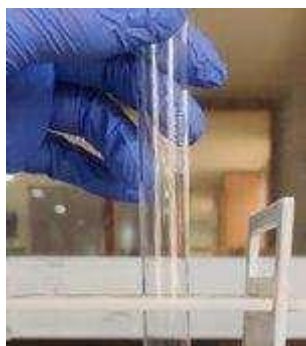
Phytochemical screening for oyster mushroom crude extract was tested and shown positive results for flavonoids, saponins, alkaloids, tannins, sterols, terpenoids and anthraquinones. The result for total phenol, flavonoids, saponins, sterols, alkaloids, terpenoids were high in crude ethanolic extract and it could be used as antibiotics for different curable and incurable diseases. The ethanol extract is used as control for performing the test.(fig 5.25). Results are interpreted in the (table 5.12)



Fig 5.25 mushroom ethanol extract

Table 5.12 chemical tests and its results

S.No	Chemical tests	Observation	Results
1.	Alkaline reagent test (fig 5.25)	Changes to colourless	Positive
2.	Test for tannins (fig 5.26)	Colour changes to yellow	Positive
3.	Test for anthraquinones(fig 5.27)	Colour changes to pale pink colour	Positive
4.	Test for terpenoids(fig 5.28)	Colour changes to reddish brown colour	Positive
5.	Test for sterols(fig 5.29)	Colour changes to reddish colour	Positive
6.	Test for saponins (fig 5.30)	Formation of foam	Positive
7.	Test for alkaloids (fig 5.31)	Presence of reddish brown colour	Positive
8.	Test for phenols(fig 5.32)	Colour changes to dark blue	Positive
9.	Test for cardiac glycoside (fig 5.33)	Colour changes to blue	Positive
10.	Ferric chloride test for flavonoids (fig 5.34)	Appearance of green- blue colour	Positive
11.	Molisch's test for carbohydrates (fig 5.35)	Formation of red/violet colour at the interface of two layers	Positive



**Alkaline reagent test
fig 5.26**



**Test for tannins
fig 5.27**



**Test for anthraquinones
fig 5.28**



**Test for terpenoids
fig 5.29**



**Test for sterols
fig 5.30**



**test for saponins
fig 5.31**



**Test for alkaloids
Fig 5.32**



**test for phenols
fig 5.33**



**test for cardiac glycoside
fig 5.34**



Ferric chloride test for flavonoids
fig 5.35

Molisch's test for carbohydrates
fig 5.36

CHAPTER 6

SUMMARY AND CONCLUSION:

The data presented above in this analysis demonstrates that the oyster mushroom has a variety of medicinal characteristics that call for more sophisticated methods for further investigation. *Pleurotus ostreatus*, an edible oyster mushroom containing a number of bioactive compounds, develops its large number of therapeutic properties. In the world, antimicrobial resistance is a huge problem, increasing death rates and length of stay in the hospital, leading to the need for specific drugs. We aim to evaluate the antibacterial, anticancer and antioxidant properties from the extract of *Pleurotus ostreatus* (Oyster mushroom). The extract was prepared with the solvent Ethanol. This analysis shows the antibacterial potential of OM. antibacterial activity against gram positive and gram negative bacteria was tested positive. The MTT assay proved that oyster mushroom extract is active against MCF7 cells - human breast cancer cells - and shows anticancer activity. The thin layer chromatography, GC-MS, and FTIR tests have shown that the extract contains bioactive compounds. The DPPH assay, phenols test, flavonoids tests proves the antioxidant properties of the oyster mushroom extract. Further exploration may lead to the discovery of new antimicrobial drugs from edible oyster mushrooms.

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