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Nutritional, Phytochemical & Antidiabetic Properties of Oyster Mushroom (Pleurotus ostreatus)

Karim, Md. Rezaul

University of Rajshahi

Ph. D.
THESIS

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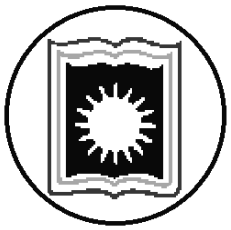

Ph. D. THESIS

A Dissertation
Submitted to the University of Rajshahi in fulfillment of the requirements for the Degree of Doctor of Philosophy (Faculty of Science)

MD. REZAUL KARIM

JUNE, 2013

Protein and Enzyme Research Laboratory
Department of Biochemistry and Molecular Biology
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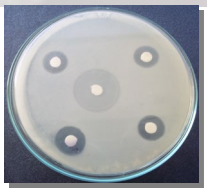
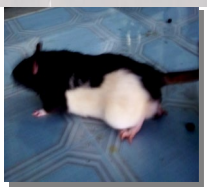



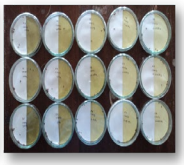

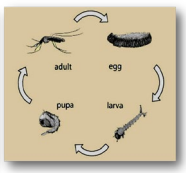
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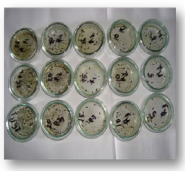

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Dedicated
To
My Beloved Parents

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All the admiration and praise to the almighty Allah, the most merciful, who has enabled me to submit this dissertation. I extremely pleased in expressing my high indebtedness, heartiest profound, deepest sense of respect, gratitude and best regards to the honorable supervisor Professor Dr. Md. Tofazzal Hossain, Department of Biochemistry & molecular Biology, University of Rajshahi, Bangladesh; who opened the door for his research to be well aquinted with modern techniques of research.

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The author

CERTIFICATE

It is my pleasure to certify that the thesis entitled, "Nutritional, Phytochemcial and Antidiabetic properties of Oyster Mushroom (*Plurotus ostreatus*)", submitted to the Department of Biochemistry and Molecular Biology, University of Rajshahi by Md. Rezaul Karim in fulfillment of the requirement for the degree of Doctor of Philosophy (Ph.D). It contains no materials previously published or written by any other person except, wherever, due references are made in the text of the thesis. I hereby clarify that the author completed his work under my direct supervision and contributed some new ideas and openings in our research field by adding most recent information in this line of the contemporary world.

I have gone through the draft of the thesis and found it acceptable for submission.

Dr. Md. Tofazzal Hossain
Professor
Department of Biochemistry and Molecular Biology
University of Rajshahi
Rajshahi-6205, Bangladesh.

DECLARATION

I hereby declare that the whole work now submitted as a thesis entitled, "Nutritional, Phytochemcial and Antidiabetic properties of Oyster Mushroom (*Plurotus ostreatus*)", in the Department of Biochemistry and Molecular Biology, University of Rajshahi for the degree of Doctor of Philosophy (Ph. D) is the result of my own investigation. The thesis contains no material which has been accepted for the award of any other degree or diploma elsewhere and to the best of my knowledge, the thesis contains no material previously published or written by another person, except where due references is made in the text.

Md. Rezaul Karim

ABSTRACT

Plants are often condemned as non-scientific and inactive. But various phytochemical and biological investigations have proved its therapeutic utility and functional properties. Now-a-days, the use of plant constituents as remedy for diseases is very promising. According to folk medicine the mushrooms *Pleurotus ostreatus* (Family: Tricholomataceae) is used in protein deficiency diseases, diabetes, anemia, cardiac diseases, typhoid fever, and diseases of blood.

Pleurotus ostreatus is locally known as Oyster mushroom and consumed by the local people as foods and vegetables. In many countries various types of foods are produced from mushrooms like burger, bread, sandwich, curry etc. In our country too, people started preparation of such types of foods. The present study included nutritional analysis, insecticidal and repellent activity against *Tribolium castaneum* adult, cytotoxicity against Brine shrimp nauplii, larvicidal activity against mosquito larvae, antimicrobial activity of some gram (+) and gram (-) pathogenic bacteria, antidiabetic, and hepatoprotective activity in long evan rats of *Pleurotus ostreatus*.

The physico-chemical and nutritional parameters changes in the three different (immature, mature and over mature) stages of Oyster mushroom (*Pleurotus ostreatus*) were studied to obtain a comparable data on their nutritional qualities. The physico-chemical parameters such as pH and moisture, contents are increased and ash and crude fiber contents are decreased with its maturity. The amount of total sugar, reducing sugar and sucrose was higher at over mature stage than immature and mature stage. Protein content was higher at mature stage than immature and over mature stage. The mentionable case is that the protein content was higher than the other nutrients. So, Oyster mushroom can be considered as good

source of protein. In this study, phytochemical investigation of fruiting body of *Pleurotus ostreatus* was performed. The extracts of fruiting body of the Oyster mushroom were obtained from its powder using methanol, ethyl acetate, chloroform, pet-ether and hot water.

In the insecticidal activity of the crude extracts dose mortality results against *T. castaneum* were found promising. Methanol extracts of *Pleurotus ostreatus* showed high mortality of the beetles and the results have been presented in table 4.5. The LD_{50} values for methanol extract were 0.480, 0.142, 0.106, 8.49E-02 mg/cm^2 for 12, 24, 36 and 48 hrs. of exposure respectively. The LD_{50} values for Chloroform extract were 0.992, 0.944, 0.719, 0.452 mg/cm^2 for 12, 24, 36 and 48 hrs. of exposure respectively. In the same way LD_{50} values for Ethyl acetate, pet-ether and hot water were 5.614, 3.322, 2.495, 2.199 mg/cm^2; 3.681, 3.225, 3.322, 2.722 mg/cm^2; and 3.77, 3.41, 2.981, 2.785 mg/cm^2 respectively.

All the extracts of *Pleurotus ostreatus* showed promising repellent activity against the adults beetles of *T. castaneum*. The P values of methanol, ethyl acetate, chloroform, pet-ether and hot water were 0.017, 0.01, 0.009, 9.38E-05 and 0.013 respectively. The extracts ethyl acetate and pet-ether showed repellent activity against *T. castaneum* adults at ($p<0.01$) level, while other extracts showed at ($p<0.05$) level.

The larvicidal activity of the crude extracts dose mortality results against clues mosquito larvae were found promising. The chloroform extract showed highest larvicidal activity and the results have been shown in table 4.7. The LC_{50} of the extract were 1224.768, 902.232, 617.195, 434.093ppm for 12, 18, 24 and 30 hrs. of exposure respectively. In the same way the LC_{50} values for methanol, ethyl acetate, pet-ether and hot water were 3677.837, 1818.165, 1258.106, 865.0ppm; 2160.578, 1855.401, 1041.842, 753.726 ppm; 1638.01, 867.305, 794.921ppm; 6856.736, 6195.057, 5888.1, 2412.954 ppm respectively.

The brine shrimp lethality tests were carried out to assess the efficacy of *Pleurotus ostreatus*. A dose mortality test was carried out on brine shrimp (*A. Salina*) nauplii. The extract of pet-ether showed highest cytotoxic activity. The LC_{50} of pet-ether extract were 1592.379, 775.952, 1592.37, 775.95ppm for 12, 18, 24 and 30 hrs. of exposure respectively. The LC_{50} of methanol, ethyl acetate, chloroform and hot water were 3113.815, 2010.848, 997.086, 626.94ppm; 4041.849, 2047.168, 1420.399, 906.229ppm; 5854.292, 2031.773, 1122.01, 584.610ppm; 5069.0, 3210.936, 2771.954, 1477.487ppm respectively.

The extracts of *Pleurotus ostreatus* showed moderate antibacterial activity against *Bacillus megatorium, Escherichia colli, Bacillus subtilis, Bacillus cerus and Shigella dysenteriae*.

Powder, methanol and ethyl acetate extracts of oyster mushroom (*Pleurotus ostreatus*) in streptozotocin induced diabetic long evan rats showed promising antidiabetic and hypolipidemic activity. Administration of powder, methanol and ethyl acetate extracts of oyster mushroom (*Pleurotus ostreatus*) and standard antidiabetic drug (Glipizide) reduced the glucose level by 16%, 21%, 31% and 47.1% respectively and total cholesterol level by 5%, 6.72%, 10% and 13% respectively when compared to diabetic control rats.

Administration of powder, methanol and ethyl acetate extracts of oyster mushroom (*Pleurotus ostreatus*) showed hepatoprotective activity in CCl_4-treated hepatic injured rats. Powder, methanol, ethyl acetate extracts and standard hepatoprotective drug (Silymarin) lowered the SGPT, level by 17.2%, 27.86%, 40.1%, 50.81% respectively; SGOT level by 14.2%, 20.7%, 27.1%, 37.85% respectively; SALP level by 8.7%, 15.43%, 21.81% and 40.6% respectively.

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CHAPTER-ONE

INTRODUCTION

1.1 General introduction:

At present the uses of the herbal products are safe in contrast to the synthetics which are known as unsafe to human and environment. Herbs have paid more attention for their medicinal, flavoring and aromatic qualities for centuries and the blind dependence on synthetics is over and people are returning to the naturals with hope of safety and security. Over three-quarters of the world population rely mainly on plants and plant extracts for health care. More than 30% of the entire plant species are used for medicinal purposes. It is estimated that world market for plant derived drugs may account for about 3,40000 crore taka. Drugs of herbal origin have been used in traditional systems of medicines such as Unani and Ayurveda since ancient times. The drugs are derived either from the whole plant or from different organs, like leaves, stem, bark, root, flower, seed, etc. Some drugs are prepared from excretory plant product such as gum, resins and latex. Even the Allopathic system of medicine has adopted a number of plant-derived drugs, which include an important segment of the modern pharmacopoeia. Some important chemical intermediates needed for manufacturing the modern drugs are also obtained from plants[1].

Plant and plant products provide not only the remedy of diseases but also the essential macro and micro nutrients. It has now been established that the plant which naturally synthesizes and accumulates

secondary metabolites like alkaloids, glycosides, tannins, saponins, volatile oil, absinthian, flavones, and organic acid and contain vitamins and minerals have herbals properties. According to WHO, plants which contain substances that can be used for therapeutic purpose are termed as medicinal plants.[2] Approximately one third of all drugs are plant based and if bacteria and fungi are also included, nearly 60% of pharmaceuticals are of plant origin [3].

In the present study, Oyster mushroom (*Pleurotus ostreatus*) has been taken as experimental sample. The *Pleurotus ostreatus* constitutes an important species of mushroom with immense medicinal value[4]. Beside this, the varied number of species exists in the genus *Pleurotus*. The most important Oyster mushrooms namely, *P. Sajor-caju*, *P. Stropharia*, *P. cystidiosus* and *P. flabellatus*. *Pleurotus ostreatus* are used as vegetables, burger, bread, sandwich, curry etc in Bangladesh, Indian subcontinent and in other countries. It contains various neutraceuticals, which have healing properties[4]. *Pleurotus ostreatus* is rich in both macro and micronutrients. So this may fill up the daily needs of nutrients especially micronutrients. In addition to these, various secondary metabolites such as alkaloids, phenolic compounds[5] and others present in *Pleurotus ostreatus* play a potent role in the prevention of many diseases[6]. The present investigations have revealed the chemical and functional properties of *Pleurotus ostreatus* on the nutritional points of view.

1.2 Information about Oyster mushroom (*Pleurotus ostreatus*):

1.2(I) Botanical information:

Local name: Oyster Mushroom

Binomial name: *Pleurotus ostreatus*

Scientific classification

Kingdom: Fungi

Phylum: Basidiomycota

Class: Agaricomycetes

Order: Agaricales

Family: Tricholomataceae

Genus: Pleurotus

Species: ***P. ostreatus***

1.2(II).The Family Tricholomataceae:

The **Tricholomataceae** is a large family of mushrooms within the Agaricales. A classic "wastebasket taxon", the Tricholomataceae is inclusive of any white-, yellow-, or pink-spored genus in the Agaricales not already classified as belonging to the Amanitaceae, Lepiotaceae, Hygrophoraceae, Pluteaceae, or Entolomataceae.

Arnolds (1986) and Bas (1990) also place the genera of the Hygrophoraceae within this family; however, this classification is not accepted by the majority of fungal taxonomists[7].

Genera within the Tricholomataceae include *Tricholoma* (the type genus), *Clitocybe*, *Pleurotus*, *Armillaria*, *Mycena*, and *Marasmius*, among others.

Molecular phylogenetic analysis has greatly aided in the demarcation of clear monophyletic groups among the Tricholomataceae. So far, most of these groups have been defined cladistically rather than being defined as formal Linnean taxa, though there have been several cases in which older proposed segregates from the Tricholomataceae have been validated by evidence coming from molecular phylogenetics. As of 2006, validly published families segregated from the Tricholomataceae include the Hydnangiaceae, Lyophyllaceae, Marasmiaceae, Mycenaceae, Omphalotaceae, Physalacriaceae, and Pleurotaceae.

The name "Tricholomataceae" is nevertheless seen as having validity in describing *Tricholoma* and its close relatives, and whatever other genera can at some future point be described as part of a monophyletic family including *Tricholoma*. To that end, the International Botanical Congress has voted on two occasions (1988 and 2006) to conserve the name "Tricholomataceae" against competing names. This decision does not invalidate the use of segregate families from the Tricholomataceae, but simply validates the continued use of Tricholomataceae[24].

1.2(III).The Genus *Pleurotus*[8]:

Pleurotus is a genus of gilled mushrooms which includes one of the most widely eaten mushrooms, the Oyster mushroom.

Like all other basidiomycetes, it is distinguished by having clamp connections along its hyphal length.

Species in the genus

- *P. acerosus*
- *P. australis* (The Brown Oyster Mushroom)
- *P. citrinopileatus* (The Golden Oyster Mushroom)
- *P. cornucopiae* (The Branched Oyster Mushroom)
- *P. cystidiosus* (The Abalone Mushroom)
- *P. djamor* (The Pink Oyster Mushroom)
- *P. dryinus*
- *P. eryngii* (The King Oyster Mushroom)
- *P. euosmus* (The Tarragon Oyster Mushroom)
- *P. ferulae*
- *P. nebrodensis*
- *P. ostreatus* (The Tree Oyster Mushroom)
- *P. pulmonarius* (The Phoenix or Indian Oyster Mushroom)
- *P. tuberregium* (The King Tuber Mushroom)
- and others

1.2 (IV).The Species: *Pleurotus ostreatus*.

1.3 Literature survey on Oyster mushroom (*Pleurotus ostreatus*):

The *Pleurotus* mushroom is generally called the Oyster mushroom because the pileus or cap is shell-like, spatulate and the stipe eccentric or lateral. The species of *Pleurotus ostreatus* has been used as food or for medicinal purposes for a long time[9].

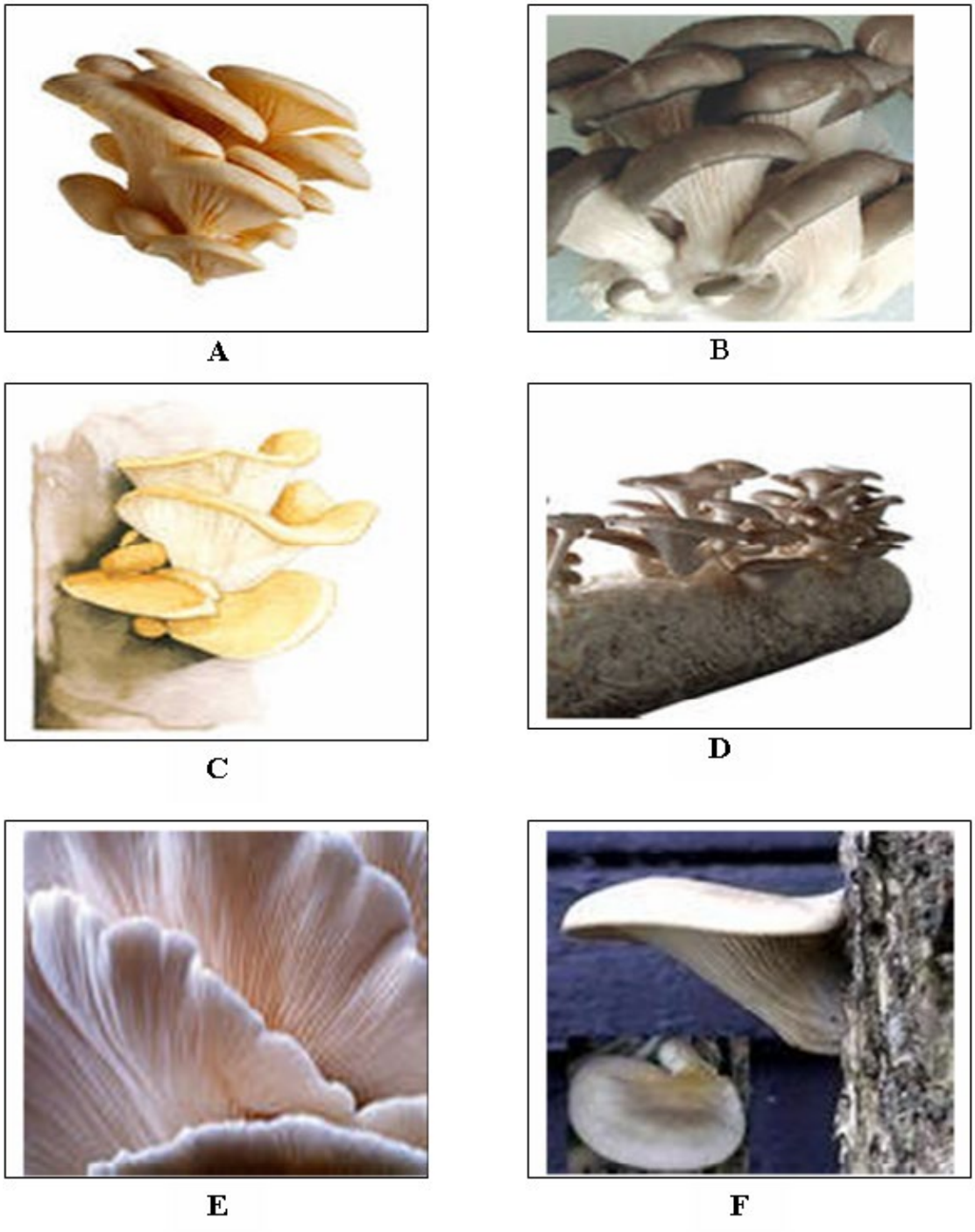


Figure 1.1: Photographs of Oyster Mushroom (*Peurotus ostreatus*). A) The Oyster mushroom is highly esteemed. B) Home fresh Oyster mushroom. C) Thin Oyster mushroom. D) Oyster mushroom Spawn. F) Gills of Oyster mushroom. E) Oyster mushroom with tree.

At present it plays an important role as a commercial edible mushroom. More than 1000 Species of the Oyster mushroom have been found throughout the world, in more than 25 related genera. However only approximately 50 valid species are recognized in the genus *Pleurotus*. *Pleurotus ostreatus* is one of the best known among the Oyster mushrooms. Other commonly cultivated Species include: *P. sajor-caju*(Fries) sing.(gray Oyster mushrooms or phoenix-tail mushroom), *P. cystidiosus* O.K. Miller (abalone mushroom), *P. ostreatus* var. florida nom.prov.Eger (white Oyster mushroom), *P.citinopileatus* Sing. (golden Oyster mushroom) *P. flabellatus* (Berk. and Br.) Sacc (pink Oyster mushroom) and *P. sapidus* (Schulzer) Kalchbremer (black Oyster mushroom). They belong to the family Tricholomataceae, order Agaricales and Class Basidiomycetes. They grow naturally mainly in the temperate zones or in the cooler seasons in subtropical areas on rotting trees such as oak, elm, maple, bass, poplar, holly and laburnum. In Bangladesh it is frequently seen in various trees like mango tree, palm tree, Banyan tree etc. They have a fragrant odor and delicious flavor. However *Pleurotus* has been reported as parasitic on several trees. Species of *pleurotus*, such as *P. cornucopiae* (Paul.:Pers.) Roll., *P. cystidiosus, P. ostreatus*, *P. tuberregium* (Fr.) Sing., are known to attack and consume living nematodes, through special structures named micro droplets, as reported by Barron and Thorn, and Hibbett and Thorn[10].

As *Pleurotus* species are comparatively easy to grow and of broad adaptability, they are cultivated worldwide and their production has increased rapidly in recent years[11]. Methods of cultivation have now been worked out and the techniques for growing them are simple and inexpensive. A wide range of plant wastes, such as sawdust, paddy straw,

bagasse, cornstalks, waste cotton, stalks and leaves of banana, can all be used for *Pleurotus* mushroom production without a requirement for costly processing methods and enrichment materials[12, 23]. It has frequently been suggested that edible mushrooms, because of their high protein content, should be produced in greater amounts to alleviate protein deficiencies in places where the prospect of starvation is a life-threatening problem. Recently, it has been suggested that the most important of the edible mushrooms that could be used in this way is the species of *Pleurotus*[9].

1.4. Medicinal uses of Oyster mushroom (*Pleurotus ostreatus*):

Different parts of this mushroom are used as medicine in the treatment of a variety of human diseases. It is known that, owing to their high protein content and dietary fibers these mushroom have positive effect on human nutrition. Besides, dietary intake of *Pleurotus* species modulates the immune system, shows hypoglycemic[13], antithrombotic and antimicrobial activity[14], inhibits tumor growth[14-15], reduce inflammation and lowers blood pressure and plasma concentration. In addition, they effectively reduce lipids[16-17] in general and specifically low-density lipoprotein (LDL) and cholesterol[18-20].

1.5. Antimicrobial and Antineoplastic Activity of Oyster mushroom *(Pleurotus Ostreatus)* [14-15]:

The submerged culture of the *pleurotus* genus has also been studied for the production of extracellular enzymes, flavoring agents, β-glycosidases, antimicrobial, vitamins, polysaccharides etc. These polysaccharides represent the major constituent that determines the rigidity and morphological properties of the cell wall and depending on

the culture condition; they can also be excreted to the culture medium. Among the polysaccharide produced, β-(1, 3) and β-(1, 6) glucans play an important role due to their medicinal properties.

These properties have gained much interest for extensive scientific studies on their hypoglycemic, antithrombotic, antitumor, anti-inflammatory and antimicrobial activities as well as for processing the ability to modulate the immune system and to reduce arterial pressure and blood cholesterol.

Investigations using polysaccharides from fruiting bodies and basidiomycete mycelium have shown that *Pleurotus Ostreatus* presents antineoplastic activities[14], which can be attributed to the polysaccharides of the cell wall components. Dietary intake of *Pleurotus* species to mice showed modulation in the immune system and also showed hypoglycemic, antithrombotic and antimicrobial activity.

1.6. Use of Oyster mushroom *(Pleurotus ostreatus*) as edible mushroom[17, 22]:

Pleurotus ostreatus has frequently been suggested as edible mushroom, because of high protein content. It should be produced in greater amounts to alleviate protein deficiencies in places where the prospect of starvation is a life-threten problem. It contains small amount of dietary fiber[21] which may reduce constipation. It is a delicious edible mushroom and is found throughout the North Temperate Zone, almost always on dead hardwood (angiosperm) trees. It can also be (relatively) easily cultivated on a variety of substrates, so it is making its way onto many supermarket shelves.

Pleurotus species are characterized by a white spore print, attached to *Pleurotus ostreatus* decurrent gills. Often with an eccentric (off center) stipe or no stipe at all. They always grow on wood on nature, usually on dead standing trees or on fallen logs. The common name "Oyster mushroom" comes from the white shell-like appearance of the fruiting body, not from the taste. The taste of the Oyster mushroom varies from very mild to very strong, sometimes sweet with the smell of anise (licorice). It varies in texture from very soft to very chewy, depending on the strain and what time of the year it should be picked, they tend to be chewier during the cold months of the year. One can make a delicious "Oyster Mushroom Rockefeller" and a variety of stir-fry dishes.

1.7. Role of Plants as Insecticides:

Botanical insecticides are considered as alternatives to the synthetic chemicals for being biodegradable, pest specific, non-hazardous to human health and environment and leaving no toxic residue in nature[25]. Plant extracts are comparatively safe to human due to their low toxicity. So derivatives of some plants have had temporary to restricted use in pest control or have been considered items of regional interest[26]. Within the past few decades the world advanced rapidly with remarkable development in medicine, pesticide and insecticide technology but there are still some problems. A serious problem was observed to human health when the microorganisms were found to resistant to the antibiotics and recently, this problem has become more evident since most of the organisms exhibit some degree of resistance to the commonly available anti-microbial and chemotherapeutic agents.

Pest control is a major issue for underdeveloped agricultural countries. More than 2000 species of field and storage pests annually destroy approximately one third of world's food production, valued US $ 100 billion among which highest losses (43% of potential production) occur in developing Asian countries[27]. Synthetic pesticides are the easy control technology for insect pest. Continuous or heavy usages of some pesticides have created undesirable changes in the gene pool for the presence of some mutagenic agents and thereby increasing pesticide resistance in insects. Heavy usages of pesticides has also created serious problem such as direct toxicity to parasites, predators, pollinators, fish and man[28-29]. It has therefore become necessary to complement our reliance on synthetic pesticides with less hazardous and biodegradable substitutes.

In the rural areas of south Asia, including Bangladesh, farmers traditionally mix leaves, bark, seeds, roots or oils of certain plants with stored grains to keep them free from insect attacks. Such technique has been inherited as part of the traditional culture[30]. However, several workers[31] have reported effective uses of plant materials as toxic substance against stored product pests. Plant-derived pesticides are more readily biodegradable. Therefore, they are less like to contaminate the environment and may be less toxic to mammals[32]. The botanical pesticides are broken down readily in soil and are not stored in plant or animal tissues. Often their effects are not as long lasting as those of synthetic pesticides. Environmentally, they are less harmful than synthetic pesticides and acting on many insects in different ways [33-34].

Locally available plants and minerals have been widely used to protect stored products against damage by insect infestation[35]. The main advantage of plant extract is that they are easily produced by farmers in small-scale industries and are less expensive. Moreover, botanical insecticides are broad-spectrum in pest control, many are safe to apply, unique in action and can be easily processed and used[36].

1.8. Aim of the present study:

In Bangladesh there are a lot of medicinal plants, which grow mostly in forest, jungles and fallow lands without any care. Oyster mushroom (*Pleurotus ostreatus*) is native to Bangladesh, India and China and commonly grown in South East Asia and South America. In our subcontinent mushroom is rarely consumed, people usually take it as foods and vegetables. Oyster mushroom (*Pleurotus ostreatus*) is rich in nutrients and is used in the prevention of some diseases, especially protein deficiency diseases.

Limited scientific research works have been done on the nutrient composition of different varieties of Oyster mushroom. Hence we have analyzed three stages of Oyster mushroom (immature, mature and over mature) to obtain comparative information on their nutritional parameters such as pH, moisture, ash, crude fiber, protein, lipid, soluble sugar, reducing sugar, non-reducing sugar and starch.

It is evident from the chemical investigations that the Oyster mushrooms contain some important sources of biologically active compounds possessing potential antimicrobial, anticancer, pesticidal, parricidal properties.

The folk medicine practitioners use this mushroom as medicine without having knowledge about their side effects and toxicity. If it is

possible to exclude the poisonous components present in the mushrooms, which are toxic to human and animal body, then we could properly use this mushroom for the treatment of various diseases. So our aim is to investigate the cytotoxic effects of this mushroom.

The goal of the present study was to investigate the nutritional quality and the attention was concentrated on the Oyster mushroom (*Pleurotus ostreatus*) for preparation of bioactive extracts with phyto-chemical screening and to evaluate the antimicrobial, insecticidal, repellent, cytotoxic, larvicidal, antidiabetic and hepatoprotective activity of these extracts.

1.9. Study protocol:

The following study protocol has been designed for the research-

1. Nutritional analysis of Oyster mushroom (*Pleurotus ostreatus*) at different maturity stages.
2. Preparation of methanol, ethyl acetate, chloroform, pet-ether and hot water extract of Oyster mushroom (*Pleurotus ostreatus*).
3. The following biochemical activities have been performed from the above crude extracts.

   I) Antibacterial activity.

   II) Insecticidal and repellent activity against *Tribolium castaneum* adults.

   III) Cytotoxicity against *A. salina* nauplii.

   IV) Larvicidal activity against mosquito larvae.

   V) Antidiabetic activity in streptozotocin-idnuced diabetic long evan rats.

   VI) Hepatoprotective activity in CCl_4-treated hepatic injured long evan rats.

CHAPTER-TWO

NUTRITIONAL ANALYSIS

1. Spon of Oyster mushroom

2. Emerging of Oyster mushroom

3. Immature

4. Mature

5. Over mature

Figure 2.1: Photographs show 1. Spon of Oyster mushroom 2. Emerging Oyster mushroom 3. Immature stage and 4. Mature stage and 5. Over mature stage of Oyster mushroom (*Pleurotus ostreatus*).

2. Nutritional Analysis:

2.1. Introduction:

Oyster mushrooms (*Pleurotus ostreatus*) were brought to the protein research laboratory of the Department of Biochemistry and Molecular Biology from the Oyster mushroom production center, Chapai nawabganj for experimental purpose. The physical and chemical parameters examined in the present study are as follows.

2.1.1. Determination of pH:

2.1.1.1. Extraction of juice from the Oyster mushroom (*Pleurotus ostreatus*):

About 70 gm of samples was taken in a mortar with small amount of water. The samples was crushed thoroughly with a pestle and then filtered through two layers of muslin cloth. The filtrate was then centrifuged for 10 minutes at 3000 r.p.m. and the clear supernatant was collected.

2.1.1.2. Standard buffer solution:

A standard buffers solutions were prepared by dissolving pH 7.0 and 4.0 buffer tablets (BDH chemicals Ltd. Poole, England) in 100 ml distilled water.

2.1.1.3. Procedure:

The electrode assembly of the pH meter was dipped into the standard buffer solution of pH 7.0 taken in a clear and dry beaker. The temperature correction knob was set to room temperature (28°C) and the fine adjustment was made by asymmetry potentially knob to pH 7.0. After washing, the electrode assembly was then dipped into a solution of standard pH 4.0 and adjusted to the required pH by fine asymmetry

potentially knob. The electrode assembly was removed from the buffer solution, washed twice with distilled water, rinsed off with juice of the Oyster mushroom and then dipped into the juice of the Oyster mushroom flesh. The pH of the Oyster mushroom flesh juice was given in the table 2.1.

2.1.2. Determination of moisture:

Moisture content was determined by the conventional procedure.

2.1.2.1. Materials:

a) Porcelain crucible

b) Electrical balance

c) Oven

d) Desecrator

2.1.2.2. Procedure:

About 5 gm of each of three stages (immature, mature and over mature) of Oyster mushroom (*Pleurotus ostreatus*) were weighed in a porcelain crucible (which was previously cleaned, heated to 100°C, cooled and weighed). The crucible with the sample was heated in an electrical oven for about six hours at 100°C. It was then cooled in a desiccator and weighed again.

2.1.2.3. Calculation:

Percent of moisture content (gm per 100 gm of *Pleurotus ostreatus*)

$$= \frac{\text{Weight of moisture obtained}}{\text{Weight of the sample}} \times 100$$

2.1.3. Determination of ash:

Ash content was determined following the method of A.O.A.C [37].

2.1.3.1. Materials:

a) Porcelain crucible

b) Muffle furnace

c) Electrical balance (Mettler H-18)

d) Desecrator

2.1.3.2. Procedure:

About 5 grams of immature, mature and over mature Oyster mushroom (*Pleurotus ostreatus*) were weighed in a porcelain crucible (which was previously cleaned and heated to about 100°C, cooled and weighed). The crucible was placed in a muffle furnace for about four hours at about 600°C. It was then cooled in a desiccator and weighed. To ensure completion of ashing, the crucible was again heated in the muffle furnace for half an hour, cooled and weighed again. This was repeated till two consecutive weights were the same. The ash was almost white in color.

2.1.3.3. Calculation:

Percent of ash content (gm per 100 gm of *Pleurotus ostreatus*)

$$= \frac{\text{Weight of ash obtained}}{\text{Weight of the sample}} \times 100$$

2.1.4. Determination of crude fiber from Oyster mushroom flesh:

Crude fiber content of the three stages (immature, mature and over mature) of Oyster mushroom (*Pleurotus ostreatus*) was determined by the following method.

2.1.4.1 Reagent:

(a) Sulfuric acid (0.225N).

(b) Sodium hydroxide (1.25%).

(c) Ethanol.

(d) Ether.

2.1.4.2 Method:

About 5 gm of dry immature, mature and over mature of Oyster mushroom (*Pleurotus ostreatus*) were taken in beaker (500ml) and 200ml of boiled 0.225N sulfuric acid was added to it. The mixture was boiled for 30 minutes, keeping the volume constant by the addition of water at frequent intervals (a glass rod was inserted in the beaker for smooth boiling). At the end of this period, the mixture was filtered through a muslin cloth and the residue was washed with hot water, till free from acid.

The material was then transferred into the same beaker and 200 ml of boiling 1.25% sodium hydroxide was added.

After boiling for 3 minutes (keeping the volume constant as before), the mixture was filtered through a muslin cloth and the residue was washed with hot water, till free from alkali, following by washing with some ethanol and ether. It was then transferred to a crucible, dried over night at 80-100^0 C and weighed. The crucible was then heated in a muffle furnace at 600^0 C for 3 hours, cooled and weighed again. The difference in the weight represented the weight of crude fiber.

The percentage of crude fiber was calculated from the formula given bellow:

2.1.4.3 Calculation:

Crude fiber content (gm per 100 gm of Oyster mushroom *(Pleurotus ostreatus*)

$$= \frac{\text{Weight of the crude fiber}}{\text{Weight of the sample}} \times 100$$

2.1.5 Determination of protein content of oyster mushroom

Protein contents of three stages of the oyster mushroom were determined by the method of Micro-Kjeldahl.

Reagents and equipments:

a) Solid potassium sulphate,

b) Concentrated sulphuric acid,

c) 5% $CuSO_4$, $5H_2O$ in distilled water,

d) 0.01 NH_2SO_4 solution,

e) Concentrated sodium hydroxide solution (5N approximately).

f) Boric acid solution containing bromocresol green (receiving fluid): 10 gm of boric acid was dissolved in hot water (250 ml.) and cooled. 1 ml of 0.1% bromocresol green in alcohol was added and diluted to 500 ml with distilled water.

g) Few quartz chips,

h) Nitrogen determination apparatus according to Paranas-Wanger made of JENA glass-all connections with inter-changeable ground joints.

Procedure:

A) Digestion

Concentrated H_2SO_4 (4-6 ml), 1.0 gm K_2SO_4 one to drops of 5% $CuSO_4$ solution (catalyst) and some quartz chips were added (to avoid

bumping) to 3-2 gm of guava in a Kjeldahl flask. The mixture was heated till it had become light green (2-3 hours).

B) Collection of ammonia

The digestion was carried out in the steam distillation chamber of the nitrogen determination apparatus. The chamber is designated to act as a micro-kjeldahl flask and can be easily detached when needed. After completion of digestion the stream distillation chamber containing the digested mixture was fitted back to the nitrogen determination apparatus. Boric acid solution (15 ml) in small flask was placed so that the top of the condenser outlet dipped below the surface of boric acid solution. Sufficient amount of concentrated sodium hydroxide solution (approximately, 3-40ml) was added to the digest in the chamber to neutralize the amount of acid present. Steam was generated from the steam-generating flask and the sample in the chamber was team distilled until 20 ml of distillate was little was collected in boric acid solution. The condenser outlet was then rinsed with little distilled water and the receiving flask was removed.

C) Titrimetric estimation of ammonia

The ammonia in boric acid solution was titrated with 0.01 NH_2SO_4 unit the solution had been brought back to its original yellow green color. The titration was repeated with a control containing only 15ml of boric acid solution diluted to approximately the final volume of the titrated sample. The volume of boric acid required was noted.

The nitrogen was calculated using the formula given below:

i) 100 ml of 1N acid ≡ 14gm of nitrogen

ii) X gm of N_2 ≡ 6.25 × gm of protein.

Calculation:

Percent of protein content (gm per 100 gm of oyster mushroom) = $\frac{\text{Weisht of the protein content}}{\text{Weight of the oyster mushroom}} \times 100$

2.1.6. Determination of water-soluble protein:

Water-soluble protein content of Oyster mushroom *(Pleurotus ostreatus*) flesh were determined following the method of Lowry *et. al.*[40]. The extraction was carried out with distilled water.

2.1.6.1. Reagents:

(a) 2% Na_2CO_3 solution in 0.1N NaOH

(b) 0.5 gm copper sulfate in 1% sodium-potassium tartarate.

(c) Folin-Ciocaltean reagent (FCR): (Diluted with equal volume of H_2O, just before use).

(d) Protein standard: Bovine serum albumin (BSA) 100 µg/ml in water.

2.1.6.2. Procedure:

1) Reagents (a) and (b) were mixed in the ratio 50:1 and diluted reagent (c) just before use.

2) To nine glass test tubes, 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.8 ml of the standard protein solution, respectively were taken and the volume was made upto 1 ml by distilled water. The sample was transferred to a 50 ml volumetric flask and the volume was made upto the mark by distilled water. Water was carefully added avoiding formation of emulsion. 1 ml of the sample was taken in a test tube and a duplicate was made. To each of the tubes 5.0 ml of (a:b) mixture was added and after 10 minutes, 0.5 ml (FCR) solution was added. Absorbance's of the solutions were recorded after 30 minutes at 650 nm. A standard curve was constructed with the data obtained from the standards and the amount of water soluble protein in the sample was calculated from the standard curve (table-2.2).

2.1.6.3. Calculation:

Percent of water soluble protein content (gm per 100gm of Oyster mushroom (*Pleurotus ostreatus*))

$$= \frac{\text{Weight of water soluble protein}}{\text{Weight of the sample}} \times 100$$

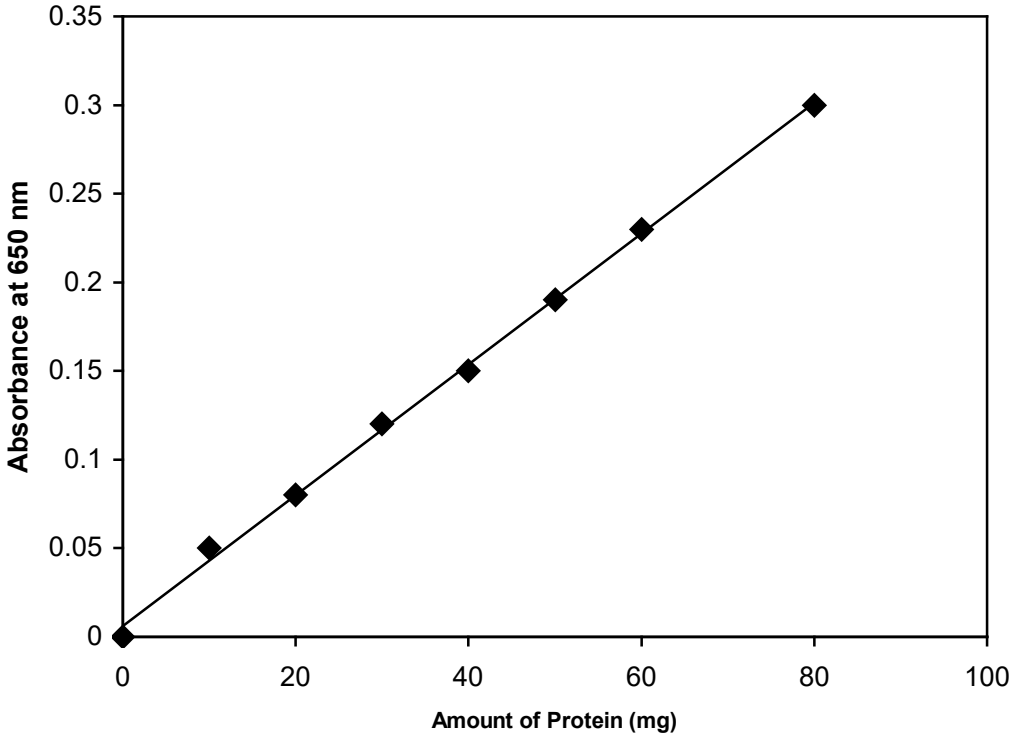


Figure 2.2: Standard curve for the determination of protein concentration.

2.1.7. Determination of lipid:

Lipid content of Oyster mushroom (*Pleurotus ostreatus*) were determined by the method of Bligh and Dyer[41].

2.1.7.1. Reagent:

A mixture of chloroform and ethanol (2: 1 V/V).

2.1.7.2. Procedure:

About 5 gm of dry immature, mature and over mature Oyster mushroom (*Pleurotus ostreatus*) were first pasted separately in mortar with about 10 ml of distilled water. The pasted flesh was transferred to a separating funnel and 30 ml of chloroform-ethanol mixture was added

and mixed well. It was then kept overnight at room temperature in the dark. At the end of this period, 20 ml of chloroform and 20 ml of water were further added and mixed. Three layers were seen. A clear lower layer of chloroform containing the entire lipid, a colored aqueous layer of ethanol with all water-soluble materials and a thick pasty inter-phase were seen.

The chloroform layer was carefully collected in a pre-weighed beaker (50 ml) and then placed on a steam bath for evaporation. After evaporation of the chloroform, the weight of the beaker was determined again. The difference in weight gives the amount of the lipid.

2.1.7.3. Calculation:

Percent of lipid content (gm per 100 gm of Oyster mushroom (*Pleurotus ostreatus*).

$$= \frac{\text{Weight of lipid obtained}}{\text{Weight of the sample}} \times 100$$

2.1.8. Determination of total soluble sugar:

Total sugar content of Oyster mushroom flesh was determined colorimetrically by the anthrone method as described in the Laboratory Manual in Biochemistry[42].

2.1.8.1. Reagents:

A) Anthrone reagent: The anthrone reagent was prepared by dissolving 2 gm of anthrone in 1 liter of concentrated H_2SO_4.

B) Standard glucose solution: A standard solution of 0.1mg/ml glucose was prepared by dissolving 10 mg of glucose in 100 ml of distilled water.

2.1.8.2. Extraction of sugar from Oyster mushroom flesh:

Extraction of sugar from Oyster mushroom *(Pleurotus ostreatus*) flesh was performed following the method described by Loomis and Shul[43].

About 5 gm of immature, mature and over mature of Oyster mushroom (*Pleurotus ostreatus*) were separately plunged into boiling ethyl alcohol and allowed to boil for 5-10 minutes (5 to 10 ml of alcohol was used per gm of flesh pulp). The extract was cooled and pasted thoroughly in a mortar with a pestle. Then the extract was filtered through two layers of muslin cloth and re-extracted the pasted tissue for three minutes in hot 80 percent alcohol, using 2 to 3 ml of alcohol per gm of sample. This second extraction ensured complete removal of alcohol soluble substances. The extract was cooled and passed through muslin cloth. Both the extracts were filtered through Whatmann No-41 filter paper.

The volume of the extract was evaporated to about one-fourth the volume over a steam bath and cooled. This reduced volume of the extract was then transferred to a 100 ml volumetric flask and made up to the mark with distilled water. Then 1 ml of the diluted solution was taken into another 100 ml volumetric flask and made up to the mark with distilled water (working standard).

2.1.8.3. Procedure:

Aliquot of 1 ml of the flesh extract was pipetted into test tubes and 4 ml of the anthrone reagent was added to each of this solution and mixed well. Glass marbles were placed on the top of each tube to prevent loss of water by evaporation. The test tubes were heated for 10 minutes in a boiling water bath and then cooled. A reagent blank was prepared by taking 1 ml of water and 4 of anthrone reagent in a tube and treated similarly. The absorbance of the blue-green solution was measured at 680 nm in a colorimeter.

A standard curve of glucose was prepared by taking 0.0, 0.1 0.2, 0.4, 0.6, 0.8 and 1 ml of standard glucose solution in different test tubes containing 0.0, 10, 20, 40, 60, 80 and 100 μg of glucose respectively and made the volume upto 1 ml with distilled water. Then 4 ml of anthrone reagent was added to each test tube and mixed well. The absorbance was measured at 680 nm using the blank containing 1 ml of water and 4 ml of anthrone reagent.

The amount of total sugar was calculated from the standard curve of glucose (figure 2.3). Finally, the percentage of total sugar present in the Oyster mushroom (*Pleurotus ostreatus*) was determined using the formula given below:

2.1.8.4. Calculation:

Percent of total sugar (gm per 100 gm of Oyster mushroom (*Pleurotus ostreatus*)). $= \frac{\text{Weight of sugar obtained}}{\text{Weight of the sample}} \times 100$

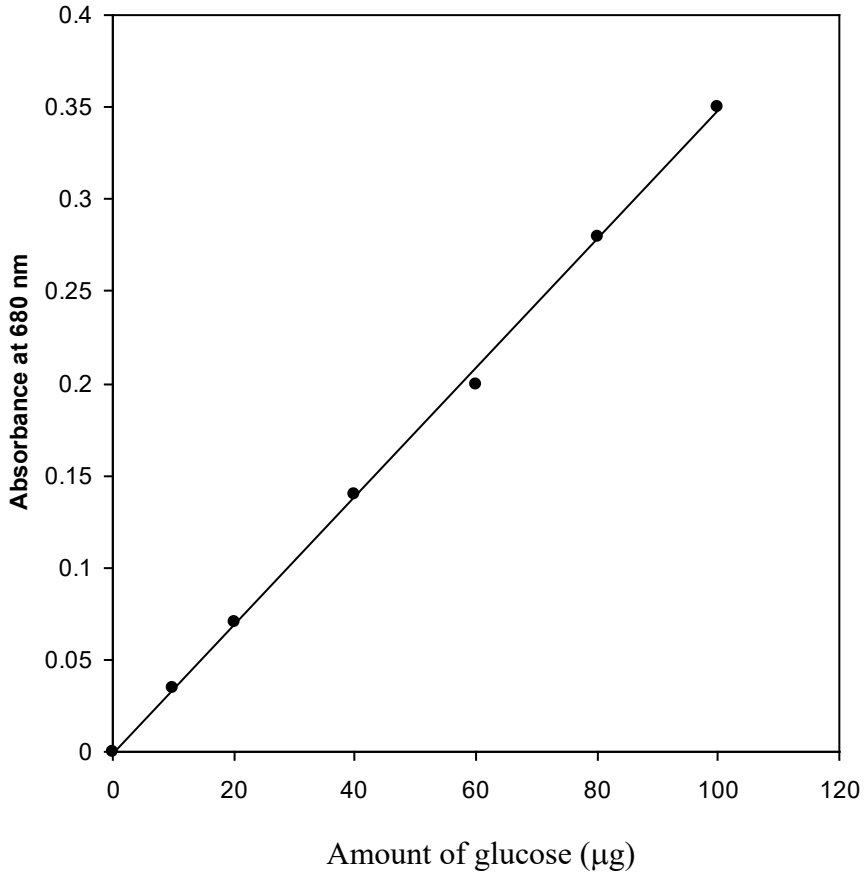


Figure 2.3: Standard curve of glucose for estimation of total soluble sugar and starch.

2.1.9. Determination of reducing sugar:

Reducing sugar content of the Oyster mushroom (*Pleurotus ostreatus*) was determined by dinitrosalicylic acid (DNS) method[44].

2.1.9.1. Reagents:

(a) Dinitrosalicylic acid (DNS) reagent. Simultaneously 1 gm of DNS, 200 mg of crystalline phenol and 50 mg of sodium sulfite were placed in a beaker and mixed with 100 ml of 1% NaOH solution by stirring.

(b) 40% solution of Rochelle salt.

2.1.9.2. Extraction of sugar extract from Oyster mushroom (*Pleurotus ostreatus*):

Sugar was extracted from of Oyster mushroom (*Pleurotus ostreatus*) by the method as described in section (2.1.8.2).

2.1.9.3. Procedure:

Aliquot of 3 ml of the extract was pipetted into test tubes and 3 ml of DNS reagent added to each of the solutions and mixed well. The test tubes were heated for 5 minutes in a boiling water bath. After developing the color, 1 ml of 40% Rochelle salt was added while the contents of the tubes were still warm. The test tubes were then cooled under a running tap water. A reagent blank was prepared by taking 3 ml of water and 3 ml of DNS reagent in a tube and treated similarly. The absorbances of the solutions were taken at 575 nm in a colorimeter.

The amount of reducing sugar was calculated from the standard curve of glucose (figure 2.3).

2.1.9.4. Calculation:

The percent of reducing sugar (gm per 100 gm of Oyster mushroom (*Pleurotus ostreatus*))

$$= \frac{\text{Weight of reducing sugar obtained}}{\text{Weight of the sample}} \times 100$$

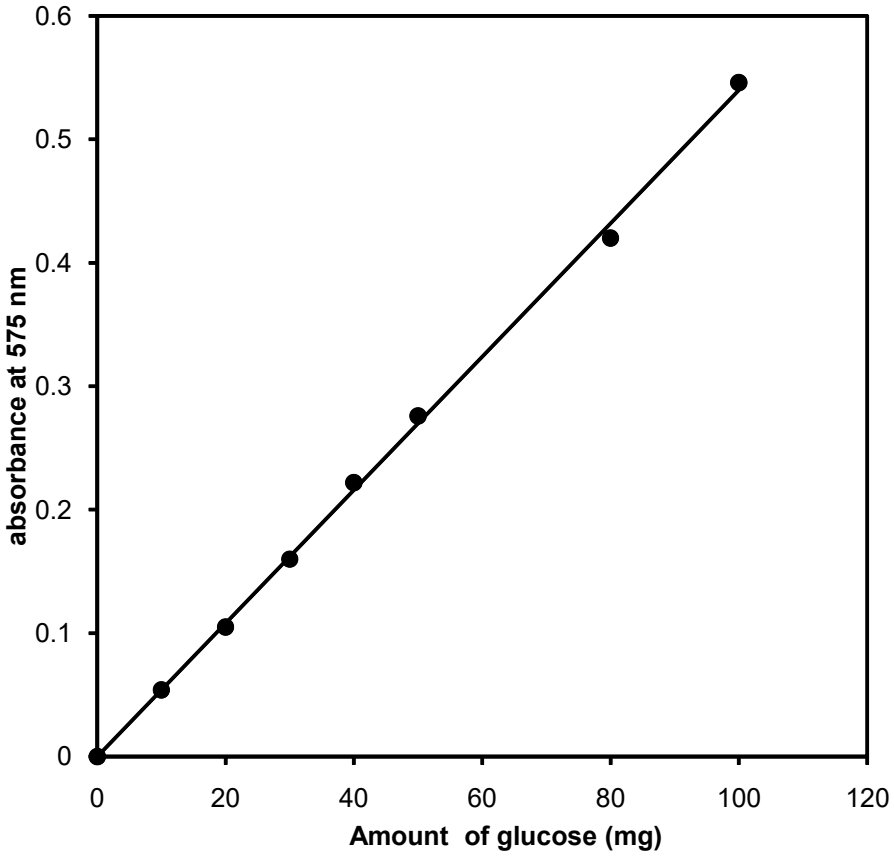


Figure 2.4: Standard curve of glucose for estimation of reducing sugar.

2.1.10. Determination of non-reducing sugar or sucrose:

Sucrose content was determined by the following formula[45].

Percent of Sucrose or non-reducing sugar = (% Total sugar - % Reducing sugar) × 0.95.

2.1.11. Determination of starch content of Oyster mushroom (*Pleurotus ostreatus*):

The starch content of the Oyster mushroom (*Pleurotus ostreatus*) was determined by the Anthrone method as described in Laboratory Manual in Biochemistry[42].

2.1.11.1. Reagents:

(a) Anthrone reagent (0.2% anthrone in conc. H_2SO_4),

(b) Standard glucose solution (10 ml/100 ml)

(c) 1M HCl

2.1.11.2. Procedure:

About 5 gm of Oyster mushroom (*Pleurotus ostreatus*) were cut into small pieces and homogenized well with 20 ml of water. The homogenate was then filtered through double layer of muslin cloth. To the filtrate, twice the volume of ethanol was added to precipitate the polysaccharide, mainly starch. Then it was kept overnight in cold; the precipitate was collected by centrifugation at 3,000 rpm for 15 minutes. The precipitate was then dried over a steam bath. Then 40 ml of 1M HCl acid was added to the dried precipitate and heated to about 70°C. It was transferred to a volumetric flask and diluted to 100 ml with 1M HCl. Then 2 ml of diluted solution was taken in another 100 ml volumetric flask and made upto the mark with 1M HCl.

An aliquot of 1 ml of the extract was pipetted into test tubes and 4 ml of anthrone reagent was added to the solution of each tube and mixed

well. Glass marbles were placed on top of each tube to prevent loss of water by evaporation. The tubes were placed in a boiling water bath for 10 minutes, then removed and cooled. A reagent blank was prepared by taking 1 ml of anthrone reagent in a test tube and treated as before. The absorbance of the blue-green solution was measured at 680 nm in a colorimeter.

The amount of starch present in the Oyster mushroom (*Pleurotus ostreatus*) was calculated from standard curve of glucose (figure- 2.2).

2.1.11.3. Calculation:

The percent of starch (gm per 100 gm of Oyster mushroom (*Pleurotus ostreatus*))

$$= \frac{\text{Weight of starch obtained}}{\text{Weight of the sample}} \times 100$$

2.1.12. Determination of Minerals

Organic matter present in ashes of different stages of Oyster mushroom was digested and K, Fe, Mn, Cu, and P were released by digestion with nitric acid. Fe, Mn, and Cu were determined by atomic absorption spectrophotometry, K was determined by flame photometry, and P was determined by spectrophotometry.

Digestion:

1. 0.500g dried mushroom were weighed and taken into each of 38 nitrogen digestion tubes. The two remaining tubes were blanks. 5 ml 68% nitric acid was added to each of all 40 tubes. The content in each tube was

mixed and left the tubes overnight. The tubes were placed in the digester and the tubes were covered with the exhaust manifold. The temperature was set to 125°C. The digester was turned on the digestion was continued for 4 hours after boiling started.

2. After cooling, the digestion mixture with distilled water was transferred to a 100 ml volumetric flask. The flask was made to volume with water and mixed. Then it was filtered through a dry filter into a dry bottle, which could be closed with a screw cap. The filtrate was kept in the closed bottle. K, Fe, Mn, Cu and P were determined in the filtrate.

2.1.13. Determination of K and P

Using a pipette, 20 ml filtrate was transferred to a 100 ml volumetric flask. The flask was made to volume with distilled water and mixed.

1. Measurement of K:

10ml diluted filtrate was transferred into a 50ml volumetric flask using a pipette. The flask was made to volume with water and mixed. The content of K was measured by flame photometer. When the reading was higher than the reading of the highest standard solution, a larger dilution was made, e.g. 5ml filtrate into a 50ml volumetric flask. In this case 1:100 diluted HNO_3 was added to the volumetric flask to make the total volume of 1:100 diluted HNO_3 and filtrate equal to 10ml.

2. Measurement of P:

5 ml diluted filtrate was transferred to a 50ml volumetric flask. 30ml water was added, mixed. 10ml ammonium molybdate-ascorbic acid

solution was added made to volume with distilled water and mix. After 15 minutes, the absorbance was measured on a spectrophotometer at 890 nm. When the absorbance was higher than that of the highest standard solution, the procedure was repeated using a smaller amount of filtrate. In this case 1:100 diluted HNO_3 was added to the volumetric flask to make the total volume of 1:100 diluted HNO_3 and filtrate equal to 5ml.

When the content of P was very high, it was diluted, the filtrate further before the transfer to the 50ml flask. The dilution was made with water. After transferred of 5 ml diluted filtrate to the 50ml volumetric flask, 5ml 1:100 diluted HNO_3 and water to approx, 30ml were added. Then 10 ml ammonium molybdate-ascorbic acid was added, the 50ml volumetric flask was made to volume with water and the absorbance were measured at 890 nm after 15 minutes.

2.1.14. Measurement of Fe, Mn and Cu

The content of these elements were measured by atomic absorption spectrometer (AAS) directly in the undiluted filtrate.

Calculations: K and P mg / kg mushroom material $\frac{a \times 25000}{b \times c}$

Where, a=mg/1 K or P measured on atomic absorption spectrometer, flame photometer or spectrophotometer,

b = ml diluted filtrate transferred into the 50 ml volumetric flask for determination of K, or P.

c = gm mushroom material weighed into the digestion tube.

Additional dilution was made before the transferred to the 50ml volumetric flask, the result was multiplied with the dilution factor.

Cu, Fe and Mn mg/kg mushroom material = $\frac{d \times 100}{c}$

Where, d=mg/1 Cu, Fe or Mn measured on atomic absorption spectrometer or spectrophotometer.

c = gm mushroom material weighed into the digestion tube.

2.1.15. Determination of total sulphur

Organic matter were destructed and sulphur was oxidized to sulphate by digestion with a mixture of nitric and perchloric acid. The sulphate .was determined by precipitation as barium sulphate.

Procedure:

1. 1.0gm mushroom material of each different stage was taken into each of 38 nitrogen digestion tubes. The two remaining tubes were taken as blanks. 5 ml of 68% nitric acid was added to each of all 40 tubes. The content were mixed in each tube and left the tubes overnight. The tubes were placed in a digester and the tubes were covered with the exhaust manifold. The temperature was set to 80°C. The digester was turned on and the digestion was continued for 1.5 hours. After turned off the digester the tubes were cooled to room temperature.

2. After complete cooling, 5ml of 70% perchloric acid was added to each tube and mixed. The temperature was set to 180°C, the digester was turned on and digestion was continued for 3 hours. After 3 hours, the digester was turned off.

3. After cooling, the digestion mixture was transferred to a 100ml volumetric flaks. The flask was made up to volume with water and mixed. They were filtered on a dry filter into a dry bottle, which was closed with a screw cap. The filtrate was kept in the closed bottle.

4. 80 ml filtrate was transferred into a 600 ml beaker. Distilled water was added to the beaker to make the volume approx. 300 ml. 20ml 1:1 hydrochloric acid was added, stirred and the solution was heated to boiling. While stirring, 20ml of 0.5 M $BaCl_2$ solution was added dropwise, and the solution was boiled for 5 minutes while stirring.

5. The beaker was covered with a watch glass and it was placed on a boiling water bath for 1 hour. The water bath turned off and the beaker leave on the water bath until the precipitate was settled. The beaker was removed from the water bath and it was cooled to room temperature.

6. The supernatant was decanted through a Whatman No. 42 filter and the precipitate was transferred completely to the filter using hot distilled water. The precipitated tends to adhere to the beaker and it may be necessary to rub some precipitate off the inside of the beaker using a glass spatula with a rubber sleeve. The precipitate was continued to wash on the filter with hot distilled water until chloride has been removed completely. The presence of chloride was checked by adding a few drops filtrate from the funnel to a few ml 0.5 % silver nitrate.

7. The filter was removed from the funnel, it fold carefully taking care that no precipitate was lost, and it was placed in a ceramic crucible which has been heated to 800°C, cooled and weighed on an analytical

balance. The filter was placed in an oven at 105°C, and it left in the oven until it dry.

8. The crucible was moved with the dry filter to a muffle furnace adjusted to 800°C. After half an hour, the crucible was removed from the muffle furnace; it was cooled in a desiccator, and weighed the crucible with precipitate on an analytical balance. The heating was repeated in the muffle furnace, cooling and weighing until the weight became constant.

Calculation:

A x 1374 %S= $\frac{A \times 1374}{M \times W}$ $\%SO_3$ =8x2.50 Where, A = Weight of $BaSG_4$ (gm) M=amount of solution transferred to beaker for precipitation of $BaSO_4$, ml W = Weight of mushroom material sample (gm).

Reagents:

I) Iron accelerator

Procured from the LECO Company.

II) Copper accelerator

Procured from the LECO Company.

III) Concentrated sulphuric acid

98% H_2SO_4, Specific gravity 1.84.

IV) Catalyst mixture

1000gm potassium sulphate (K_2SO_4) and 100gm copper (11) sulphate ($CuSO_4.5H_2O$ in a mortar) was crushed and mixed.

V) 33% sodium hydroxide

Sodium hydroxide solution was prepared by dissolving 1667g NaOH in water in a 5 litter volumetric flask. After complete dissolution, the flask was filled to volume with water, and the content was mixed.

VI) 0.05 M sodium hydroxide

The content of 1 vial hydrochloric acid (3.645g) was transferred to a 2 liter volumetric flask, filled to volume with water and mixed.

VII) 0.05 M hydrochloric acid

The content of 1 vial hydrochloric acid (3.645g) was transferred to a 2 litre volumetric flask, filled to volume with water and mixed.

VIII) Methyl red-methylene blue indicator solution

0.667g methyl red was dissolved in 500ml 96% ethanol. 0.625g methylene blue was also dissolved in 500ml 96% ethanol. The two solutions were mixed equal volume.

IX) Nitric acid, 68 %

X) **1:20 diluted HNO_3**

20ml 68% HNO_3 was transferred to a 2000ml volumetric flask, the flask was made to volume with water and mixed.

XI)5MHNO_3

700ml concentrated HNO_3 (65%) was transferred to a 2 litter. volumetric flask.

XII) $LaCl_3$ solution

435g $LaCl_3.7H_2O$ weighed into a beaker. 100ml 5M HNO_3 more, and transferred the solution was added to a 5 litter volumetric flask. Made to volume with water and mixed. The solution was contained 3.25% La.

XIII) Calcium stock solution 1

2.502g $CaCO_3$ was weighed into a 1000ml volumetirc flask. Some water and 15ml 37 % HC1 were added. After complete dissolution of the $CaCO_3$, made to volume with water and mixed. The solution was contained 5.00 cmol (+) Ca per 1.

XIV) Calcium stock solution 2

100ml calcium stock solution 1 was pipetted into a 500 ml volumetric flaks. The flask were made to volume with water and mixed. The solution contained 1.00 cmol (+) Ca per 1.

XV) Calcium standard solutions

0-5-10-15-20ml calcium stock solution 2 was pipetted into 5200ml volumetric flasks. 20 ml $LaCl_3$ solution and 80 ml 1:100 diluted HNO_3 were added to each flask, made to volume with water and mixed. The solutions were contained 0.00-5.01-10.02-15.03-20.4 mg/1 Ca.

XVI) Potassium stock solution 1

3.859gm KC1 was weighed into a 1000 ml volumetric flaks. 500 ml water was added. After complete dissolution of the salt, made to volume with water and mixed. The solution was contained 2000 mg/1 K.

XVII) Potassium stock solution 2

100ml potassium stock solution 1 was pipetted into a 500ml volumetric flask and made to volume with water and mixed. The solution contained 400mg/1 k.

XVIII) Potassium standard solution

0-5-10-15-20 ml potassium stock solution 2 was pipetted into 5200 ml volumetric flask. 40 ml 1:100 diluted HNO_3 were added to each flask, made to volume with water and mixed. The solutions contained 0.00-10.0-20.0-30.40.0 mg/1 K.

XIX) 24.0g ammonium molybdate $(NH_4)_6Mo_7O_{24}.4H_2O$) was dissolved in 500 ml water in a beaker.

In another beaker, 0.5816g potassium antimony tartrate ($C_4H_4O_7KSb$) was dissolved in 100 ml water. 2 liter water was transferred to a 5 liter volumetric flask, it was added slowly, while shaking 300 ml concentrated H_2SO_4 was added. Caution: The mixture became very hot, and it might be necessary to stop the addition of sulfuric acid, allowed some cooling, and then addition of sulfuric acid. After the addition of sulfuric acid has been completed, the solution allowed to cool to room temperature. The solutions of ammonium molybdate and potassium antimony tartrate was transferred to the flask, made to volume with water and mixed. The solution was stored in a dark bottle in a refrigerator.

XX) Ammonium molybdate-ascorbic acid solution

4.1g ascorbic acid was dissolved in 1 liter ammonium molybdate solution. This solution was not stable and used on the same day as it was prepared. Hence, a fresh solution might be prepared daily.

XXI) P stock solution 1,100 mg/I P

0.4394 g KH_2PO_4 were weighed into a 1000 ml volumetric flask. Some water was added. When the salt was completely dissolved, made to volume with water and mixed.

XXII) P stock solution 2,100 mg/1 P

10ml P stock solution 1 was transferred into a 1000 ml volumetric flask. Made to volume with water and mixed.

XXIII) P standard solutions, 0.0-0.1-0.2-0.3-0.4 mg/1 P

5ml 0.5 M $NaHCO_3$ and 5ml 0.3 M H_2SO_4 were transferred into each of 550ml volumetric flasks. During 30 minutes to complete effervescence mixed and shake frequently. 0-5-10-15-20ml P stock solution 2 was added to the flasks and water to approximately 30ml. 10 ml ammonium molybdate-ascorbic acid solution were added to each flask, made to volume with water and mixed. After 15 minutes, the absorbance was measured on a spectrophotometer at 890nm.

RESULTS AND DISCUSSION

2.2. pH of Oyster mushroom (*Pleirotus ostreatus*):

pH of two stages of Oyster mushroom are shown in the table 2.1. The pH of Oyster mushroom fruting body was in the acidic range of the pH scale. The results indicate that the acidity of *Pleurotus ostreatus* decreases gradually with the changes of its maturity.

2.3. Moisture and ash content:

Moisture is necessary for most of the physiological reaction in plant tissue and without moisture, plants become dry, life does not exist. The moisture contents were found to be slightly varied in three stages of Oyster mushroom (90%, 91% and 92%) as shown in table 2.1. The results reveal that the moisture content of Oyster mushroom increases with its maturity. Most of the inorganic constituents or minerals are present in ash. The ash content of three-stages of Oyster mushroom is given in the table 2.1.

2.4. Crude fibers content of Oyster mushroom:

Crude fibers are composed of the substances which are insoluble but become soluble upon boiling in 1.25% H_2SO_4 and in 1.25% NaOH. The main components are cellulose and lignin and it has pronounced effect on digestion and absorption of nutrients[21]. The fibers content decreased with the maturity as shown in table 2.1.

Table 2.1: pH, moisture, ash and crude fibers content of Oyster mushroom flesh.

Parameter	Immature (%)	Mature (%)	Over mature (%)
pH	6.14 ± 0.09	6.23 ± 0.07	6.3±0.08
Moisture (gm%)	90 ± 0.05	91 ± 0.09	92±0.08
Ash (gm%)	0.82 ± 0.06	0.73 ± 0.06	0.61±0.08
Crude fiber (gm%)	1.4 ± 0.04	0.96 ± 0.07	0.88±0.06

The amount of crude fibers at the immature, mature and over mature stage were 1.4%, 0.96% and 0.88% respectively.

2.5. Protein content of Oyster mushroom:

Protein content of Oyster mushroom at three stages are shown in table 2.2.

Mature stage of Oyster mushroom contained highest amount of protein i.e 3.71%. While immature and over mature stage contained 3. 5% and 3.59% respectively.

2.6. Water-soluble protein content of Oyster mushroom flesh:

The amounts of water-soluble protein present in the Oyster mushroom flesh of the three stages of Oyster mushroom are shown in the table 2.2.

Table 2.2: Protein, water-soluble protein, lipid, total soluble sugar, reducing sugar, non-reducing sugar and starch present in the oyster mushroom flesh.

Parameter	Immature (%)	Mature (%)	Over Mature (%)
Protein	3.51±0.08	3.71±0.05	3.59±0.07
Water-soluble protein (gm%)	2.50 ± 0.07	2.67 ± 0.07	2.41±0.08
Total Lipid (gm%)	0.19 ± 0.06	0.21 ± 0.04	0.22±0.08
Total soluble sugar (gm%)	2.8 ± 0.06	2.91 ± 0.05	2.98±0.06
Reducing sugar (gm%)	2.35 ± 0.02	2.44 ± 0.03	2.48±0.05
Non-reducing sugar (gm%)	0.427 ± 0.03	0.446 ± 0.02	0.5±0.04
Starch content (gm%)	1.4 ± 0.05	1.5 ± 0.04	1.6±0.06

As given in table 2.2, the water-soluble protein content of Oyster mushroom slightly decreases with the change of maturity.

2.7. Lipid content of Oyster mushroom flesh:

Fats are concentrated form of energy and are important as carrier of certain fat-soluble vitamins such as A, D, E and K[37]. Lipid contents of three different stages of Oyster mushroom are presented in table 2.2.

The present data of three-stages of Oyster mushroom clearly indicate that they contained very little amount of lipid. So it may not be

considered as a source of lipid also. The total lipid content was highest at the over mature stage i.e. 0.22% while immature and mature stage contained 0.19% 0.21% respectively.

2.8. Total soluble sugar content of Oyster mushroom:

Total sugar content of three stages of Oyster mushroom are given in table 2.2.

As shown in table 2.2, the amount of total sugar present in the Oyster mushroom increases with the maturity of the stage. The highest amount of total soluble sugar found in over mature stage was 2.98%.

2.9. Reducing and non-reducing sugar content of Oyster mushroom:

The reducing and non-reducing sugar contents of three-stages of Oyster mushroom are shown in table 2.2.

Over mature Oyster mushroom contained 2.48% reducing sugar and 0.5% non reducing sugar, mature stage contained 2.44% reducing sugar and 0.446% non-reducing sugar or sucrose, immature contained 2.35% reducing sugar and 0.427% non-reducing sugar. The increases in reducing sugar were due to enzymatic conversion of starch to reducing sugar and also conversion of some non-reducing sugar.

2.10. Starch content of Oyster mushroom:

Starch content of immature, mature and over mature Oyster mushroom are shown in table 2.2.

Starch is a stored carbohydrate of chlorophyll containing plants[37]. In plant, the starch is land down in the cells in granules. It is formed by

α-glycoside chain. In the over mature stage, the highest amount of starch was present i.e. 1.6% and mature stage contained 1.5% and the immature stage contained 1.4%. The result suggested that starch content of Oyster mushroom increased gradually with the maturity.

2.11 Mineral content

The amount of some minerals content present in the three different (immature, mature and over mature) stages of mushroom as shown in the table 2.3.

Table-2.3: Fe, K, P, S, Cu and Mn content of mushroom.

Stages	Content (mg/100gm)					
	K	**P**	**S**	**Fe**	**Cu**	**Mn**
Immature	328	135	630	15	1	1
Mature	340	160	504	14	2	1.5
Over mature	346	165	756	19	3	2

The micronutrients of Oyster mushroom such as Cu and Mn content of gradually increased with its advancement of maturity. K and P is also increased with the advancement of its maturity.

But in case of S and Fe content, mature stage contains lowest amount compared with immature and over mature stage.

CHAPTER-THREE

ANTIBACTERIAL ACTIVITY

3.1 Preparation of mushroom crude extract:

Oyster mushroom (*Pleurotus ostreatus*) was collected and dried in the sun and then crushed into powder. The selected extracts were then prepared in the following way. These extracts were used for screening of antibacterial, insecticidal, repellent, cytotoxic, larvaecidal, antidiabetic and hepatoprotective activity.

3.2 Extraction procedures:

3.2.1. Extraction procedure for organic (methanol, ethyl acetate, chloroform and pet-ether) solvents

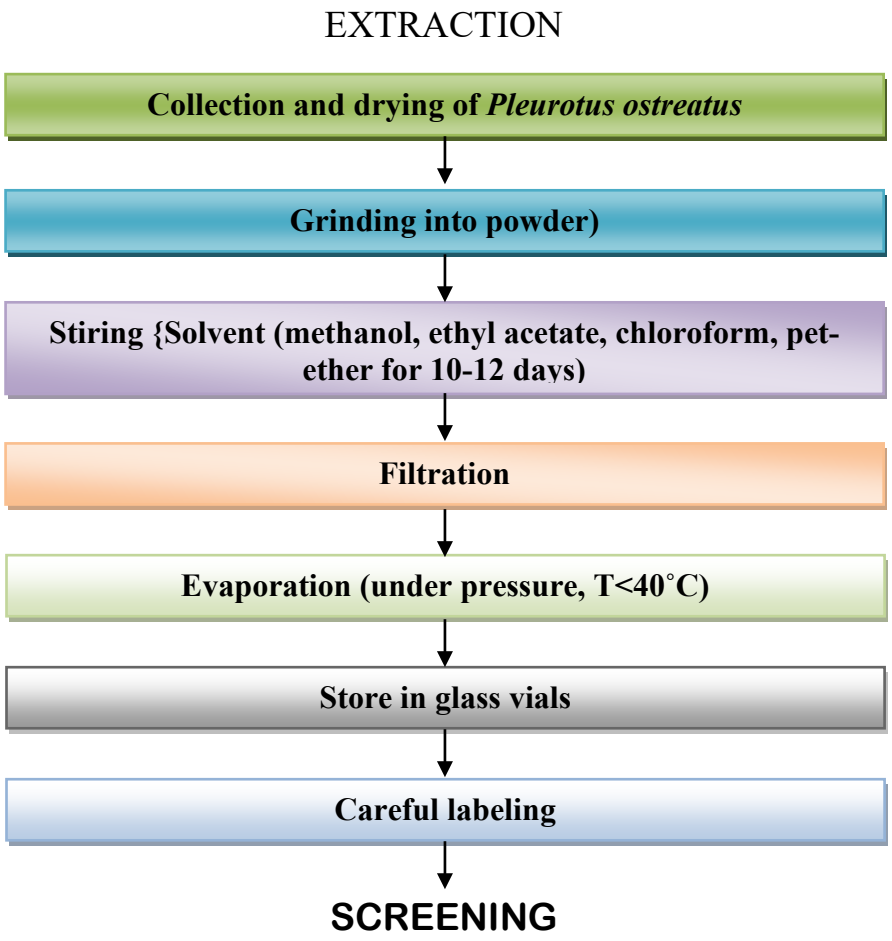


Figure-3.1: Pathway of extraction (organic solvents).

3.2.2. Extraction procedure for Distilled Water solvent

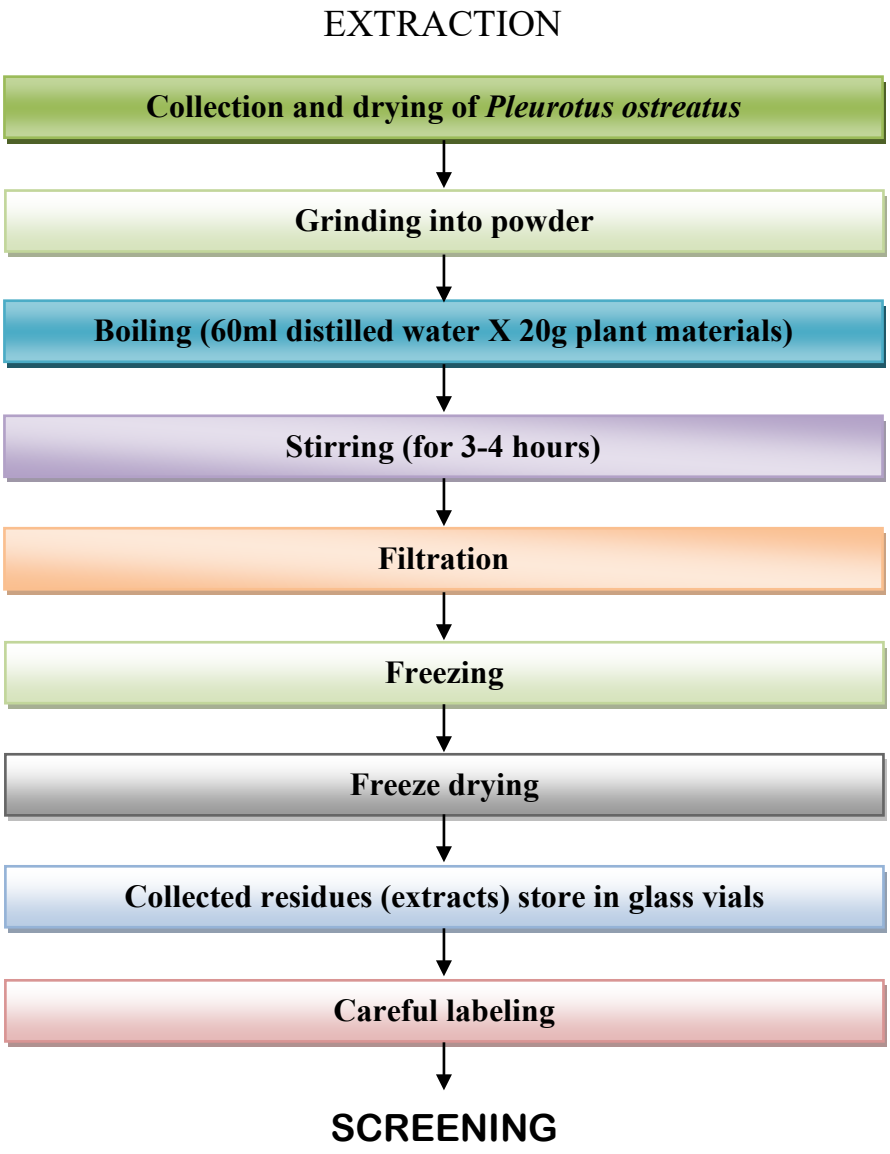


Figure-3.2: Pathway of extraction (water solvent).

3.3. Introduction:

Plants have been a growing source for medicinal compounds since pre-historic time. Different parts of plants were used in Ayurvedic, Unani and Allopathic systems of medicine for the treatment of human diseases such as wound, infections, typhoid, dysentery, skin disease etc. Chemical substances or biological agents that either destroy or suppress the growth of micro-organism is called antimicrobial agent. Antimicrobial activity can be detected by observing the growth response of various microorganisms to the different plant extracts or isolated compounds from them, which are placed in contact with them. The susceptibility of micro-organisms to antimicrobial agents can be measured in vitro by a number of techniques. Among these the disc diffusion method[68] widely acceptable for the preliminary evaluation of antibacterial activity was adopted for the present study.

3.4. Apparatus and reagents:

(i) Standard disc (Amoxycillin-30µg/disc)

(ii) Ethyl acetate

(iii) Ethanol (95%)

(iv) Filter paper discs (sterilized)

(v) Petridishes

(vi) Inoculating loop

(vii) Sterile cotton

(viii) Test tubes

(ix) Sterile forceps

(x) Micropipette

(xi) Spirit burner

(xii) Nutrient agar (Difco)

(xiii) Laminar airflow unit (Bio-Craft & Scientific Industries, India)

(xiv) Incubator (OSK-9639A, Japan)

(xv) Refrigerator (Aritstion, Italy)

(xvi) Autoclave (ALP Co. Ltd. KT-30L, Tokyo)

3.5. Test organisms:

Eight pathogenic bacteria were selected for the antibacterial activity test, three of them were gram positive and the remaining were gram negative. The pure cultures were collected from the Protein and Enzyme Research Laboratory of the Department of Biochemistry and Molecular Biology, University of Rajshahi. The bacterial strains used for this investigation are listed in the following table.3.1.

Table-3.1: List of test bacteria.

Gram positive	Gram negative
(i) Bacillus megaterium QL-38	*(i) Shigella dysenteriae AL35587*
(ii) Bacillus subtilis QL-40	*(ii) Salmonella typhi*
(iii) Staphylococcus aures	*(iii) Bacillus cerus*
	(iv) Escherichia coli FPFC-1407
	(v) Shigella sonnei AJ-8992

3.6. Sterilization Procedure:

Antibacterial screening was carried out in a laminar airflow unit and all types of precautions were highly maintained to avoid any contamination during the test. UV light was switched on one hour earlier of the start of the experiment to avoid contamination. Petridishes and other glassware were sterilized by an autoclave machine at a temperature of 121°C and pressure of 15 lb/sq inch for 30 minutes. Blank discs were also sterilized and kept in laminar hood under UV light for 30 minutes.

3.7. Preparation of the culture media:

For demonstrating the antibacterial activity and subculture of the test organisms, nutrient agar media (Difco) was used. For preparation of 100 ml nutrient agar media, 0.5 gm peptone, 1 gm yeast extract, 0.5 gm sodium chloride and 2gm agar were dissolved in distill water. This composition of the nutrient was maintained constant throughout the work.

3.8. Preparation of fresh culture:

The nutrient agar medium was prepared and dispersed in a number of clean test tubes to prepare slants (5 ml in each test tube). The test tubes were plugged with cotton and sterilized for 30 minutes. After sterilization, the test tubes were kept in an inclined position (45°) for solidification. These were then incubated at 37.5°C to ensure sterilization. The test organisms were transferred to the agar slants from the supplied pure cultures with the help of an inoculating loop in an aseptic condition. The loop was red heated carefully after each transfer of micro-organism to avoid contamination. The inoculated slants were then incubated at

37.5°C for 24 hours to assure the growth of test organisms. These fresh cultures were used for the sensitivity test.

3.9. Preparation of the test plates:

The test plates were prepared according to the following procedure.

(i) 15 ml previously prepared nutrient agar medium was poured in each of the clean test tubes and plugged with cotton.

(ii) The test tubes and a number of Petridishes were sterilized in an autoclave for 30 minutes and were transferred into laminar air flow unit and then allowed to cool about 45°C to 50°C.

(iii) The test organism was transferred from the fresh subculture to the test tube containing 15 ml autoclaved medium with the help of an inoculating loop in an aseptic condition. Then the test tube was gently shaken to get a uniform suspension of the organism.

(iv) The bacterial suspensions were immediately transferred to the sterile petridishes in an aseptic area. The petridishes were rotated several times, first clockwise and then anticlockwise to assure homogenous distribution of the test organisms.

The media were poured into Petridishes in such a way as to give a uniform depth of approximately 4 mm.

(v) Finally, the medium was cooled to room temperature under laminar airflow and was stored in a refrigerator (~4°C).

3.10. Preparation of discs:

Three types of discs were prepared for antibacterial screening. These are-

1. Sample discs:

Sterilized (BBL, U.S.A) filter paper discs (5 mm in diameter) were prepared with the help of punch machine and were taken in a blank petridish. Sample solution of desired concentration (10µl/disc) was applied on the discs with the help of a micropipette in an aseptic condition.

2. Standard discs:

These were used to compare the antibacterial activity of test material. In our investigation, Amoxicillin (30µg/discs) was used as standard disc.

3. Solvent control discs:

These were prepared using same filter paper (5 mm diameter) and same volume of residual solvent without sample following the same process and condition. These were used as negative control to ensure that the residual solvent and the filter paper themselves was not active.

3.11. Preparation of test sample:

20 mg and 40 mg of hot water (W), methanol (M), Pet-ether (P) and chloroform (C) extracts were dissolved in 1 ml respective solvent in separate glass vial. Thus the concentrations were 20µg/µl and 40µg/µl, respectively for each extract.

3.12. Placement of the discs and incubation:

The following procedure was adopted for the placement of the discs:

(i) The dried crude extract discs and standard discs were placed gently on the solidified agar plates seeded with the test organisms with the help of a pair of sterile forceps to ensure contact with the medium.

(ii) Then the plates were kept in a refrigerator at 4°C for 24 hours in order to provide sufficient time to diffuse the antibiotics into the medium.

(iii) Finally, the plates were incubated at 37.5°C for 24 hours in an incubator.

3.12.1. Precaution:

The discs were placed in such a way that they were separated from each other about 20 mm and 15 mm far from the edge of the plate to prevent overlapping the zones of inhibition.

3.12.2. Measurement of the zones of inhibition:

After incubation, the antibacterial activities of the test samples were determined by measuring the diameter of inhibitory zones in term of mm with a transparent scale.

RESULTS AND DISCUSSION

The antibacterial activity of the crude hot water (W), methanol-(M), pet-ether (P) and chloroform (C) extracts were tested against eight bacteria at concentrations of 200µg/disc and 400µg/disc. Standard antibiotic disc Amoxicillin 30µg/disc was used for comparison. The results obtained are shown in table 3.2 and 3.3.

The produced zone of inhibition for M extract of *Pleurotus ostreatus* against *Bacillus megatorium, Shigella dysenteriae, Bacillus cerus* were 13, 11, 11mm at 400µg/disc dose respectively. At 200 µg/disc dose, the produced zones of inhibition against the same bacteria were 10, 9 and 9 mm, respectively but this extract had no activity against the other bacteria. These data indicate that the antibacterial activity of (M) against the above bacteria increases dose dependently.

On the other hand, P extract had the activity against *Bacillus megatorium* only 8mm and12mm at 200µg/disc and 400µg/disc dose respectively.

The produced zone of inhibition for C extract of *Pleurotus ostreatus* against *Bacillus megatorium, Shigella dysenteriae, Bacillus cerus*, *Escherichia coli* and *Bacillus subtilis* were 9, 9, 9, 11 and 8 mm at 400µg/disc dose respectively. At 200µg/disc dose, the produced zone of inhibition against the same bacteria were 8, 7, 7, 8 and 7 mm respectively but this extract had no activity against the other bacteria. These data indicate that the antibacterial activity of C against the above bacteria increases dose dependently.

Table-3.2: *In vitro* antibacterial activity of Methanol (M) extract, Pet-ether (P) extract and Amoxicillin.

Test bacteria	**Methanol extract of *Pleurotus ostreatus* (µg/disc)**		**Pet-ether extract of *Pleurotus ostreatus* (µg/disc)**		**Amoxicillin (µg/disc)**
	200	**400**	**200**	**400**	**30**
	Zone of inhibition (diameter in mm.)				
Bacillus megatorium	10	13	8	12	28
Shigella Dysenteriae	9	11	-	-	27
Salmonella typhi	-	-	-	-	25
Bacillus cerus	9	11	-	-	21
Escherichia colli	-	-	-	-	27
Bacillus subtilis	-	-	-	-	28
Staphylococcus aureus	-	-	-	-	24
Shigella sonnei	-	-	-	-	27

Symbol: "-" indicate no sensitivity.

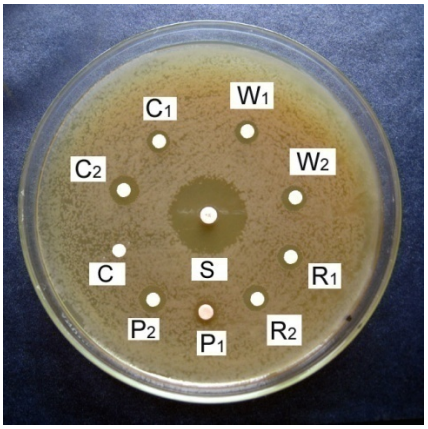
Table-3.3: *In vitro* antibacterial activity of Chloroform (C) extract, Hot water (W) extract and Amoxicillin**.**

Test bacteria	**Chloroform extract of *Pleurotus ostreatus* (µg/disc)**		**Hot water extract of *Pleurotus ostreatus* (µg/disc)**		**Amoxicillin (µg/disc)**
	200	**400**	**200**	**400**	**30**
	Zone of inhibition (diameter in mm.)				
Bacillus megatorium	8	9	6	6	28
Shigella Dysenteriae	7	9	8	10	27
Salmonella typhi	-	-	-	-	25
Bacillus cerus	7	9	-	-	21
Escherichia colli	8	11	-	-	28
Bacillus subtilis	7	8	-	-	28
Staphylococcus aureus	-	-	-	-	24
Shigella sonnei	-	-	-	-	27

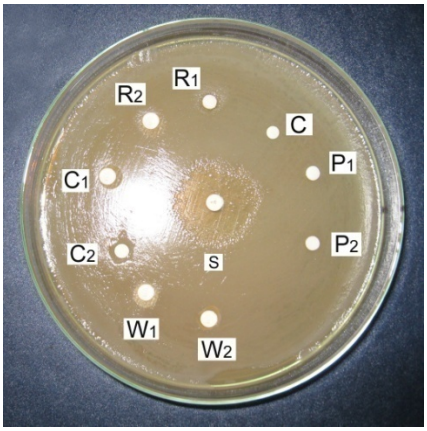
Symbol: "-" indicate no sensitivity.

On the other hand, W extract had the activity against *Bacillus megatorium* and *Shigella dysenteriae* 6, 8 mm at 200µg/disc and 8, 10 mm at 400µg/disc dose respectively.

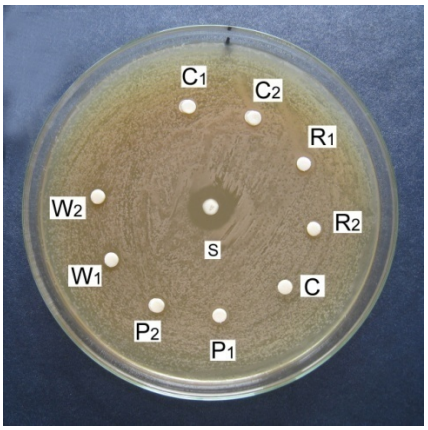
From the above experiment it has been found that the extracts might have some compounds, which are responsible for the antibacterial activity. Finally we can conclude that *Pleurotus ostreatus* may be used as the medicine against boil, abscess, typhoid fever and dysentery.



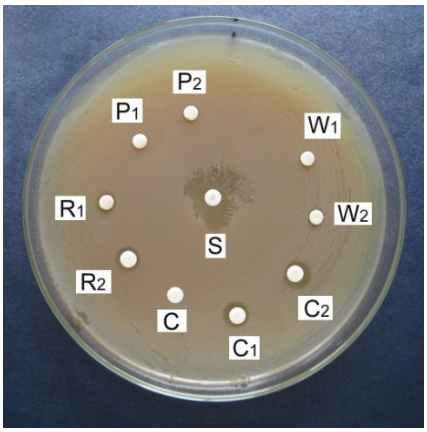
Bacillus Megatorium



Shigella Dysenteriae

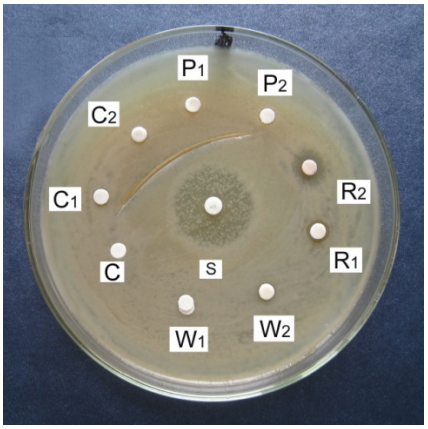


Salmonella typhi

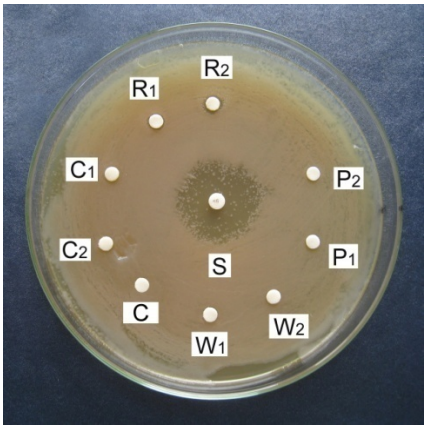


Bacillus cerus

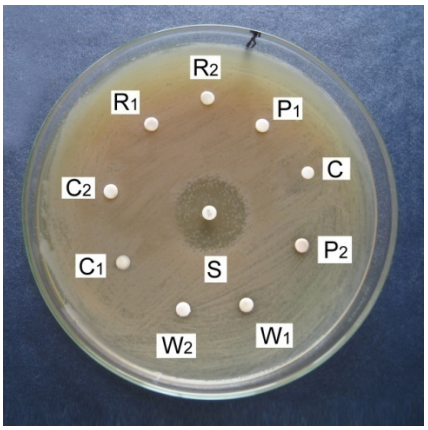
Phtograph-3.1: *S= Standard (Kanamycine-30µg/disc); C = Control; M_1 = Methanol. (200 mg/disc); M_2 = Methanol. (400 mg/disc)*
P_1 = Pet.-Ether (200 mg/disc); P_2 = Pet.-Ether (400 mg/disc); C_1 = Chloroform (200 mg/disc); C_2 = Chloroform (400 mg/disc); W_1 = Hot Water (200 mg/disc); W_2 = Hot Water (400 mg/disc).



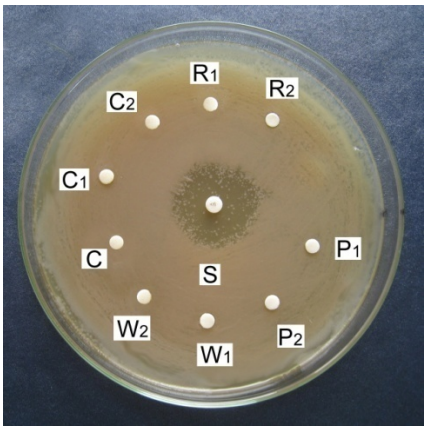
Escherichia colli



Bacillus subtilis



Staphylococcus aureus



Shigella sonnei

Phtograph-3.2: *S= Standard (Kanamycine-30µg/disc); C = Control; M_1 = Methanol. (200 mg/disc); M_2 = Methanol. (400 mg/disc)*

P_1 = Pet.-Ether (200 mg/disc); P_2 = Pet.-Ether (400 mg/disc); C_1 = Chloroform (200 mg/disc); C_2 = Chloroform (400 mg/disc); W_1 = Hot Water (200 mg/disc); W_2 = Hot Water (400 mg/disc);

3.13. Determination of minimum inhibitory concentrations (MIC)[68]:

3.13.1. Introduction:

The minimum inhibitory concentration (MIC) may be defined as the lowest concentration of the test sample or drug at which it shows the highest activity against the growth of the pathogenic micro-organisms. There are two methods for determining the minimum inhibitory concentration (MIC) values. They are as follows:

(i) Serial tube dilution technique or turbidimetric assay.

(ii) Paper disc plate technique or agar diffusion assay.

Here "serial tube dilution technique" was followed using nutrient broth medium to determine the MIC values of different extracts and isolated compounds against the following two gram positive and three gram negative bacteria.

Table-3.4: List of bacteria used for determination of MIC (Collected from the Protein and Enzyme Laboratory of the Department of Biochemistry and Molecular Biology, University of Rajshahi).

Gram positive	**Gram negative**
Bacillus megatorium QL-38	*Shigella dysenteriae AL-35587*
Bacillus subtilis QL-40	*Bacillus cerus*
	Escherichia coli FPFC-1407

3.13.2. Preparation of inoculums:

Fresh cultures of the test organisms were grown at 37.5°C for over night on nutrient agar media. Bacterial suspensions were then prepared in sterile nutrient broth medium in such a manner so that the suspension contains 10^7 CFU/ml. These suspensions were used as inoculums.

3.13.3. Preparation of the sample solution:

The crude hot water (W), methanol (M), pet-ether (P) and chloroform(C) extracts were measured accurately (2.048mg each) and were taken in different vials. Then 2% DMSO solution (2 ml) was added to each of the vials and mixed well to make sample solution whose concentration becomes 1024µg/ml.

3.13.4. Procedure of serial tube dilution technique:

1. Twelve autoclaved test tubes were taken, nine of which were marked 1, 2, 3, 4, 5, 6, 7, 8 and 9 the rest three were assigned as C_M(Media), C_{MS}(Media + sample) and C_{MI}(Media + inoculumn).

2. To each of twelve test tubes, sterile nutrient broth media (1 ml) was added.

3. These test tubes were cotton plugged and sterilized in an autoclave for 30 minutes at 121°C temperature and 15lbs/sq. inch pressure.

4. After cooling, 1 ml of the sample solution was added to the 1st test tube and mixed well. Then 1 ml of this content was transferred to the 2nd test tube.

5. The content of the 2nd test tube was mixed uniformly. Again 1 ml of this mixture was transferred to the 3rd test tube. This process of serial dilution was continued up to 9th test tube.

6. Then 10µl of properly diluted inoculums was added to each of the nine test tubes and mixed well.

7. To the control test tube C_{MS}, 1 ml of the sample was added, mixed well and 1 ml of this mixed content was discarded to check the clarity of the media in presence of diluted solution of the sample.

8. To the control test tube C_{MI}, 10µl of inoculums was added to observe the growth of the organism in the media used.

9. The control test tube CM, containing media only was used to confirm the sterility of the media.

10. All the test tubes were incubated at 37°C for 18-24 hours.

RESULTS AND DISCUSSION

The MIC values of methanol (M), petroleum ether (P), chloroform(C) and hot water (W) extracts were determined by serial dilution technique against two gram positive bacteria (*Bacillus megatorium* & *Bacillus subtilis)* and three gram negative bacteria (*Shigella dysenteriae, Bacillus cerus* & *Escherichia coli*). These values were shown in table 3.5.

For the crude methanol (M) extract the growth was observed in the test tube containing 256μg/ml of extract against *Bacillus megatorium*, *Shigella dysenteriae* and *Bacillus cerus.*

Table-3.5: MIC values of methanol (M), Pet-ether (P), chloroform (c) and hot water (W) extracts of *Pleurotus ostreatus*.

Test Bacteria	Methanol extract (µg/ml)	Pet-ether extract (µg/ml)	Chloroform extract (µg/ml)	Hot water extract (µg/ml)
Bacillus megatorium	256	256	256	256
Shigella dysenteriae	256	-	512	512
Bacillus cerus	256	-	512	256
Escherichia coli	-	-	256	-
Bacillus subtilis	-	-	256	-

Symbol: "-" indicate no sensitivity.

So the MIC values of M extract for *Bacillus megatorium*, *Shigella dysenteriae* and *Bacillus cerus* were 256µg/ml.

For pet-ether extract the growth was observed in the test tube containing 256µg/ml of extract against *Bacillus megatorium*. So the MIC value of PE was 256µg/ml against *Bacillus megatorium*.

For chloroform extract the growth was observed in the test tube containing 512µg/ml against *Shigella dysenteriae*, *Bacillus cerus* and 256µg/ml against *Bacillus megatorium*, *Escherichia coli*, *Bacillus subtilis.* So the MIC values of chloroform extract were 512µg/ml against *Shigella dysenteriae*, *Bacillus cerus* and the MIC values of chloroform

extract were 256µg/ml against *Bacillus megatorium*, *Escherichia coli*, *Bacillus subtilis*.

For hot water extract, the growth was observed in the test tube containing 512µg/ml of extract against *Shigella dysenteriae* and 256µg/ml of extracts against *Bacillus megatorium, Bacilus cerus*. So the MIC values of W extract were 512µg/ml for *Shigella dysenteriae* and 256µg/ml for *Bacillus megatorium, Bacilus cerus* .

Since the MIC values of P and M were found to be less than that of W and C. So the P and M might have more bioactive compounds than that of W and C, yet to be isolated.

CHAPTER-FOUR

BIOASSAY

4.1. Background information on the test organisms:

The whole thesis work has been designed to carry on screening of the crude extracts of the test plant species on several test organisms for the detection of biological activity keeping an option to show extent of activity by analyzing the data statistically that read on various parameters during the course of the work. The following test agents have been taken into consideration:

Table 4.1: List of the test agents.

Test agents	Types of test
T. castaneum	Dose mortality test
	Repellency test
Artemia salina	Brine shrimp lethality test
Mosquito larvae	Larvicidal test

4.2. 'Rust-red flour beetle' *(T. castaneum* Hbst.):

T. castaneum (Hbst.), the red flour beetle is an Indo-Australian origin and is found in temperate region, but survives in the winter in protected places, especially where there is central heat[96]. In the United States, it is found primarily in the southern states. *T. castaneum* is a worldwide and commonest pest of wheat-flour. It is commonly known as

'Rust-red flour beetle'. It is an insect of the family 'Tenebrionidae' under the order 'Coleoptera'.

It is one of the serious pests of stored products. Mouthparts of this pest insect are not adapted to feed on hard whole grains and they are thus found in almost any kind of flour, cracked grains etc. The specific foods of *T. castaneum,* which include whole-wheat flour, bran, rice flour, cornmeal, barley flour and oat meals. It also feeds upon dried fruits, dried plant roots, nuts, chocolates, drugs, snuff, cayenne pepper, pulses and prepared cereal foods such as corn flakes[97]. Not only pulses and millets, but also cereals are also been attacked by this beetle[98]. *T. castaneum*, attack meal, crackers, beans, spices, pasta, cake mix, dried pet food, dried flowers and even dried museum specimens[99,100]. Although small beetles, about one-fourth of an inch long, the adults are long-lived and may live for more than three years. The red flour beetle is reddish brown in color and its antennae end is a three segmented club[101]. The head of the red flour beetle is visible from above, does not have a beak and the thorax has slightly curved sides.

Scientific classification:

Kingdom: Animalia

Phylum: Arthropoda

Class: Insecta

Order: Coleoptera

Family: Tenebrionidae

Genus: *Tribolium*

Species: *T. castaneum* Hbst.

Plate-4.1: Photograph of the *T. castaneum* Hbst.

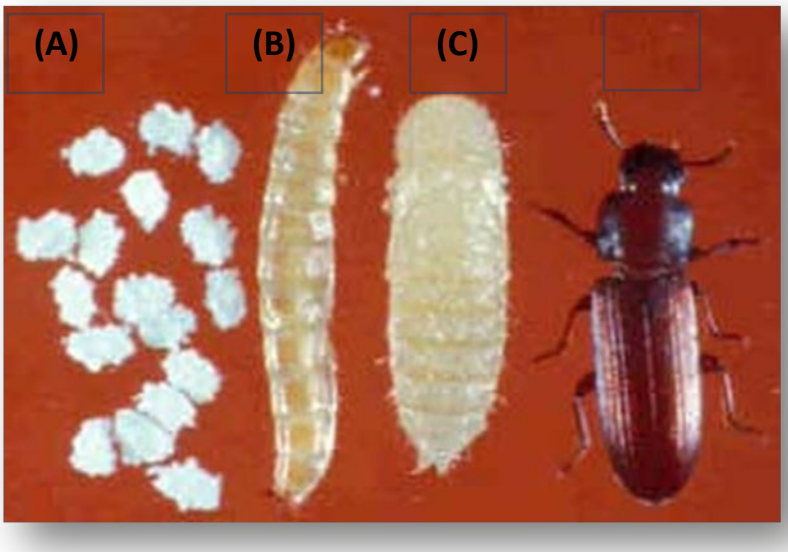


Plate-4.2: Photograph of the life cycle of the *T. castaneum* Hbst.

Table-4.2: Developmental rates of *T. castaneum* (Hbst.)

Rearing temperatures	**30°C**	**34°C**
Egg	3 days	2 days
Larva	20 days	15 days
Pupa	4 days	3 days
Reproductive maturation	5 days	4 days
Total time from egg to egg	32 days	24 days

The eggs are white, microscopic and often have bits of flour stuck to their surface. The slender larvae are creamy yellow to light brown in color. They have two dark pointed projections on the last body segment. The young larva is yellowish white and measures 1 mm in length. As it matures, it turns reddish yellow, becomes hairy and measures over 6mm in length. Its head, appendages and the last abdominal segment are darker. The adult is a small reddish-brown beetle, measuring about 3.5 mm in length and 1.2 mm in width. Its antennae are bent and bear a distinct club formed by the three enlarged terminal joints. The last antennal segment is transversely rounded. It was commonly found in wild state in rotting wood and in loose bark of trees in India. This insect is now widely distributed all over the world mainly through commerce.

The red flour beetle may be present in large numbers in infested grain, but are unable to attack sound or undamaged grain. Both the larva and the adults cause damage. They are found in great numbers on infested materials and caused serious losses and considerable damage to flour and

grains that have previously been attacked by other pests. Much of the damage done by *T. castaneum* is directly to kernels (germplasm and endoplasm). In case of severe infestation flour or other materials invaded may have a characteristics pungent odor as a result of the gaseous secretion exuded by the beetle. Such flour has an exceedingly low viscosity and its elasticity is markedly affected which may cause gastric disturbance if used as food. In severe infestation, the flour turn grayish and moldy and has a pungent, disagreeable odor making it unfit for human consumption[73]. Infested material will show many elongate reddish brown beetles, about 1/7 inch long crawling over the material when it is disturbed and brownish white (somewhat flattened) six-legged larval bedding on the inside of the grain kernels and crawling over the infested seeds. They are generally known among millers as "bran bugs". *T. castaneum* contaminates more than they consume.

According this contamination results[72] from:

- The presence of living or dead insects or insect parts;
- Cast exuvae, eggshell and pupal cases;
- Fecal and persistent odor; and
- Webbing of food.

Tribolium species are major pests of stored grains and grain products in the tropics. Control of these insects relies heavily on the use of synthetic insecticides and fumigants, which has led to problems such as disturbances of the environment, increasing costs of application, pest resurgence, pest resistance to pesticides and lethal effects on non-target organisms in addition to direct toxicity to users[86]. Thus, repellents,

fumigants, feeding deterrents and insecticides of natural origin are rational alternatives to synthetic insecticides.

4.3. Brine shrimp (*A. salina*) nauplii:

Brine shrimp lethality bioassay is a recent development in the bioassay for the bioactive compounds, which indicates cytotoxicity, as well as, a wide range of pharmacological activities (e.g. anticancer, antiviral, pesticidal, anti-AIDS, etc.) of the compounds. Bioactive compounds are almost always toxic in high doses. Pharmacology is simply toxicology at a lower dose or toxicology is simply pharmacology at a higher dose. Brine shrimp lethality bioassay is a bench top bioassay method for evaluating anticancer, anti-microbial and pharmacological activities of natural products. Natural product extracts, fractions or pure compounds can be tested for their bioactivity by this method. Here *in vivo* lethality of a simple monitor for screening a fractionation in the discovery of new bioactive natural products.

The Brine shrimp belongs to a genus of very primordial crustacean (crawfish-crayfish) the *Anostraca* (Fairy shrimp). Crawfish of this genus just have a divided exoskeleton made of Chitin enhanced protein, no usual crust of chitin (escutcheon) as other crawfish have. There are many species within the genus of *Anostraca*, but the *Artemia salina* is very nice to grow, since the rate of successful hatches is very high. To carry out toxicity tests of certain materials these nauplii are very easy to grow from its marketed cysts and to set experiments thereby.

Systemic position:

Kingdom: Animalia

Phylum: Arthropoda

Subphylum: Crustacea

Class: Branchiopoda

Order: Anostraca

Family: Artemiidae

Genus: *Artemia*

Species: *A. salina* L. (1758)

Plate-4.3: Photograph of *A. salina* L.

4.4. Mosquito larvae:

There are over 2500 different species of mosquitoes throughout the world. *Culex, Aedes, Anopheles* etc. are the most common genus of mosquitoes. Mosquitoes are very important to serve as vectors of important diseases, such as West Nile virus, dengue, filariasis, Japanese encephalitis, St. Louis encephalitis and avian malaria. Mosquito have complete metamorphosis in their life cycle with four successive stages including: egg, larva, pupa and adult. Egg, larval and pupal stages are aquatic.

Systemic position:

Kingdom: Animalia

Phylum: Arthropoda

Class: Insecta

Order: Diptera

Family: Culicidae

Genus: *Culex*

Habit: All mosquitoes must have water in which to complete their life cycle. This water can range in quality from melted snow water to sewage effluent and it can be in any container imaginable. The type of water in which the mosquito larvae is found can be an aid to the identification of which species it may be. Also, the adult mosquitoes show a very distinct preference for the types of sources in which to lay their eggs. They lay their eggs in such places such as tree holes that periodically hold water, tide water pools in salt marshes, sewage effluent ponds, irrigated pastures, rain water ponds, etc. Each species therefore has unique environmental requirements for the maintenance of its life cycle.

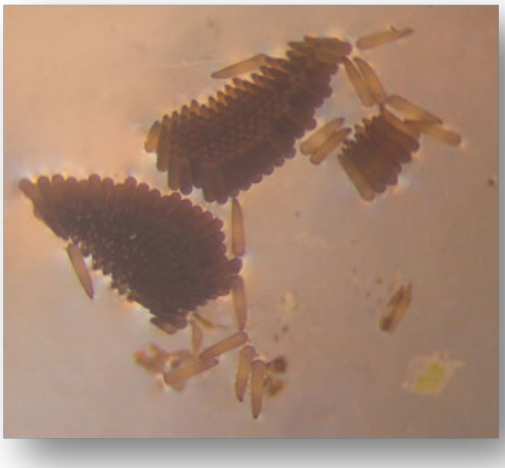
The feeding habits of mosquitoes are quite unique in that it is only the adult females that bite man and other animals. The male mosquitoes feed only on plant juices. Some female mosquitoes prefer to feed on only one type of animal or they can feed on a variety of animals. Female mosquitoes feed on man, domesticated animals, such as cattle, horses, goats, etc; all types of birds including chickens; all types of wild animals including deer, rabbits; and they also feed on snakes, lizards, frogs, and toads.

Most female mosquitoes have to feed on an animal and get a sufficient blood meal before she can develop eggs. If they do not get this blood meal, then they will die without laying viable eggs. However, some species of mosquitoes have developed the means to lay viable eggs without getting a blood meal.

The flight habits of mosquitoes depend on the species. Most domestic species remain fairly close to their point of origin while some species known for their migration habits are often an annoyance far from their breeding place. The flight range for females is usually longer than that of males. Many times wind is a factor in the dispersal or migration of mosquitoes. Most mosquitoes stay within a mile or two of their source. However, some have been recorded as far as 75 miles from their breeding source.

Life cycle: The length of life of the adult mosquito usually depends on several factors: temperature, humidity, sex of the mosquito and time of year. Most males live a very short time, about a week; and females live about a month depending on the above factors. The mosquito goes through four separate and distinct stages of its life cycle and they are as

follows: Egg, Larva, pupa, and adult. Each of these stages can be easily recognized by their special appearance. There are four common groups of mosquitoes living in the Bay Area. They are *Aedes, Anopheles, Culex,* and *Culiseta*.



(A)

(B)

Plate-4.4: Mosquito egg raft (A) and larvae (B) in natural condition.

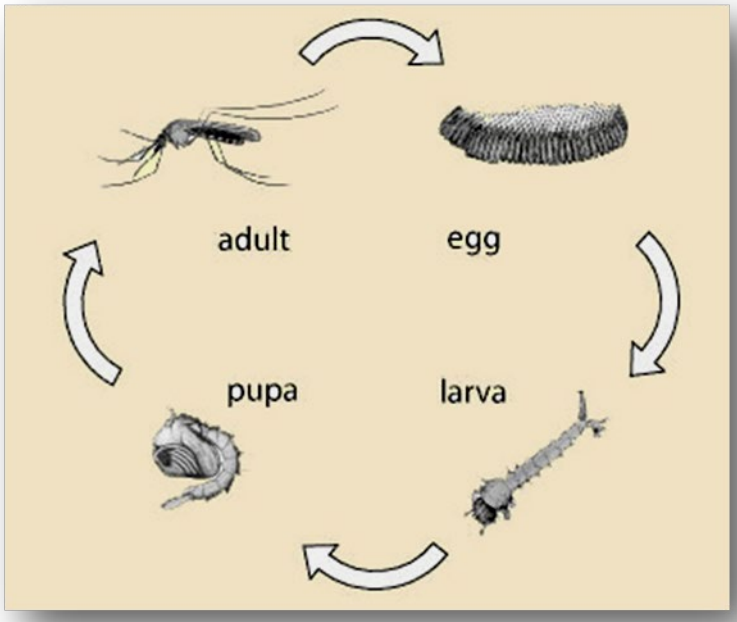


Plate-4.5: Life cycle of mosquito.

Egg: Eggs are laid one at a time and they float on the surface of the water. In the case of *Culex* and *Culiseta* species, the eggs are stuck together in rafts of a hundred or more eggs. *Culex, Culiseta,* laid their eggs on water. Most eggs hatch into larvae within 48 hours.

Larvae: Mosquito larvae are commonly referred to as "Wrigglers", these newly hatched insects can be seen wriggling up and down from the surface of the water. The larva lives in the water and come to the surface to breathe. They shed their skin four times growing larger after each molting. Most larvae have siphon tubes for breathing and hang from the water surface. The larva feed on micro-organisms and organic matter in the water. On the fourth molt the larva changes into a pupa.

Pupa: The pupal stage is a resting, non-feeding stage. This is the time the mosquito turns into an adult. It takes about two days before the adult is fully developed. When development is complete, the pupal skin splits and the mosquito emerges as an adult.

Adult: The newly emerged adult rests on the surface of the water for a short time to allow itself to dry and all its parts to harden. Also, the wings have to spread out and dry properly before it can fly. The adult mosquito can measure from 4–10 mm and morphologically has the three body parts common to insects: head, thorax, and abdomen.

The egg, larvae and pupae stages depend on temperature and species characteristics as to how long it takes for development. For instance, *Culex tarsalis* might go through its life cycle in 14 days at 70 F and take only 10 days at 80 F. (Also, some species have naturally adapted to go through their entire life cycle in as little as four days or as long as one month.

MATERIALS AND METHODS

4.5. Collection of test organisms:

Source of test insects *T. castaneum,* used in the present experiment were taken from the stock cultures of the Crop Protection and Toxicology Laboratory, University of Rajshahi, (RU) Rajshahi-6205, Bangladesh; and reared as subcultures to be used in the experimentation. Mosquito eggs (raft) were collected from different drains of RU campus. Brine shrimp cysts were collected from (different Aquarium shops) Dhanmondi, Dhaka, Bangladesh. Eggplant aphids were collected from the cultivated field of Fourth Science Building, University of Rajshahi.

4.5.1. *T. castaneum* Hbst.:

4.5.1.1. Culture of test insect *T. castaneum:*

Mass cultures were maintained in plastic containers (1200ml) and sub-cultures in beakers (1000 ml) with the food medium. The beakers were kept in an incubator at 30^0C ± 0.5^0C without light and humidity control. Each container and beaker contained 250g and 150g of food respectively. About 200 adults in each container and 100 adults in each beaker were introduced. The cultures were checked in regular intervals and eggs and larvae were separated to increase properly. A crumpled filter paper was placed inside each container and beaker for easy movement of the beetles. The containers and beakers were covered with pieces of muslin cloth tightly fixed with the help of rubber bands to avoid possible escape of the beetles.

4.5.1.2. Preparation of food medium:

The whole-wheat flour was used as the food medium for the insect species. The flour was sterilized at 60°C for 24 hours in an oven. A standard mixture of whole wheat flour with powdered dry yeast in a ratio of 19:1[69,70,71] was used as food medium throughout the experimental period. Both the flour and the powdered dry yeast were sterilized at 60°C for six hours in an oven. Food was not used until at least 15 days after sterilization to allow its moisture content to equilibrate with the environment[72].

4.5.1.3. Collection of eggs:

About 500 beetles were placed in a 500ml beaker containing food medium. The beaker was covered with a piece of cloth and kept in an incubator at 30°C±5°C. In regular interval the eggs were collected by sieving the food medium by two sieves of 500 and 250 mesh separating the adults and eggs respectively following the methods of Khan and Selman[72]. Eggs were then transferred to petridish (90mm in diameter) and incubated at the same temperature.

4.5.1.4. Collection of newly hatched larvae:

After 3-5 days, larvae hatched out in described conditions. Newly hatched larvae were then collected with a fine pointed camel hair brush and then shifted to the fresh food medium for culture. The larvae are yellowish white in color and long cylindrical shape. It appears 1 mm in length after hatching and become 6-7mm at maturation.

4.5.1.5. Collection of mature larvae:

Most larvae had six instars as reported by Good[73]. According to Good, the larval instars were determined by counting the number of exuviae (larval skin) deposited in the food medium. Two days old larvae was considered as first instar larva while second, third, fourth, and fifth instar larvae were considered on fourth, seventh, tenth and thirteenth day from hatching respectively. Depending on these days according to larval instar sixteen days old larva have been considered as a mature larva. Larval cultures were maintained in an incubator in the same procedure at 30°C±5°C without light and humidity control. The food medium was replaced by three days interval to a fresh one to avoid conditioning by the larvae[75].

4.5.1.6. Collection of adults:

A huge number of beetles were thus reared to get a regular supply of the newly formed adults. When sufficient adults produced in the sub-cultures, they were collected from the food medium. For this purpose some pieces of filter paper were kept inside the beaker on the food. Adults crawled upon the paper and then the paper was taken out with a forceps. Beetles were then collected in a small beaker (100 ml) with the help of a fine camel-hair brush.

4.6. *A. salina:*

4.6.1. Culture of *A. salina:*

As the *A. salina* is marine crustacean, this is not easy to culture like *T. castaneum* in lab conditions. But, they can be reared in a short edition. To carry out toxicity tests of certain materials these nauplii are very easy to grow from its marketed cysts and to set experiments thereby.

4.6.2. Preparation of environment:

Since the lethality test involves the culture of brine shrimp nauplii. The nauplii should be grown in the seawater. Seawater contains 3.8% of NaCl. Accordingly 3.8% sodium chloride solution was made by dissolving sodium chloride (38g) in distilled water (1000ml) and was filtered off. Brine water was taken in a small tank and *A. salina* cysts (1.5 g/L) were added to one side of the perforated divided tank with constant oxygen supply. Constant temperature (37˚C), sufficient light and aeration were maintained.

4.6.3. Collection of newly hatched nauplii:

After 24 hours, nauplii were collected and used in the experiments.

4.7. Mosquito larvae:

4.7.1. Preparation of environment and culture of mosquito larvae:

Mosquito eggs were hatched in stagnant water. They were collected from damp drains with special collecting spoon. Collected mosquito eggs (rafts) were placed into a new beaker containing pond water and kept it in a dark place of the lab to hatch.

4.7.2. Collection of newly hatched larvae:

After 24 hours, hatched larvae were collected from the hatching tank and used in the experiment.

4.8. Bioassays for activity of the selected extracts:

Crucial to any investigation of plants with biological activities is the availability of suitable bioassays for monitoring the required effects. In order to cope with the number of extracts a high sample throughput is necessary. The test systems should ideally be simple, rapid, reproducible,

and inexpensive. If active principles are only present at low concentration in the crude extract then bioassay is to be high enough sensitive for their detection. Another factor of special relevance to plant extracts is the solubility of the sample. Finding a suitable system can overcome the problems.

For the selection of bioassays to employ in research on plant constituents, the first step is to choose suitable target organisms. The complexity of the bioassay has to be designed as a function of the facilities and resources available. A list of bioassays taken in this investigation is shown in table-4.1.

4.9. Bioassay with residual film/surface film experiments:

4.9.1. Experiments for surface film test by *T. castaneum* adults:

All extracts were diluted with the solvents in which they were extracted and the actual amount of extracted matter in a dose was recorded. The application of dose was carried out by residual film method[67]. For each dose 1ml of mixture was dropped on a petridish (50 mm) in such a way that it made a uniform film over the petridish. Then the petridishes were air-dried leaving the extraction on it. The actual extract present in 1 ml mixture was calculated and dividing the value by the area of the petridish the dose per square centimeter was calculated. After drying 10 red flour beetles (3-5 day old) were released in each petridish with 3 replications. A control batch was also maintained with the same number of insects after preparing the petridish by applying and evaporating the solvent only. The treated beetles were placed in the incubator at the same temperature as reared in stock cultures and the

mortality of the beetles was counted after 30 min and every 12 hours up to 48 hours of treatment.

This is also one basic application method for doses of toxic substances to any insect population. The test material was dissolved in an organic solvent with a certain concentration to apply to a petridish of known surface area. After application being volatile the solvent evaporates out immediately simply with the atmospheric temperature. Thus the ingredient goes to make film on the surface of the petridish. Released insects within this captivity might have contact with the substance distributed evenly on the floor. However, being covered with the upper lid of the petridish there could have a captive environment with the extract distributed even in the air inside and may cause mortality by suffocation. Mortality due to suffocation may cause promptly if there was any volatile bioactive principles in the test material.

4.9.2. Preparation of doses with the crude extracts for the surface film test:

In this investigation dose-mortality efficiency was evaluated through surface film experiment with series of doses applied on *T. castaneum* adults. All five (methanol, ethyl acetate, chloroform, pet-ether and hot water) of *pleurotus ostreatus* were applied against *T. castaneum* adults. For each samples, a 'pilot' test was done before final experimentation. 50 mg extract sample was weighed and taken in a small glass vial, and then 1 ml of the same chemical was added to dissolve initially for preparing 2.547 mg/cm^2 the dose. Concentrations for methanol extract 10.19, 0.509, 0.254, 0.127, 0.063 and 0.031 mg/cm^2; for ethyl acetate, pet ether and hot water 4.584, 3.565, 3.056, 2.547 and 2.037

mg/cm^2; for chloroform 2.528, 1.019, 0.509, 0.254, 0.127 and 0.063 mg/cm^2 were selected for final experiment. Each of the doses was maintained in three replications.

Plate-4.6: Bioassay with mushroom extracts on *T. castaneum* adults by surface film method.

4.9.3. Application of doses in the surface film test:

To conduct surface film activity test 50 mm petridishes were taken for all doses and their replications, 1 ml of each of the doses were poured into the lower part of the petridishes and allowed them to dry out. Being volatile the solvent was evaporated out within a few minutes. Ten insects were released in each of the treated petridish. A control experiment by applying the only solvent into the petridish was also set at the same time under the same condition.

4.9.4. Observation of mortality in surface film tests:

After completing all the arrangements treated petridishes were placed in a secured place at room temperature. The whole experiment was

observed from time to time and mortality was observed by after 30 min and every 12 hours and the data was recorded up to 48 hours. A simple microscope was used to check each and every beetle by tracing natural movement of its organs. In some cases hot needle was taken closer to the bodies (without movement) to confirm death. Attention was also paid to recovery of the insects if occurred.

4.9.6. Statistical analysis:

The mortality records of the residual film experiments done on *T. castaneum* adults were corrected by the Abbott's formula[76]:

$$P_r = \frac{P_o - P_c}{100 - P_c} \times 100$$

Where,

P_r = Corrected mortality (%)

P_o = Observed mortality (%)

P_c = Control mortality (%), sometimes called natural mortality (%).

Then mortality percentages were subjected to statistical analysis according to Finney[66] and Busvine[67] by using 'computer software'. The dose-mortality relationship was expressed as a median lethal dose (LD_{50}).

4.10. Experiments for repellent activity of the extracts:

The repellency test used was adopted from the method (No.3) of McDonald *et al*. with some modifications by Talukder and Howse[102]. No significant difference was detected between the repellency of only solvent impregnated and untreated filter papers/areas in tests designed to check for any possible influence of different solvents. The average of the counts

was converted to percentage repellency (PR) using the formula of Talukder and Howse[102]:

$$PR = (Nc - 5) \times 20$$

Where, Nc is the average hourly observation of insects on the untreated half of the disc. Positive & negative values expressed for repellency & attractant activity respectively.

4.10.1. Preparation of doses with the crude extracts for repellency test:

A general concentration for each of the plant extracts was selected as stock dose for repellency application against *T. castaneum* adults to make other successive doses by serial dilution to give 0.508, 0.254, 0.127, 0.063 and 0.031mg/cm^2 concentrations.

4.10.2. Application of doses in the repellency test:

4.10.2.1. Application of doses against *T. castaneum:*

Half filter paper discs (Whatman No. 40, diameter 9cm) were prepared and selected doses of all the extracts separately applied onto each of the half-disc and allowed to dry out as exposed in the air for 10 minutes. Each treated half-disc was then attached lengthwise, edge-to-edge, to a control half-disc with adhesive tape and placed in a petridish (diameter 9cm), the inner surface of which was smeared with fluon to prevent insects escaping. Three replications were maintained same as the surface film test. Being volatile the solvent was evaporated out within a few minutes. Then ten insects were released in the middle of each filter paper circle. The orientation of the same was changed in the replica to avoid the effects of any external directional stimulus affecting the

distribution of the test insects. Each concentration was tested five times. Insects that settled on each half of the filter paper disc were counted after 1h and then at hourly intervals up to 5h.

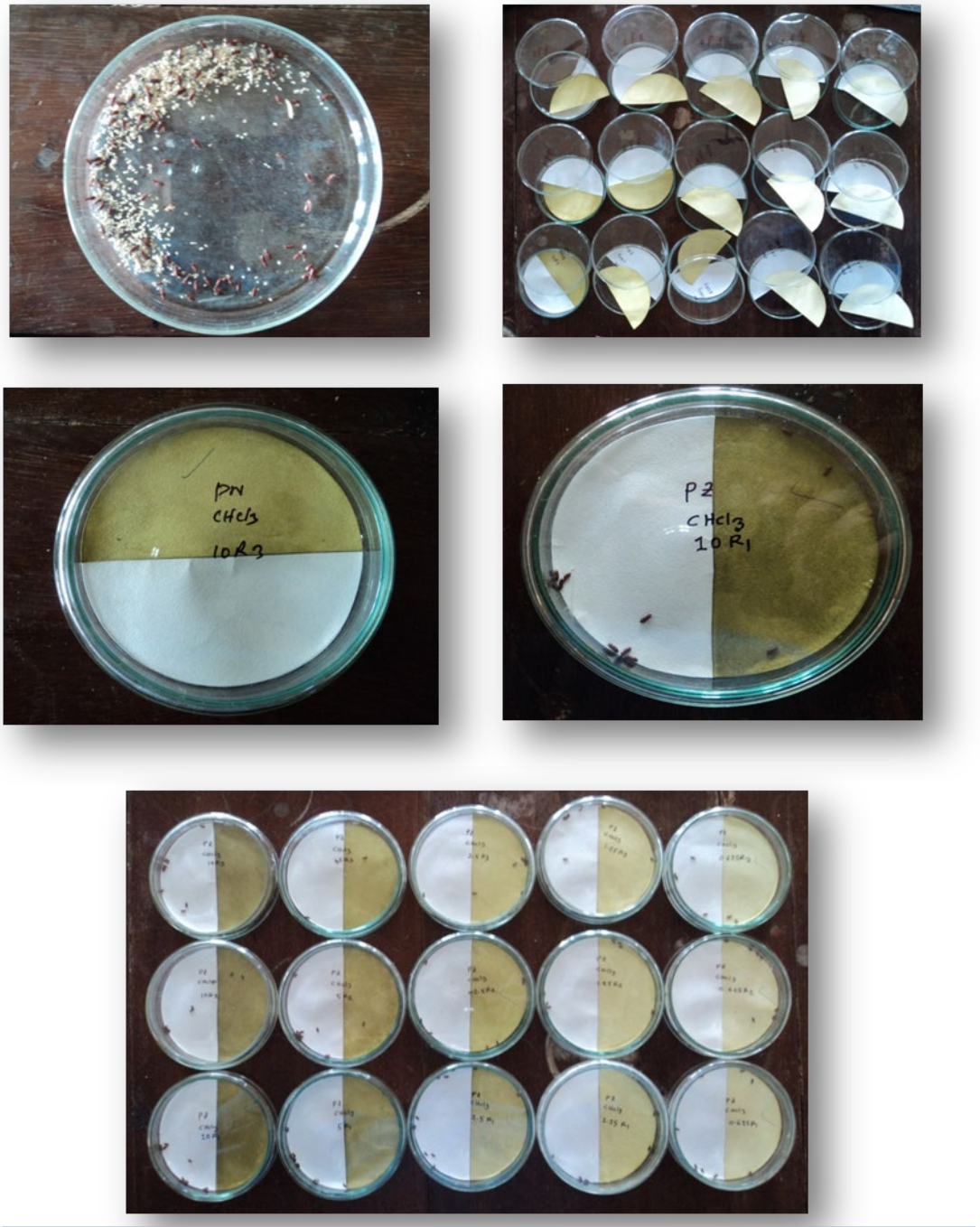


Plate-4.7: Photographs of repellency test of the mushroom extracts on *T. castaneum* adults by filter paper disc method.

4.10.2.2. Observation and analyses of repellency data:

Repellency was observed for one-hour interval and up to five successive hours of exposure, just by counting the number of insects from the non-treated part of the filter paper spread on the floor of the 90 mm petridish (for *T. castaneum*) and non-treated part of the restricted circle (36 mm).

The values in the recorded data were then calculated for percent repellency, which was again developed by arcsine transformation for the calculation of analysis of variance (ANOVA).

4.11. Lethality test against the brine shrimp nauplii:

4.11.1. Experimental design for lethality test:

Brine shrimp cysts were hatched in simulated seawater to get nauplii. Test samples were prepared by the addition of calculated amount of DMSO (Dimethyl sulfoxide) for obtaining desired concentration of test sample. The nauplii were counted by visual inspection and were taken in Test-tubes containing 5 ml of simulated seawater. Then samples of different concentrations were added to the premarked test-tubes through pipette. The test-tubes were left for 30 hours and then the nauplii were counted again to find out the cytotoxicity of the test agents and compared to the results with positive control.

Test materials:

- *salina* (Brine shrimp cysts)
- Sea salt (Non-ionized NaCl)
- Small tank/beaker to hatch the shrimp
- Pipette (1 ml and 5 ml)
- Test tubes (20 ml)
- Magnifying glass

4.11.2. Preparation of simulated seawater (brine water) and hatching of brine shrimp nauplii:

Since the lethality test involves the culture of brine shrimp nauplii that is, the nauplii should be grown in the seawater. Seawater contains 3.8% of NaCl. Accordingly 3.8% sodium chloride solution was made by dissolving sodium chloride (38 g) in normal pond water (1000 ml) and was filtered off.

Brine water was taken in a small tank and *A. salina* cysts (1.5 g/L) were added. Constant temperature (37˚C) and sufficient light and aeration were maintained. After 24 hours, matured shrimp as nauplii was collected and used for the experiment.

Plate-4.8: Hatching beaker.

Plate-4.9: Bioassay with mushroom extracts on *A. salina* nauplii by brine shrimp lethality test.

4.11.3. Experimentation of lethality test:

All the five (methanol, ethyl acetate, chloroform, pet-ether and hot water) of *pleurotus ostreatus* extracts were applied against brine shrimp nauplii. For each samples, a 'pilot' test was done before final experimentation. 2 mg extract sample was weighted and taken in a small glass vial, and then 1-2 drops of pure Dimethyl sulfoxide (DMSO) added to dissolve initially. 1 ml of pond water was taken into the vial to mix up the sample extract with water to prepare 200 ppm dose. When it mixed up completely it was added to the test-tube (10ml marked) for conducting tests. This process was also maintained during final experiment. Separate vials were taken for each dose. For each dose three replications were made.

For methanol extracts of *Pleurotus ostreatus* following concentrations 1200, 1000, 800, 600, 400, and 200ppm; for ethyl acetate 1000, 800, 600, 400, 200 and 100ppm; for chloroform and pet-ether extracts 1000,800, 600, 400, 200 and 100ppm and for hot water 1500, 1200, 1000, 800, 600 and 400ppm. were made for final application.

4.11.4. Analysis of data:

The dose mortality data were analyzed statistically by Probit analysis. The mortality records of the experiments were corrected by the Abbott's (1925) formula:

$$P_r = \frac{P_o - P_c}{100 - P_c} \times 100$$

Where,

P_r = Corrected mortality (%)

P_o = Observed mortality (%)

P_c = Control mortality (%), sometimes called natural mortality (%).

Then mortality percentages were subjected to statistical analysis according to Finney[66] and Busvine[67] by using 'computer software'. The dose-mortality relationship was expressed as a median lethal concentration (LC_{50}).

4.12. Larvicidal test against mosquito larvae:

4.12.1. Experimental design for larvicidal test:

Mosquito eggs were hatched in stagnant water. Test samples were prepared by the addition of calculated amount of DMSO (Dimethyl sulfoxide) for obtaining desired concentration of test samples. The larvae were counted by visual inspection and were taken in test-tubes containing 5 ml of pond water. Then samples of different concentrations were added to the premarked test-tubes through pipette. The test-tubes were left for 30 hours and then the larvae are counted again after 6h intervals to find out the lethality of the test agents and compared to the results with positive control.

Test materials:

- Mosquito eggs
- Small beaker with pond water to hatch the eggs
- Pipette (1 ml and 10 ml)
- Test tubes (20 ml)

4.12.2. Preparation of environment for the hatching of eggs:

Collected mosquito eggs were placed into a new beaker containing pond water and kept it in a dark place of the lab to hatch. After 24 hours, hatched larvae are collected and used for the experiment.

4.12.3. Experimentation of larvicidal test:

All the five (methanol, ethyl acetate, chloroform, pet-ether and hot water) of *Pleurotus ostreatus* extract samples were applied against mosquito larvae. 'Pilot' test was done before final experimentation. For each sample, 2mg extract sample was weighed and taken in a small glass vial, and then 1-2 drops of pure Dimethyl sulfoxide (DMSO) was added to dissolve initially. 1 ml of pond water was taken into the vial to mix up the sample extract with water to prepare 200ppm dose. When it mixed up completely, it was added to the test-tube (10ml marked) for conducting tests. This process was also maintained during final experiment. Separate vials were taken for each dose. For each dose three replications were made.

For methanol extract 1500, 1200, 1000, 800, 600 and 500ppm; for ethyl acetate 1000, 800, 600, 400, 300 and 200ppm; for chloroform 600, 500, 400, 300, 200 and 100ppm; for pet-ether 800, 600, 400, 300, 200 and 100ppm and for hot water 2000, 1800, 1500, 1200, 1000 and 800ppm concentrations were made for final application.

Plate-4.10: Hatching beaker of mosquito larvae.

Plate-4.11: Bioassay with mushroom extracts on mosquito larvae by larvicidal activity test.

4.12.4. Analysis of data:

The dose mortality data were analyzed statistically by Probit analysis. The mortality records of the experiments were corrected by the Abbott's (1925) formula:

$$P_r = \frac{P_o - P_c}{100 - P_c} \times 100$$

Where,

P_r = Corrected mortality (%)

P_o = Observed mortality (%)

P_c = Control mortality (%), sometimes called natural mortality (%).

Then mortality percentages were subjected to statistical analysis according to Finney[66] and Busvine[67] by using 'computer software'. The dose-mortality relationship was expressed as a median lethal concentration (LC_{50}).

RESULTS AND DISCUSSIONS

4.14. Bioassay on *T. castaneum* adults:

4.14.1. Effect of Oyster mushroom (*Pleurotus ostreatus*) extracts against *T. castaneum* adults by residual film assay:

All the extracts (methanol, ethyl acetate, chloroform, pet-ether, hot water) of the *Pleurotus ostreatus* were tested against the *T. castaneum* adults through residual film assay. For the five extracts following doses– 1.019, 0.509, 0.254, 0.127, 0.063, 0.031; 4.584, 3.565, 3.056, 2.547, 2.037; 1.528, 1.019, 0.509, .254, 0.127, 0.063; 4.075, 3.565, 3.056, 2.547, 2.037, 1.528; 4.075, 3.565, 3.056, 2.547, 2.037 and 1.528 mg/cm^2 respectively were made and applied on the inner surface of the petridishes, where the test insects were released to observe mortality or any sort of abnormality due to efficacy of the extracts compared to the controls. The results have been presented in the table-4.3 and appendix table I-IV. To trace acute toxicity an observation of mortality was made just after 30 minutes of application of the doses.

The data were then subjected to probit analyses and the LD_{50} values were shown in table-4.3; along with the chi-squared values and 95% confidence limits and it confirmed the sequence of efficacy in the same order mentioned above. Methanol extract was found the highest active one, for 12h, 24h, 36h and 48h of exposures by giving the LD_{50} values 0.480, 0.142, 0.106, 8.49E-02 respectively; while the regression equations were Y = 4.884+8.581x, y=1.499+6560x, y=1.831+6.67x, y=2.146+6.414x with the χ^2 values along with their df were 2.335(3), 2.565(4), 7.401(4) and 0.946(3) respectively. In the same way ethyl acetate, chloroform, pet-ether and hot water for 12h, 24h, 36h and 48h of

exposures by giving the LD_{50} values 5.614, 3.322, 2.495, 2.199; 0.992, 0.944, 0.719, 0.452; 3.681, 3.225, 3.322, 2.722; 3.77, 3.41, 2.981 and 2.785 respectively, while the regression equations were y=2.685+3.089x, y=2.684+4.440x, y=2.699+5.792x, y=3.729+3.711x; y=2.519+2.488x, y=2.792+2.264x, y=3.165+2.140x, y=1.313+2.226; y=0.140+8.584x, y=1.142+7.5683x, y=1.716+6.298, y=1.808+7.337x; y=2.046+1.756x, y=0.269+4.098x, y=0.954+3.940x and y=1.938+3.294x respectively with the χ^2 values along with their df were 0.624(4), 0.899(4), 5.143(4) and 2.688(2); 7.036(3), 0.229(2), 0.965(2), 0.797(3); 2.742(3), 4.900(4), 1.508(3), 2.340(3); 2.873(3), 2.657(1), 0.022(1) and 1.244(4) respectively.

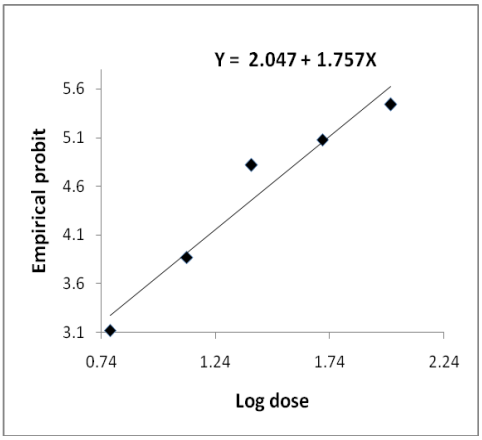


Fig. 4.1: Probit mortality line of the methanol extracts of *Pleurotus ostreatus* against *T. castaneum* after 12h of exposure.

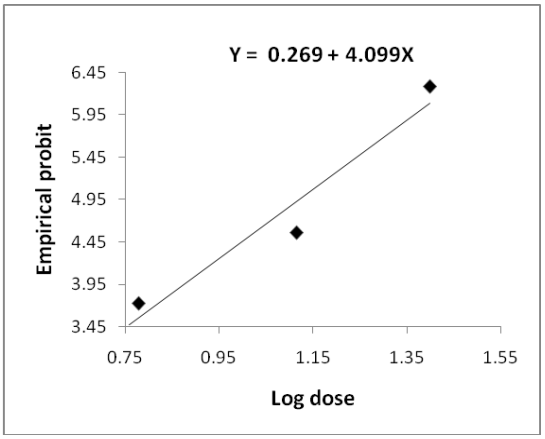


Fig. 4.2: Probit mortality line of the methanol extracts of *Pleurotus ostreatus* against *T. castaneum* after 24h of exposure.

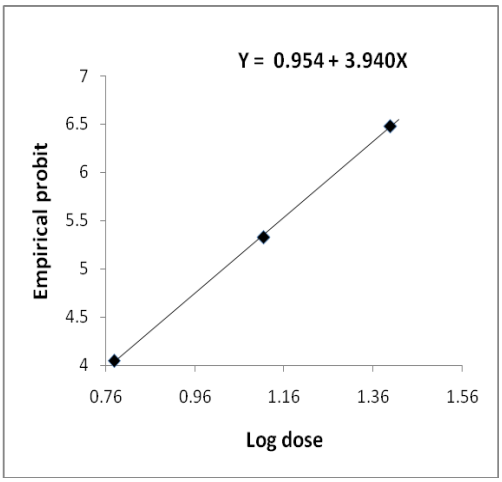


Fig. 4.3: Probit mortality line of the methanol extracts of *Pleurotus ostreatus* against *T. castaneum* after 36h of exposure.

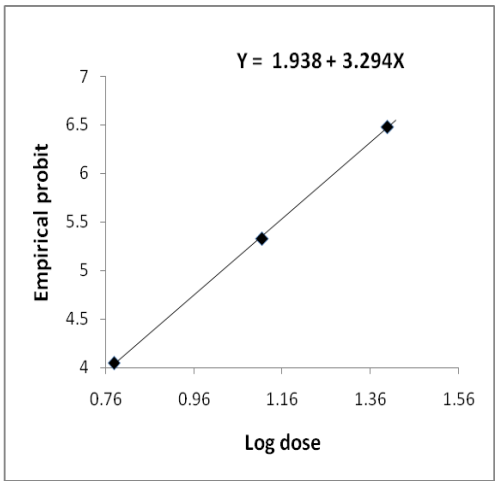


Fig. 4.4: Probit mortality line of the methanol extracts of *Pleurotus ostreatus* against *T. castaneum* after 48h of exposure.

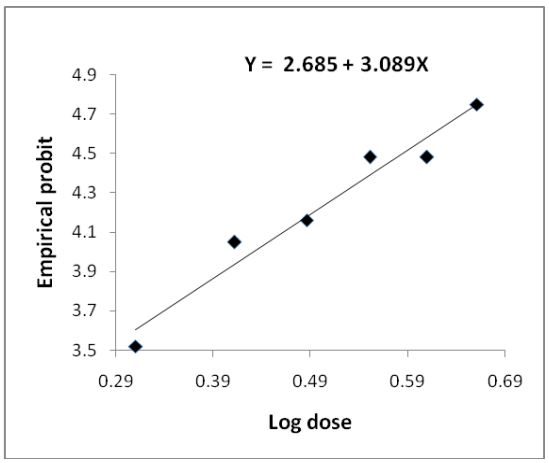


Fig. 4.5: Probit mortality line of the ethyl acetate extracts of *Pleurotus ostreatus* against *T. castaneum* after 12h of exposure.

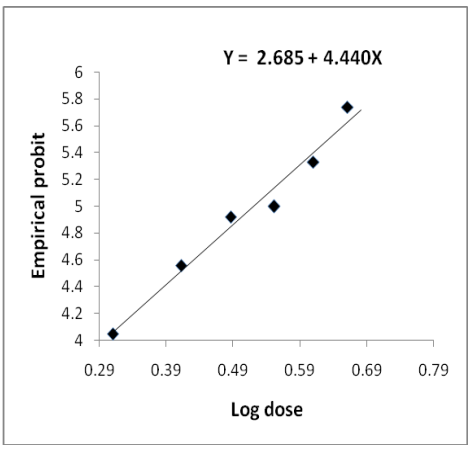


Fig. 4.6: Probit mortality line of the ethyl acetate extracts of *Pleurotus ostreatus* against *T. castaneum* after 24h of exposure.

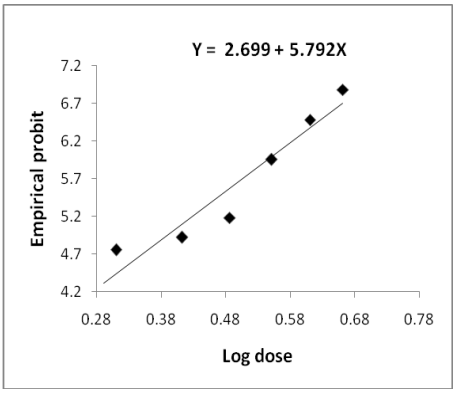


Fig. 4.7: Probit mortality line of the ethyl acetate extracts of *Pleurotus ostreatus* against *T. castaneum* after 36h of exposure.

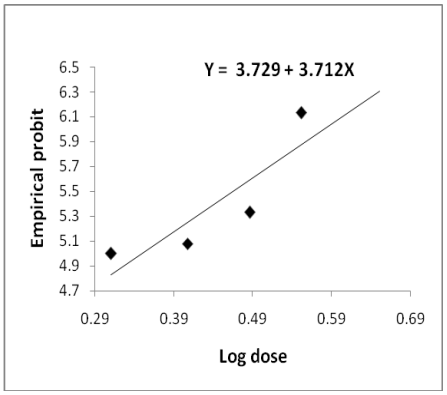


Fig. 4.8: Probit mortality line of the ethyl acetate extracts of *Pleurotus ostreatus* against *T. castaneum* after 48h of exposure.

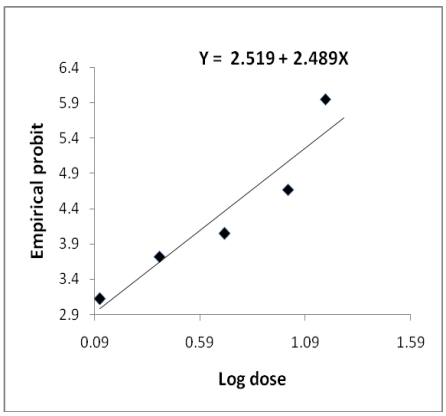


Fig. 4.9: Probit mortality line of the chloroform extracts of *Pleurotus ostreatus* against *T. castaneum* after 12h of exposure.

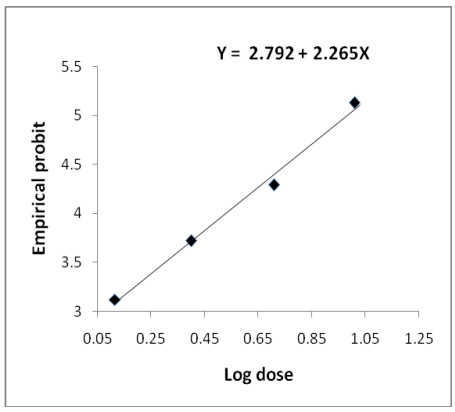


Fig. 4.10: Probit mortality line of the chloroform extracts of *Pleurotus ostreatus* against *T. castaneum* after 24h of exposure.

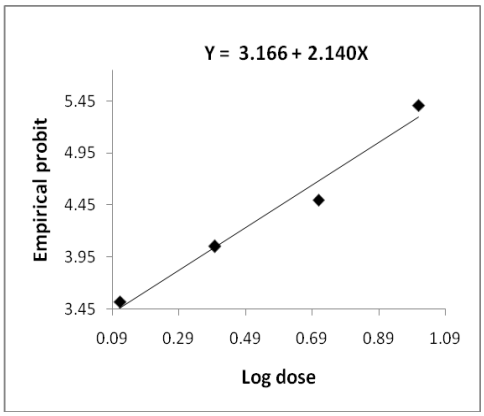


Fig. 4.11: Probit mortality line of the chloroform extracts of *Pleurotus ostreatus* against *T. castaneum* after 36h of exposure.

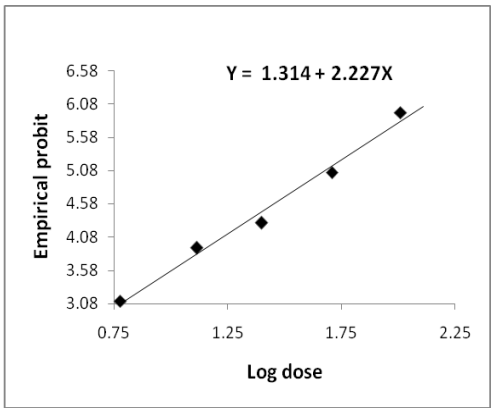


Fig. 4.12: Probit mortality line of the chloroform extracts of *Pleurotus ostreatus* against *T. castaneum* after 48h of exposure.

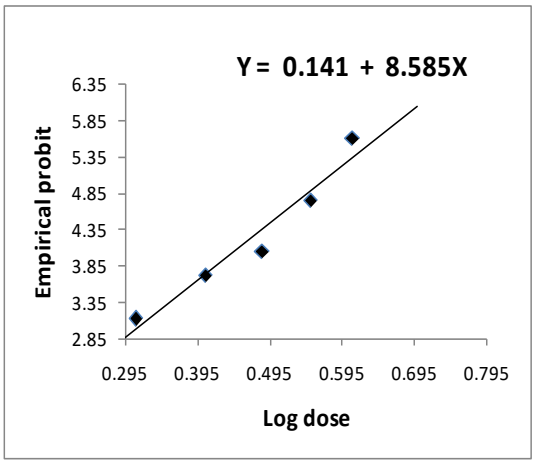


Fig. 4.13: Probit mortality line of the pet-ether extracts of *Pleurotus ostreatus* against *T. castaneum* after 12h of exposure.

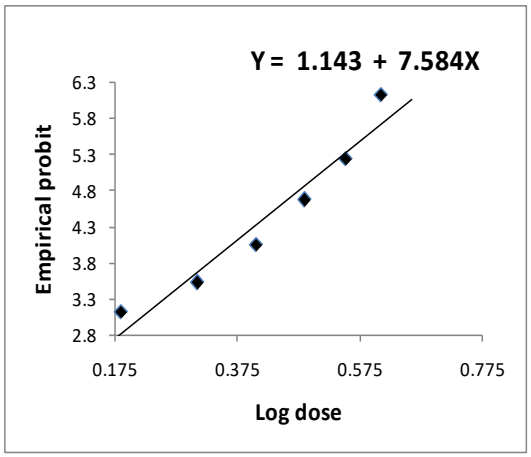


Fig. 4.14: Probit mortality line of the pet-ether extracts of *Pleurotus ostreatus* against *T. castaneum* after 24h of exposure.

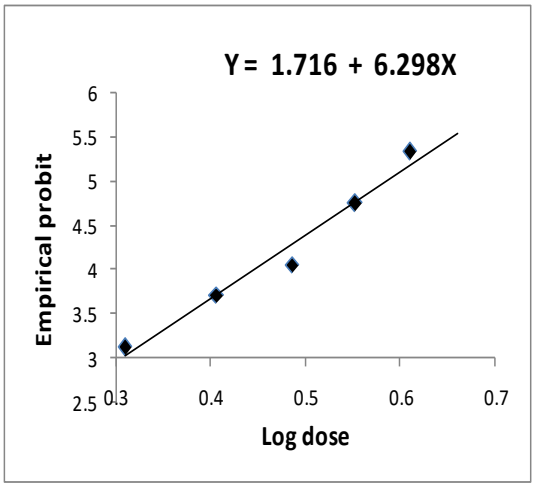


Fig. 4.15: Probit mortality line of the pet-ether extracts of *Pleurotus ostreatus* against *T. castaneum* after 36h of exposure.

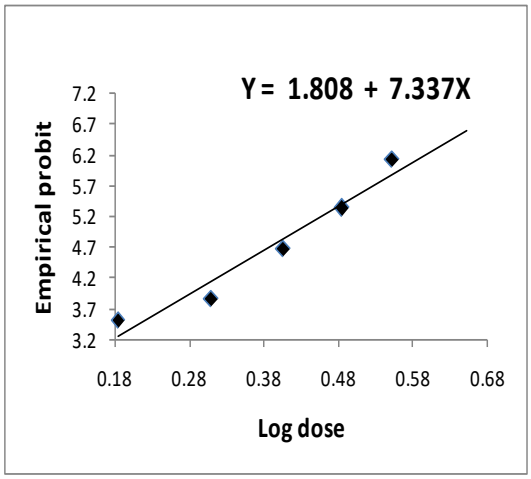


Fig. 4.16: Probit mortality line of the pet-ether extracts of *Pleurotus ostreatus* against *T. castaneum* after 48h of exposure.

Table-4.3: LD_{50} values, 95% confidence limits, Regression equations, and χ^2 values of *Pleurotus ostreatus* extracts against *T. castaneum* adults.

sample	Extract types	Exposures	LD_{50} mg/cm^2	95% confidences limits (mg/cm^2)		Regression equations	χ^2 values (df)
				Lower	Upper		
Pleurotus ostreatus	Methanol	30min	-	-	-	-	-
		12h	0.480	3.488	4.085	y=4.884+8.581x	2.335(3)
		24h	0.142	3.141	3.717	y=1.499+6.560x	2.565(4)
		36h	0.106	2.767	3.211	y=1.831+6.67x	7.401(4)
		48h	8.49E=02	2.562	3.027	y=2.146+6.414x	0.946(3)
	Ethyl acetate	30min	-	-	-	-	-
		12h	5.614	3.974	7.930	y=2.685+3.089x	0.624(4)
		24h	3.322	3.004	3.672	y=2.684+4.440x	0.899(4)
		36h	2.495	2.250	2.766	y=2.699+5.792x	5.143(4)
		48h	2.199	1.791	2.700	y=3.729+3.711x	2.688(2)
	Chloroform	30min	-	-	-	-	-
		12h	0.992	0.775	1.270	y=2.519+2.488x	7.036(3)
		24h	0.944	0.643	1.385	y=2.792+2.264x	0.229(2)
		36h	0.719	0.516	1.002	y=3.165+2.140x	0.965(2)
		48h	0.452	0.348	0.588	y=1.313+2.226x	0.797(3)
	Pet-ether	30min	-	-	-	-	-
		12h	3.681	3.417	3.965	y=0.140+8.584x	2.742(3)
		24h	3.225	3.010	3.456	y=1.142+7.583x	4.900(4)
		36h	3.322	2.973	3.711	y=1.716+6.298x	1.508(3)
		48h	2.722	2.530	2.930	y=1.808+7.337	2.340(3)
	Hot water	30min	-	-	-	-	-
		12h	3.77	0.346	0.664	y=2.046+1.756x	2.873(3)
		24h	3.41	0.119	0.169	y=0.269+4.098x	2.657(1)
		36h	2.981	0.088	0.127	y=0.954+3.940x	0.022(1)
		48h	2.785	0.101	7.004	y=1.938+3.294x	1.244(2)

So, the overall assessment of *T. castaneum* insectidal test proved that *Pleurotus ostreatus* extracts were high promising.

4.15. Repellent effect of the test mushroom:

4.15.1 Repellent effect of the test mushroom on *T. castaneum* adults:

All the extracts of *Pleurotus ostreatus* were tested against *T. castaneum* adults for their repellent activity. The extracts of *Pleurotus ostreatus* were found to show repellent activity against the adults of *T. castaneum*. The data was read with 1h interval for up to 5 hours of exposure and was subjected to ANOVA after transforming them into arcsine percentage values which are given in appendix table XXXXXXVIII-XII. The results were given in the table 4.5 and the appendix table XXXXXXIII-VII. The extract of ethyl acetate and pet-ether were highly active ($p<0.01$) but extract of methanol, chloroform and hot water were weak active ($p<0.05$) against the *T. castaneum* adults.

Table-4.4: ANOVA results of repellency by selected extracts against *T. castaneum*.

Extracts	Sources of Variation (df)			F-ration with level of significance		P-value	
	Between doses	Between time intervals	Error	Between doses	Between time intervals	Between doses	Between time intervals
Methanol	4	4	16	4.129*	1.723	0.017	0.194
Ethyl acetate	4	4	16	11.603**	1.725	0.001	0.193
Chloroform	4	4	16	4.841*	0.614	0.009	0.614
Pet-ether	4	4	16	12.272**	4.110	9.38E-05	0.017
Hot water	4	4	16	4.388*	0.919	0.013	0.476

** = ($p <0.01$), * = ($p <0.05$)

4.16. Bioassay on *A. salina* nauplii:

4.16.1. Effect of *Pleurotus ostreatus* extracts against A. salina nauplii by brine shrimp lethality test:

All the extracts (methanol, ethyl acetate, chloroform, pet-ether and hot-water) of *Pleurotus ostreatus* were tested against the 1 day aged Brine shrimp nauplii through lethality test. For the methanol extract following concentrations 1200, 1000, 800, 600, 400 and 200ppm; for hot water 1500, 1200, 1000, 800, 600 and 400ppm; for ethyl acetate, chloroform and pet-ether extracts 1000, 800, 600, 400, 200 and 100ppm were made and applied in the test-tubes, where the test organisms were released to observe lethality or any sort of abnormality due to efficacy of the extracts compared to the controls. The results have been presented in the table 4.5 and appendix table XXXXIII-XXII. To trace out toxicity an observation of lethality was made after 6hrs of application of the dose.

The data were then subjected to probit analyses and the LC_{50} values were shown in the table 4.5; along with the chi-squared values and 95% confidence limits and it confirmed the sequence of efficacy in the same order mentioned above. Pet-ether extract was found the highest active one for 12h and 18h of exposures by giving the LC_{50} values were 1592.37 and 775.95 respectively, while the regression equations were y= 1.107+1.217x and y=0.949+1.401x with the χ^2 values along with their df were 1.324(4) and 0.504(4) respectively. In the same way the other extracts showed promising cytotoxic activity against brine shrimp nuaplii as shown in the table 4.5.

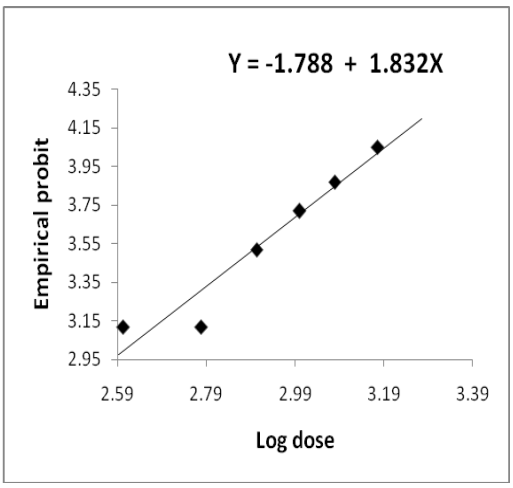


Fig. 4.18: Probit mortality line of methanol extracts of *Pleurotus ostreatus* against *A. salina* after 12h of exposure.

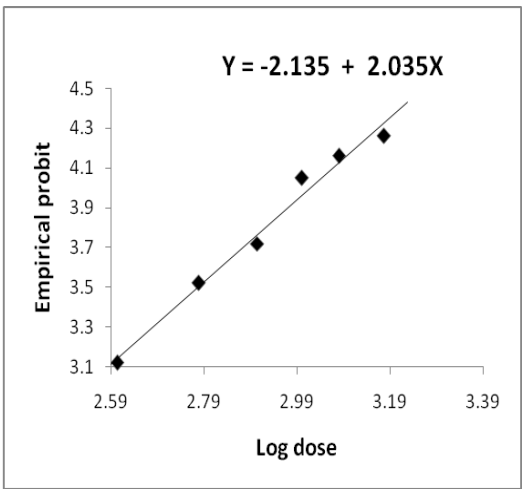


Fig. 4.19: Probit mortality line of methanol extracts of *Pleurotus ostreatus* against *A. salina* after 18h of exposure.

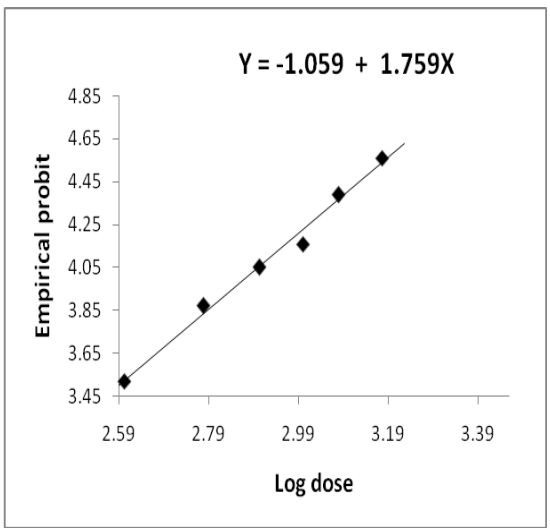


Fig. 4.20: Probit mortality line of methanol extracts of *Pleurotus ostreatus* against *A. salina* after 24h of exposure.

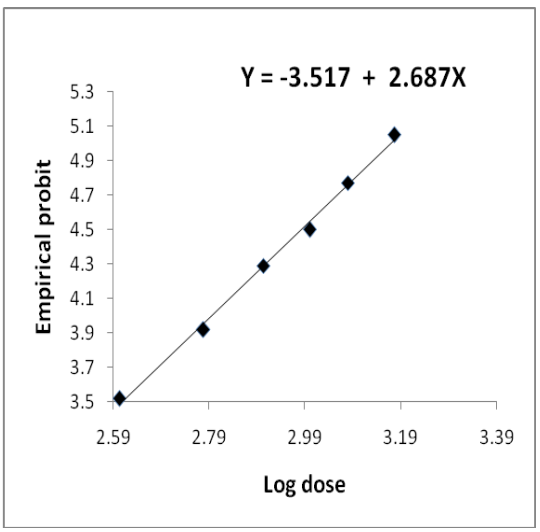


Fig. 4.21: Probit mortality line of methanol extracts of *Pleurotus ostreatus* against *A. salina* after 30h of exposure.

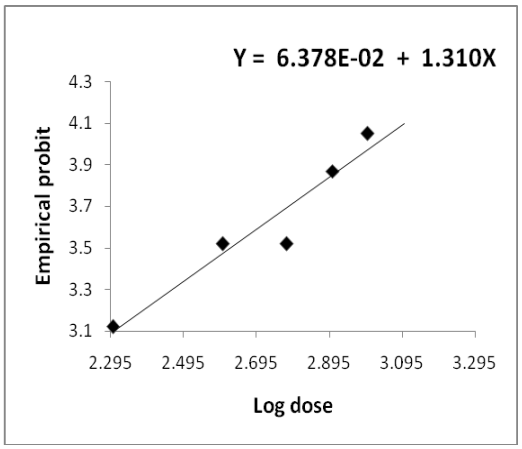


Fig. 4.22: Probit mortality line of ethyl acetate extracts of *Pleurotus ostreatus* against *A. salina* after 12h of exposure.

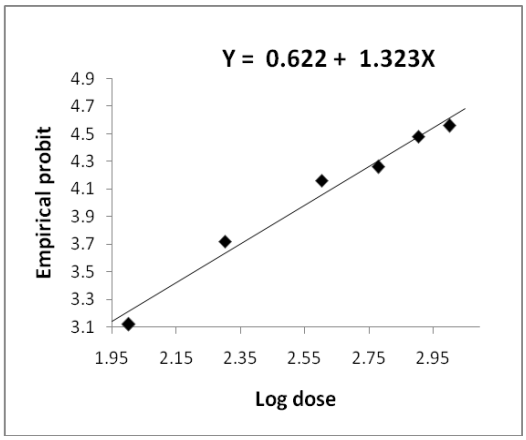


Fig. 4.23: Probit mortality line of ethyl acetate extracts of *Pleurotus ostreatus* against *A. salina* after 18h of exposure.

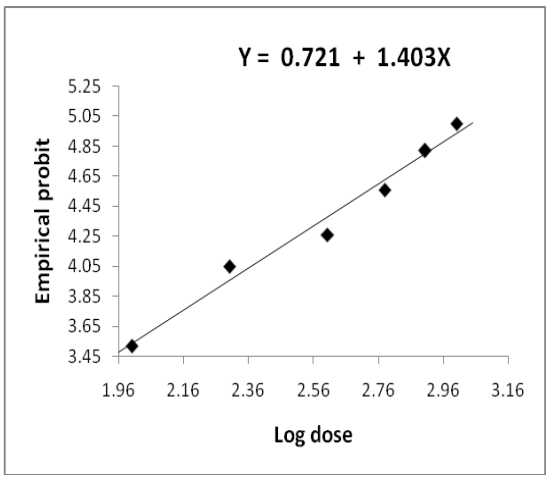


Fig. 4.24: Probit mortality line of ethyl acetate extracts of *Pleurotus ostreatus* against *A. salina* after 24h of exposure.

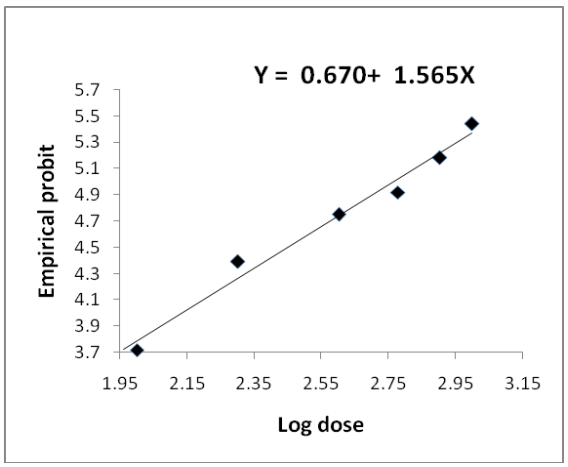


Fig. 4.25: Probit mortality line of the ethyl acetate extracts of *Pleurotus ostreatus* against *A. salina* after 30h of exposure.

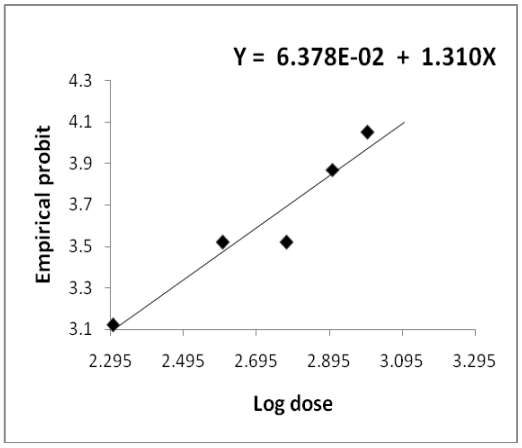


Fig. 4.26: Probit mortality line of chloroform extracts of *Pleurotus ostreatus* against *A. salina* after 12h of exposure.

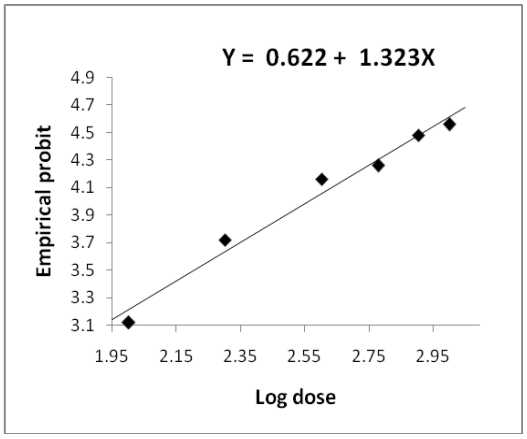


Fig. 4.27: Probit mortality line of chloroform extracts of *Pleurotus ostreatus* against *A. salina* after 18h of exposure.

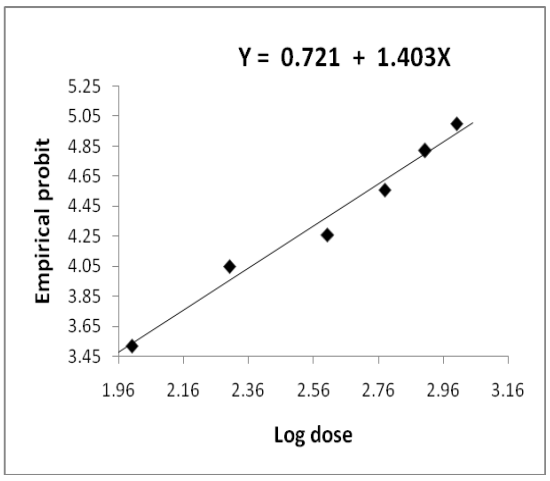


Fig. 4.28: Probit mortality line of chloroform extracts of *Pleurotus ostreatus* against *A. salina* after 24h of exposure.

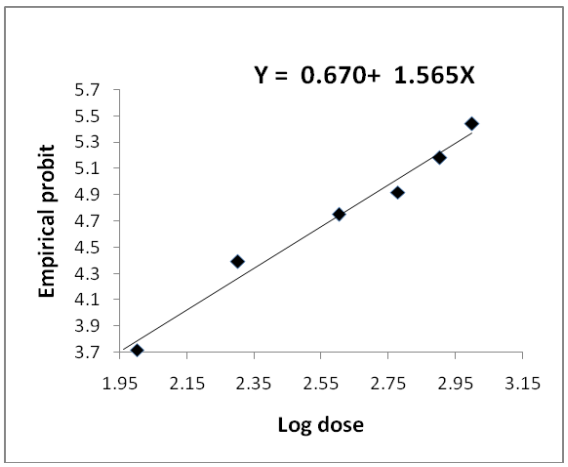


Fig. 4.29: Probit mortality line of the chloroform extracts of *Pleurotus ostreatus* against *A. salina* after 30h of exposure.

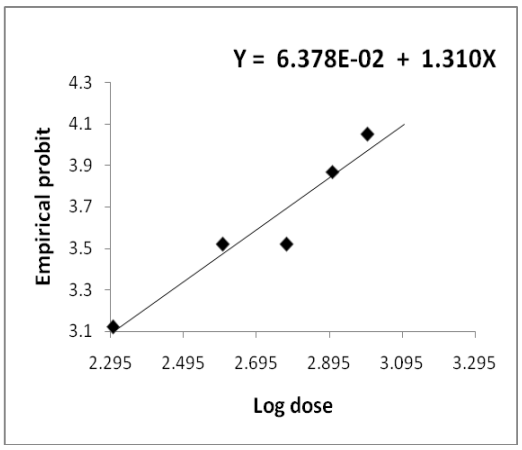


Fig. 4.30: Probit mortality line of pet-ether extracts of *Pleurotus ostreatus* against *A. salina* after 12h of exposure.

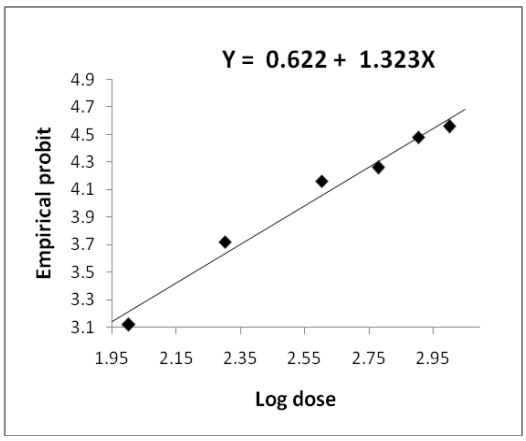


Fig. 4.31: Probit mortality line of pet-ether extracts of *Pleurotus ostreatus* against *A. salina* after 18h of exposure.

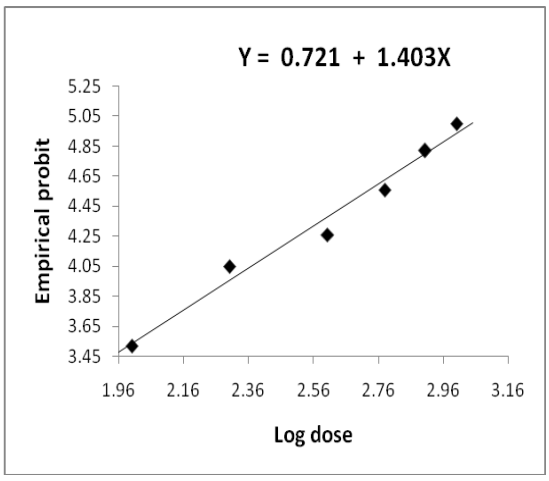


Fig. 4.32: Probit mortality line of pet-ether extracts of *Pleurotus ostreatus* against *A. salina* after 24h of exposure.

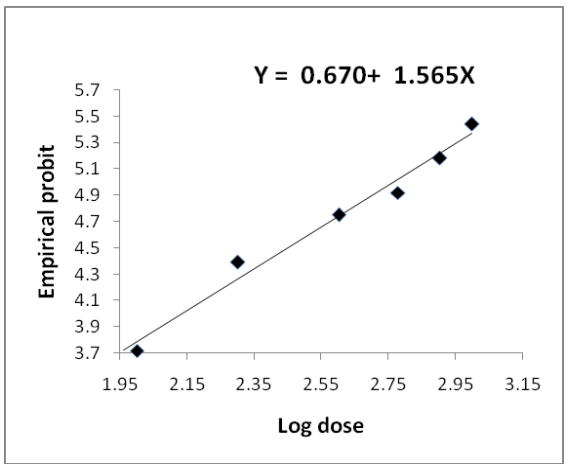


Fig. 4.33: Probit mortality line of the pet-ether extracts of *Pleurotus ostreatus* against *A. salina* after 30h of exposure.

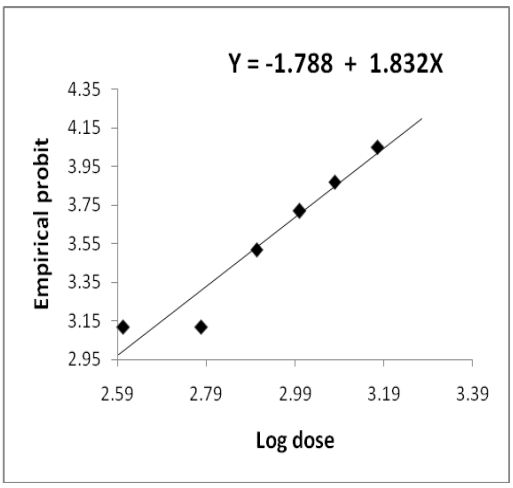


Fig. 4.34: Probit mortality line of hot water extracts of *Pleurotus ostreatus* against *A. salina* after 12h of exposure.

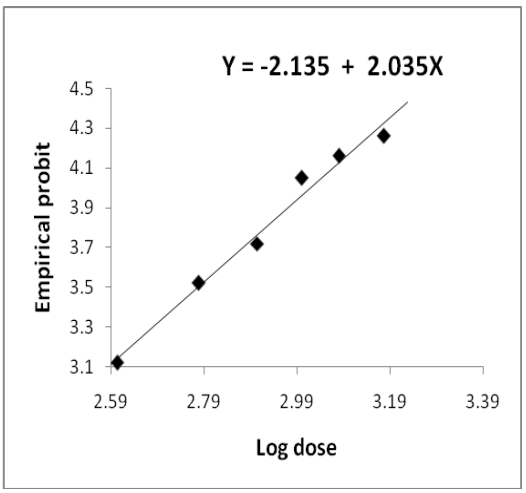


Fig. 4.35: Probit mortality line of hot water extracts of *Pleurotus ostreatus* against *A. salina* after 18h of exposure.

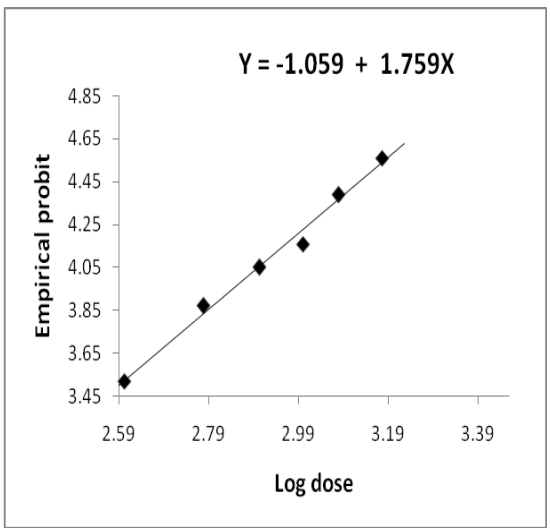


Fig. 4.36: Probit mortality line of hot water extracts of *Pleurotus ostreatus* against *A. salina* after 24h of exposure.

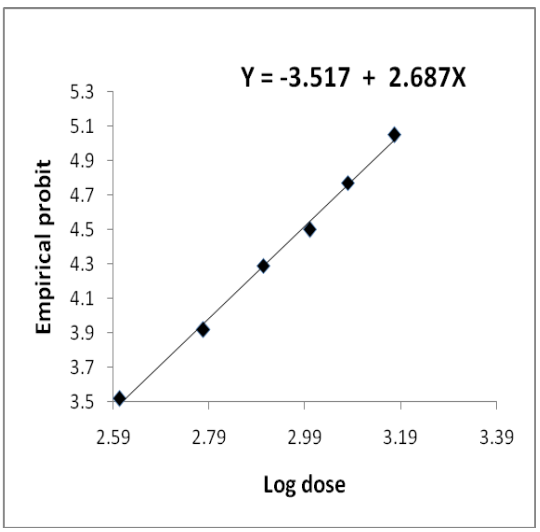


Fig. 4.37: Probit mortality line of hot water extracts of *Pleurotus ostreatus* against *A. salina* after 30h of exposure.

Table-4.5: LC_{50} values, 95% confidence limits, regression equations, and χ^2 values of *Pleurotus ostreatus* extracts against *A. salina* nauplii.

Plant	Extract types	Exposures	LC_{50} ppm	95% confidences limits (mg/cm^2)		Regression equations	χ^2 values (df)
				Lower	Upper		
Pleurotus ostreatus	Methanol	6hrs	-	-	-	-	-
		12hrs	3113.815	634.260	15286.84	y=-3.753+2.505x	0.298(2)
		18hrs	2010.848	1102.036	3669.131	y=-2.218+2.185x	1.473(4)
		24hrs	997.086	791.314	1256.366	y=-2.352+2.451x	1.663(4)
		30hrs	626.94	529.613	742.165	y=-2.57+2.706x	2.277(4)
	Ethyl acetate	6hrs	-	-	-	-	-
		12hrs	4041.849	359.787	45406.1	y=-1.586+1.826x	3.568(2)
		18hrs	2047.168	862.481	4859.113	y=-1.113+1.846x	0.211(3)
		24hrs	1420.399	804.007	2509.127	y=-6.127+1.605x	0.258(4)
		30hrs	906.229	619.987	1324.628	y=0.114+1.652x	2.331(4)
	Chloroform	6hrs	-	-	-	-	-
		12hrs	5854.292	483.129	70939.13	y=6.378+1.310x	0.377(3)
		18hrs	2031.773	848.529	4865.007	y=0.622+1.323x	0.323(4)
		24hrs	1122.01	666.568	1888.638	y=0.720+1.401x	0.495(4)
		30hrs	584.610	431.108	792.768	y=0.670+1.564x	0.498(4)
	Pet-ether	6hrs	-	-	-	-	-
		12hrs	1592.379	739.299	3429.83	y=1.107+1.217x	1.324(4)
		18hrs	775.952	521.527	1154.497	y=0.949+1.401x	0.504(4)
		24hrs	1592.37	739.299	3429.83	y=1.107+1.217x	1.324(4)
		30hrs	775.95	521.527	1154.497	y=0.949+1.401x	0.504(4)
	Hot water	6hrs	-	-	-	-	-
		12hrs	5069.0	1100.691	23352.88	y=-1.788+1.832x	0.277(4)
		18hrs	3210.936	1308.101	7881.742	y=-2.135+2.034x	0.223(4)
		24hrs	2771.954	1219.288	6301.815	y=-1.058+1.759x	0.119(4)
		30hrs	1477.487	1126.617	1937.631	y=-3.516+2.687x	0.114(4)

The overall assessment of brine shrimp lethality test proved that *Pleurotus ostreatus* extracts have lethal activity.

4.17. Boassay on mosquito larvae:

4.17.1. Effect of *Pleurotus ostreatus* extracts against mosquito larvae by larvicidal assay:

All the extracts (methanol, ethyl acetate, chloroform, pet-ether and hot-water) of the *Pleurotus ostreatus* were tested against the 1 day aged mosquito larvae through larvicidal activity test. For the methanol extracts following concentrations 1500, 1200, 1000, 800, 600 and 400ppm; for ethyl acetate 1000, 800, 600, 400, 300 and 200ppm; for chloroform 600, 500, 400, 300, 200 and 100ppm; for pet-ether 800, 600, 400, 300, 200 and 100ppm; for hot water 2000, 1800, 1500, 1200, 1000 and 800ppm concentrations were made and applied in the test-tubes, where the test organisms were released to observe lethality or any sort of abnormality due to efficacy of the extracts compared to the controls. The results have been presented in the table 4.7 and appendix table XXI-XXXXII. To trace acute toxicity an observation of lethality was made after 6hrs of application of the doses.

The data were then subjected to probit analyses and the LC_{50} values were shown in the table 4.7; along with the chi-squared values and 95% confidence limits and it confirmed the sequence of efficacy in the same order mentioned above. Chloroform extract was found the highest active one for 6h, 12h, 18h, 24h and 30h of exposures by giving the LC_{50} values 1431.852, 1224.768, 902.232, 617.195, 434.093 respectively, while the regression equations were y=-5.497+3.326x, y=-1.934+2.245x, y=-1.108+2.067x, y=-0.875+2.105x and y=-0.329+2.020 respectively, with the χ^2 values along with their df were 3.923(1), 0.220(3), 0.494(3), 0.543(4) and 2.361(4) respectively.

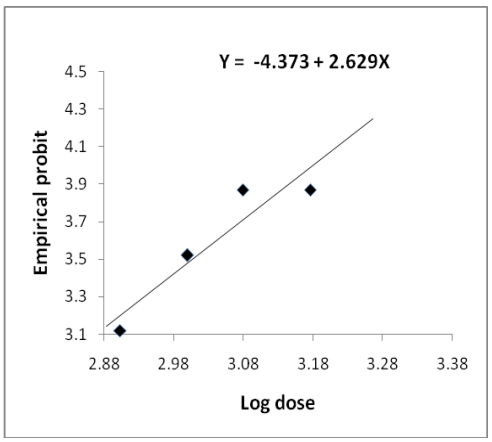


Fig. 4.38: Probit mortality line of the methanol extract of *Pleurotus ostreatus* against Mosquito larvae after 12h of exposure.

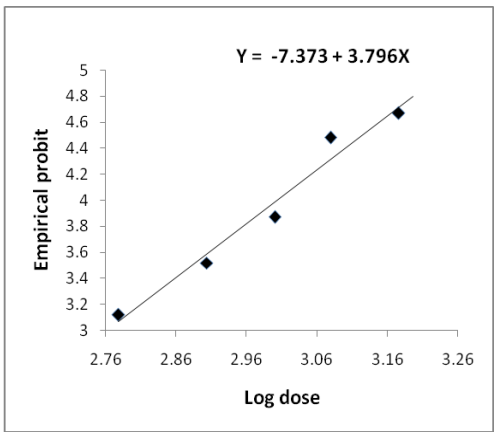


Fig. 4.39: Probit mortality line of the methanol extracts of *Pleurotus ostreatus* against Mosquito larvae after 18h of exposure.

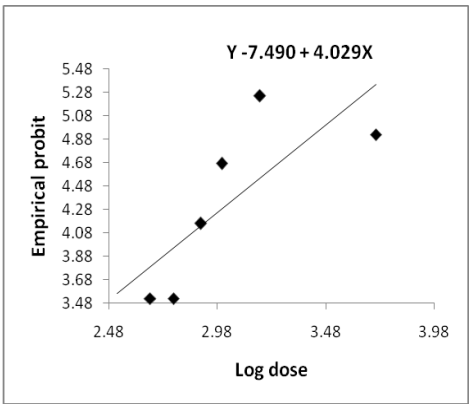


Fig. 4.40: Probit mortality line of the methanol extracts of *Pleurotus ostreatus* against Mosquito larvae after 24h of exposure.

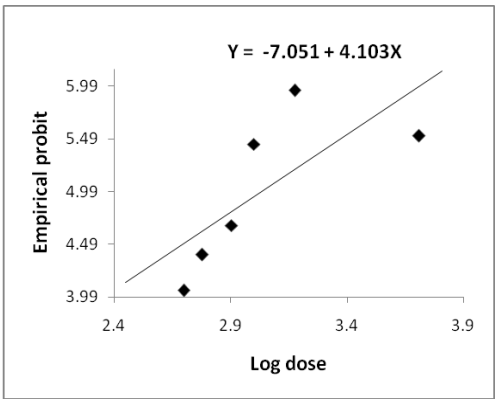


Fig. 4.41: Probit mortality line of the methanol extracts of *Pleurotus ostreatus* against Mosquito larvae after 30h of exposure.

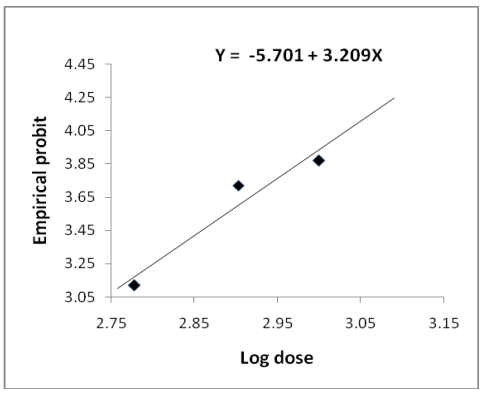


Fig. 4.42: Probit mortality line of ethyl acetate extracts of *Pleurotus ostreatus* against Mosquito larvae after 12h of exposure.

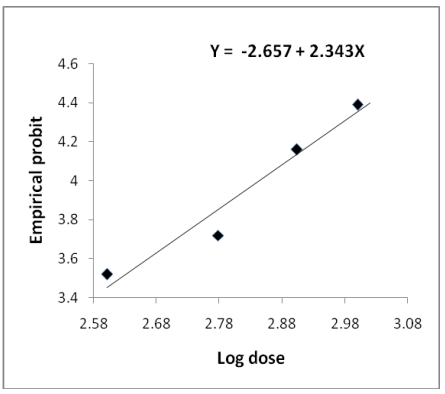


Fig. 4.43: Probit mortality line of ethyl acetate extracts of *Pleurotus ostreatus* against Mosquito larvae after 18h of exposure.

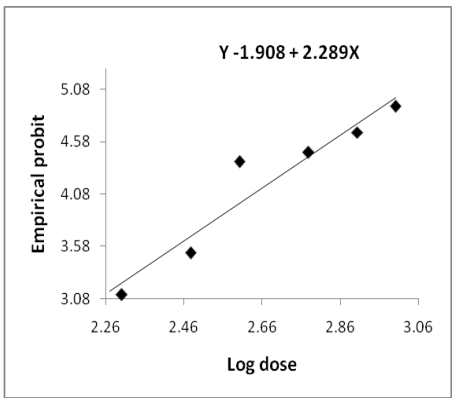


Fig. 4.44: Probit mortality line of ethyl acetate extracts of *Pleurotus ostreatus* against Mosquito larvae after 24h of exposure.

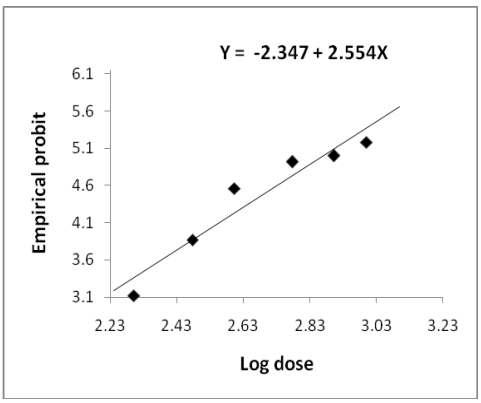


Fig. 4.45: Probit mortality line of ethyl acetate extracts of *Pleurotus ostreatus* against Mosquito larvae after 30h of exposure.

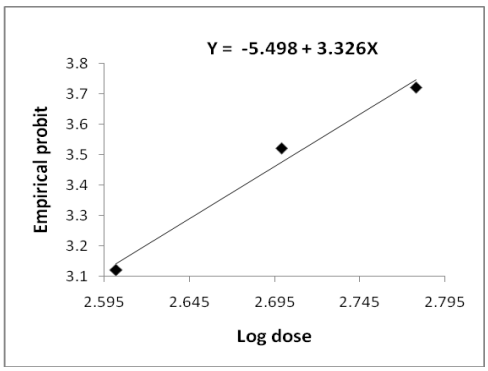


Fig. 4.46: Probit mortality line of chloroform extracts of *Pleurotus ostreatus* against Mosquito larvae after 6h of exposure.

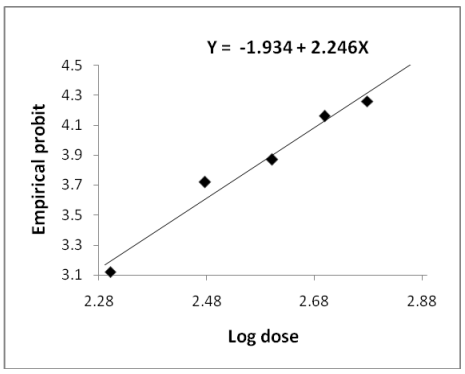


Fig. 4.47: Probit mortality line of chloroform extracts of *Pleurotus ostreatus* against Mosquito larvae after 12h of exposure.

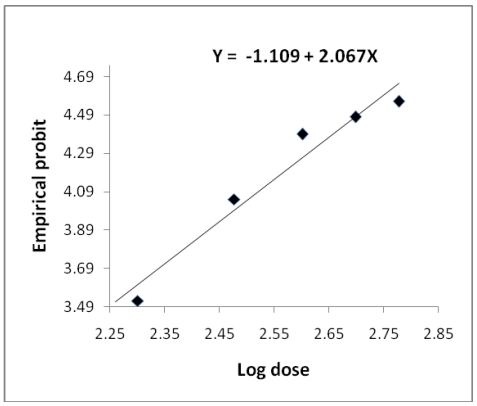


Fig. 4.48: Probit mortality line of chloroform extracts of *Pleurotus ostreatus* against Mosquito larvae after 18h of exposure.

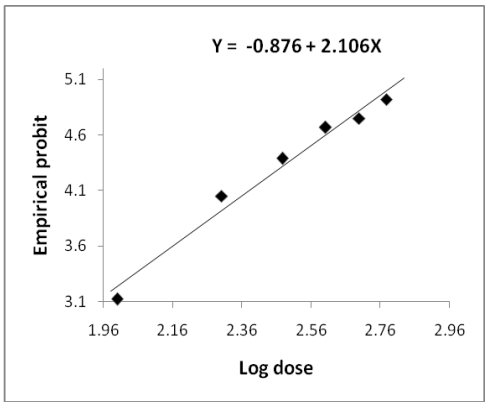


Fig. 4.49: Probit mortality line of chloroform extracts of *Pleurotus ostreatus* against Mosquito larvae after 24h of exposure.

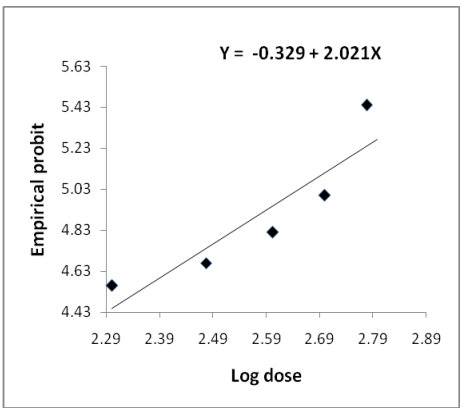


Fig. 4.50: Probit mortality line of chloroform extracts of *Pleurotus ostreatus* against Mosquito larvae after 30h of exposure.

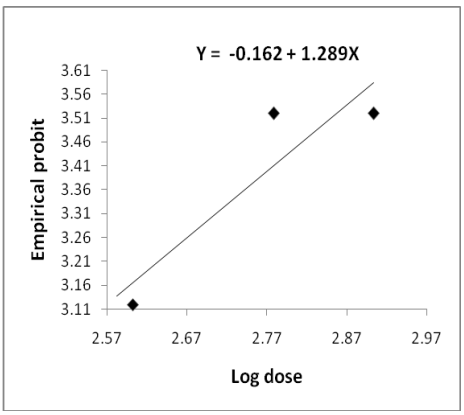


Fig. 4.51: Probit mortality line of petroleum extracts of *Pleurotus ostreatus* against Mosquito larvae after 6h of exposure.

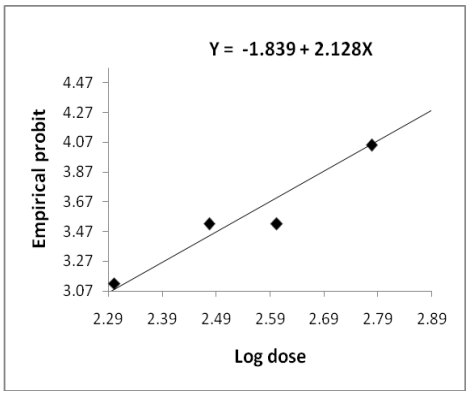


Fig. 4.52: Probit mortality line of petroleum extracts of *Pleurotus ostreatus* against Mosquito larvae after 12h of exposure.

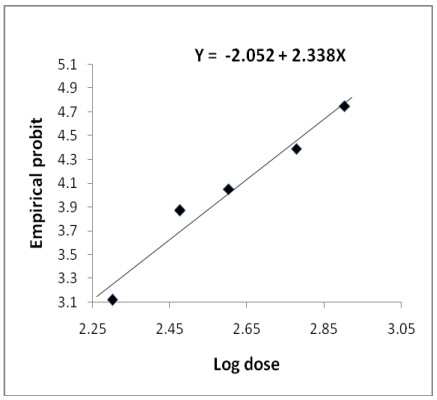


Fig. 4.53: Probit mortality line of petroleum extracts of *Pleurotus ostreatus* against Mosquito larvae after 18h of exposure.

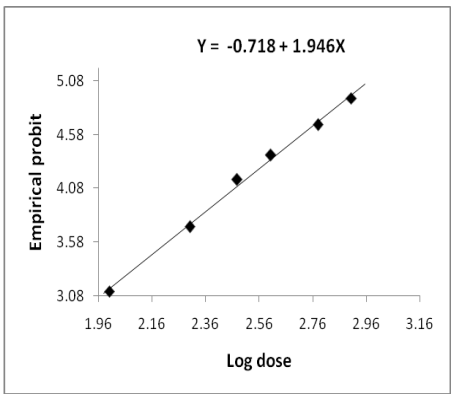


Fig. 4.54: Probit mortality line of petroleum extracts of *Pleurotus ostreatus* against Mosquito larvae after 24h of exposure.

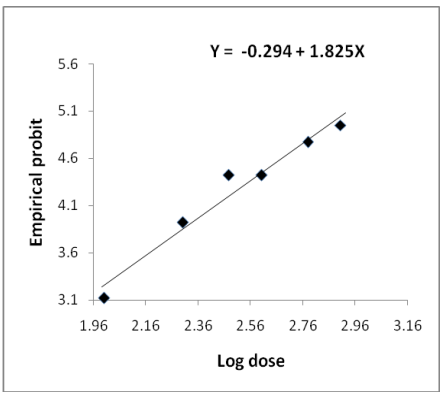


Fig. 4.55: Probit mortality line of petroleum extracts of *Pleurotus ostreatus* against Mosquito larvae after 30h of exposure.

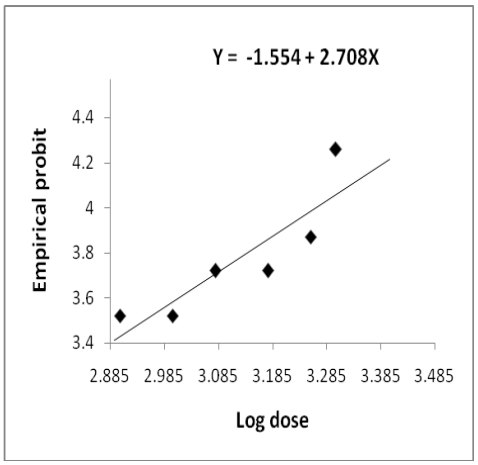


Fig. 4.56: Probit mortality line of hot water extracts of *Pleurotus ostreatus* against Mosquito larvae after 12h of exposure.

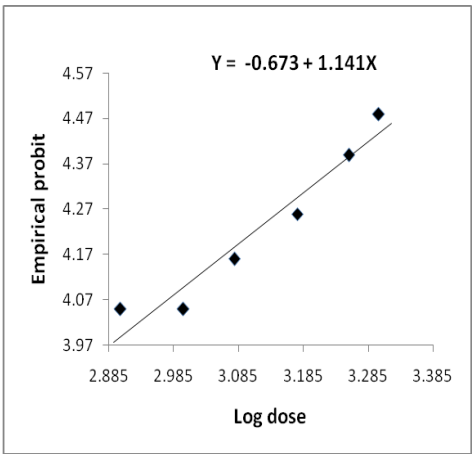


Fig. 4.57: Probit mortality line of hot water extracts of *Pleurotus ostreatus* against Mosquito larvae after 18h of exposure.

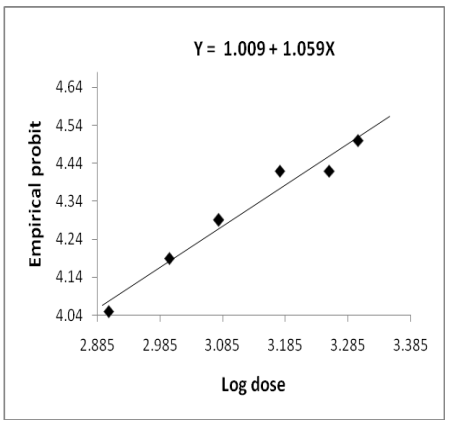


Fig. 4.58: Probit mortality line of hot water extracts of *Pleurotus ostreatus* against Mosquito larvae after 24h of exposure.

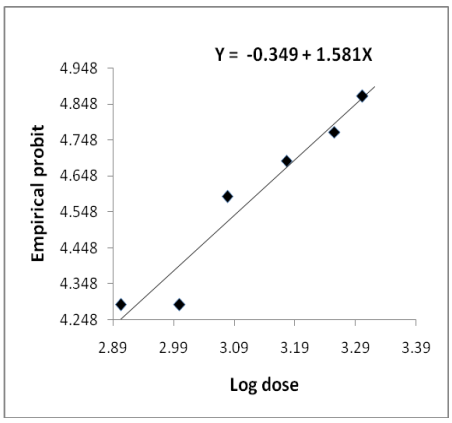


Fig. 4.59: Probit mortality line of hot water extracts of *Pleurotus ostreatus* against Mosquito larvae after 30h of exposure.

In the same way methanol, ethyl acetate, pet-ether, hot water showed larvicidal activity against mosquito larvae as shown in the table 4.6.

Table-4.6: LC_{50} values, 95% confidence limits, regression equations, and χ^2 values of *Pleurotus ostreatus* extracts against *Culex* mosquito larvae.

Plant	Extract types	Exposures	LC_{50} ppm	95% confidences limits (mg/cm^2)		Regression equations	χ^2 values (df)
				Lower	Upper		
Pleurotus ostreatus	Methanol	6hrs	-	-	-	-	-
		12hrs	3677.837	798.897	16931.45	y=-4.372+2.628x	0.524(2)
		18hrs	1818.165	1357.219	2435.657	y=-7.372+3.795x	1.173(4)
		24hrs	1258.106	1083.315	1461.099	y=-7.490+4.029x	0.572(4)
		30hrs	865.0	774.161	966.701	y=-7.051+4.103x	1.537(4)
	Ethyl acetate	6hrs	-	-	-	-	-
		12hrs	2160.578	527.072	8856.671	y=-5.701+3.209x	0.192(1)
		18hrs	1855.401	772.727	4455.01	y=-2.657+2.342x	0.282(2)
		24hrs	1041.842	743.370	1460.15	y=-1.907+2.289x	0.999(4)
		30hrs	753.726	605.925	937.579	y=-2.347+2.553x	3.339(4)
	Chloroform	6hrs	1431.852	213.085	9621.512	y=-5.497+3.326x	3.923(1)
		12hrs	1224.768	497.570	3014.761	y=-1.934+2.245x	0.220(3)
		18hrs	902.232	480.316	1694.765	y=-1.108+2.067x	0.494(3)
		24hrs	617.195	440.831	864.115	y=-0.875+2.105x	0.543(4)
		30hrs	434.093	338.542	556.613	y=-0.329+2.020x	2.361(4)
	Pet-ether	6hrs	10078.75	2.592	3.918	y=-0.162+1.289x	0.161(1)
		12hrs	1638.01	693.343	3869.786	y=-1.0839+2.127x	0.431(3)
		18hrs	1040.185	644.496	1678.806	y=-2.052+2.337x	0.611(3)
		24hrs	867.305	571.213	1316.879	y=-0.717+1.946x	0.132(4)
		30hrs	794.921	530.290	1191.613	y=-0.293+1.825x	0.927(4)
	Hot water	6hrs	-	-	-	-	-
		12hrs	6856.736	1181.732	39784.63	y=-1.553+1.708x	1.126(4)
		18hrs	6195.057	784.348	48930.7	y=0.673+1.140x	0.179(4)
		24hrs	5888.162	724.183	47875.2	y=1.009+1.058	0.111(4)
		30hrs	2412.954	1343.762	4332.87	y=-0.348+1.581x	0.255(4)

So, the overall assessment of mosquito larvae mortality test proved that *Pleurotus ostreatus* extracts were promising.

4.18. Summary of the experimentation:

For the detection of bioactive potentials in methanol, ethyl acetate, chloroform, pet-ether and hot water extracts of *Pleurotus ostreatus* insecticidal, insect repellency, larvicidal and brine shrimp lethality tests have been carried out. A total outcome of the bioassays that carried out is presented in the table 4.7 given below.

Table-4.7: Summary of the biological activity of the selected extracts.

	Test samples	**Test agents (tests)**			
		T. castaneum		***A. Salina* nauplii**	**Mosquito larvae**
		Dose mortality	**Repellency**	**Brine shrimp lethality**	**Larvicidal activity**
Pleurotus ostreatus	Methanol	+	-	+	+
	Ethyl acetate	+	+	+	+
	Chloroform	+	+	+	+
	Pet-ether	+	-	+	+
	Hot water	+	-	+	+

(+ = active, - = not active)

CHAPTER-FIVE

ANTIDIABETIC ACTIVITY

PART-A: MATERIALS AND METHODS

5.1. Materials:

5.1.1. Chemicals:

1. Glucose estimation kit.
2. Total bilirubin kit.
3. ALT/GPT reagent kit.
4. ALP reagent kit.
5. AST/GOT reagent kit.
6. Urea reagent kit.
7. Triglycerides reagent kit.
8. Cholesterol kit.
9. HDL cholesterol kit.
10. LDL Cholesterol kit.
11. Creatinine reagent kit.

5.1.2. Working Instruments:

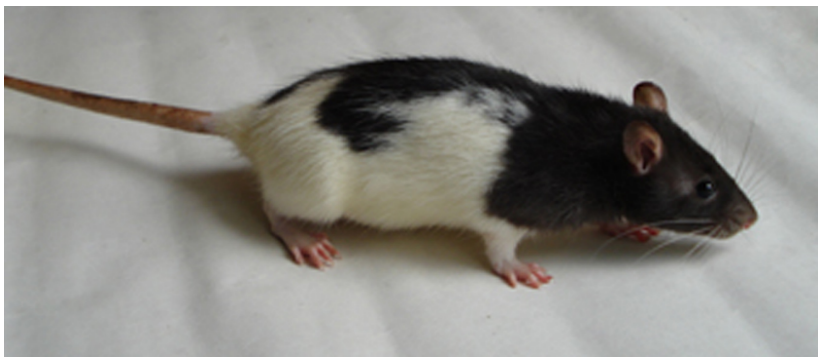
1. Bio-analyzer (Model: Microlab 200).
2. Eppendorf centrifuge 5415C.
3. Refrigerator.
4. Electric Balance.
5. Micro pipette.

5.1.3. Experiment of antidiabetic activity of Oyster mushroom powder and extracts (Methanol and Ethyl Acetate):

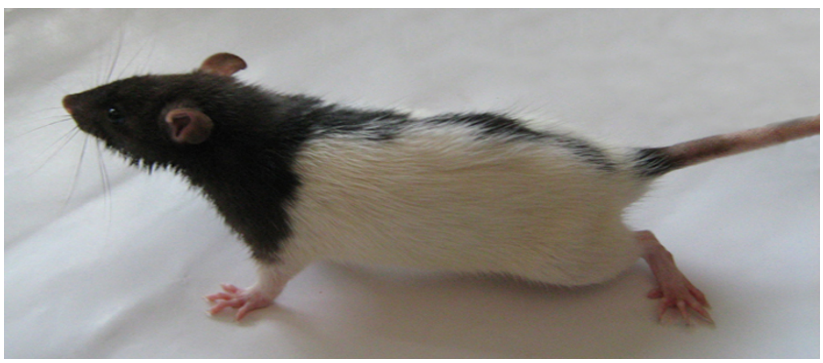
Mushrooms have been valued throughout the world as both food and medicine for thousand of years. In the present study we will observe the antidiabetic effect of mushroom powder and two extracts i.e. Methanol and Ethyl acetate in Streptozotocin- induced diabetic rats.

5.1.4. Test Animal:

Long Evan rats were selected as experimental animal to carry out this study. Rats were collected from the Animal Resource Division of ICDDR'B Mohakhali, Dhaka, weighing 100-180 gm.



Phtograph-5.1(a): Photograph of normal long Evan rat.



Phtograph-5.1(b): Photograph of diabetic long Evan rat.

5.1.5. Methods:

5.1.5.1. Maintenance of Animal:

Forty two (42) rats were randomly divided into seven groups. Each rat was numbered with a permanent marker for experimental purpose, weighed and recorded; seven cages (containing 6 rats) were kept in the departmental animal house. The animals were fed on standard laboratory diet with water and kept at room temperature. Rats were acclimatized to the laboratory conditions for one 1 week before experimental work was undertaken.

5.1.5.2. Induction of Diabetics:

Diabetes Mellitus was induced by single intraperitoneal injection of freshly prepared Streptozotocin (70mg/kg body weight) in 0.1M citrate buffer (pH-4.5) in a volume of 1ml/kg body weight where as the control rats received equivalent amounts of buffer intraperitoneally.

Rats were allowed to drink 5% glucose solution overnight to overcome the drug-induced hypoglycemia. Diabetics were developed and stabilized in these streptpzptocin treated rats over a period of 7 days[82]. After 7 days of streptozotocin administration, plasma glucose levels of each rat were determined. Rats with a fasting blood glucose level greater than 10mmol/L were considered as diabetic and included in the present study.

Table-5.1: Grouping of experimental rats

Group	No of Rats	Average bodyweight (gm)	Age (week)	Dose mg/kg bodyweight
Gr-I (Normal)	6	110.80	6-7	Normal diet
Gr-II (Normal control)	6	112.20	6-7	50 gm/kg.b.wt of mushroom powder
Gr-III (Diabetic control)	6	114.50	6-7	Normal Diet
Gr-IV (Experimental powder)	6	107.30	6-7	50 gm/kg.b.wt of mushroom powder
Gr-V (Experimental Extract)	6	113.20	6-7	Methanol extract (200mg/ kg.b.wt)
Gr-VI (Experimental Extract)	6	109.30	6-7	Ethayl acetate extract(200mg/kg .b.wt)
Gr-VII (Standard)	6	111.60	6-7	Glipzide (0.6 mg/kg.b.wt)

5.1.5.3. Preparation of Standard drug Solution for oral Administration:

About 6 mg of Glipizide (Trade name Diactin, contain Glipizide BP 5mg/tablet; Beximco pharmaceuticals Ltd.) was suspended uniformly in 5ml distilled water and mixed well with a vortex mixture. The drug was not completely dissoleved but dispersed in water. This dispersed drug was fed orally daily once with the help of a dropper to the experimental rats at a dose of 0.6 mg/kg.b.wt.

5.1.5.4. Preparation of mushroom powder and extracts (methanol and ethyl acetate) for oral administration to long evan rats:

Fifty gm of mushroom powder, 950gm of normal diet and 2 lits distilled water was uniformly mixed, and made to small pill (about 6 gms) and dried in the sun. 200 mg of methanol and 200mg ethyl acetate extracts were individually dissolved in 5 ml of distilled water. Though

these extracts were not completely dissolved but dispersed in water. After shaking these dispersed extracts, at once these rats were orally administered daily once with the help of individual dropper.

5.1.5.5. Collection of Blood for determination of various parameters:

Blood of the rats were collected from their tail (by cutting the edge of tails) at the 1st, 7^{th} and 15^{th} days of the experiment. After 30 days of treatment, finally rats were sacrificed and their blood was collected for the estimation of blood parameters. At every weeks of oral administration, rats were kept fasting for over night, and the fasting blood was collected. Serum was obtained immediately by centrifugation (15min at 4000r.p.m), which was used for the measurement of various biochemical parameters.

5.1.5.6. Estimation of serum biochemical parameters:

a. Alanine Aminotransterase (ALT/GPT):

Principle of reaction:

L-Alanine + *a*-ketoglutarici acid $\longrightarrow$ Pyruvic acid + L-glutamic acid

Pyruvic acid + NADH + H^+ $\longrightarrow$ L-lactic acid + NAD^+ + H_2O

Procedure:

One bottle of R_1 was dissolved with a ration of R_2 and used as working reagent. The working reagent was allowed to equilibrate to the reaction temperature prior to processing.

	Blank	**Sample**
Working reagent	1.00ml	1.00ml
Distilled water	0.10ml	------
Sample	------	0.10ml

Then it was mixed thoroughly and incubated at reaction temperature for 60 seconds.

The initial absorbance and the absorbance changed in every minute were taken for 3 minutes separately. The change of absorbance was determined per minute (ΔA/min).

Calculation:

$ALT\ (U/L)=(\Delta A_{Sample}/min-\Delta A_{blank}/min) \times F$

$F = V_1\ (V_s\text{-extinction coefficient}) \times 1000= 1746$

V_1- the total volume, V_s=the volume of sample

The extinction coefficient of NADH at 340nm is 6.3/mmol.

b) Urea Assay Kit (BUN):

Principle:

$$Urea + H_2O \longrightarrow 2NH_4^+ + CO_2$$

$$2NH_4^+ + 2\alpha\text{-Ketoglutarate} + 2NADH \longrightarrow 2L\text{-Glutamate} + 2NAD^+ + 2HO_2$$

Procedure:

A bottle R_1 was dissolved by R_2 and used as working reagent.

	Blank	**Calibrator**	**Sample**
Working reagent	1.0ml	1.0ml	1.0ml
Blank	0.01ml	—	—
Calibrator	—	0.01ml	—
Sample	—	—	0.01ml

Then it was mixed thoroughly and absorbance was taken, A1 at 30 seconds and A2 at 20-60 seconds.

Calculation:

$$\Delta A_{\text{standard}} = (\text{A1-A2})_{\text{standard}} - (\text{A1-A2})_{\text{blank}}$$

$$\Delta A_{\text{sample}} = (\text{A1-A2})_{\text{sample}} - (\text{A1-A2})_{\text{blank}}$$

$$\text{Urea} = \frac{\Delta A_{\text{sample}}}{\Delta A_{\text{standard}}} \times \text{Calibrator conc. (mmol/L or mg/dl)}$$

c) Aspartate Aminotransferase Assay Kit (AST/GOT):

Principle:

L-aspartic acid + α - ketoglutaric acid $\xrightarrow{\text{AST}}$ Oxalacetic acid + L-glutamate.

Oxalacetic acid + NADH + H^+ $\xrightarrow{\text{MDH}}$ L-malic acid+ NAD + H_2O.

Procedure:

One bottle of R_1 was dissolved with R_2 and used as working reagent. The working reagent was allowed to equilibrate to the reaction temperature prior to processing.

	Blank	**Sample**
Working reagent	1.00ml	1.00ml
Distilled water	0.10ml	------
Sample	------	0.10ml

Then it was mixed thoroughly and incubated at reaction temperature for 60 seconds. The absorbance change was determined per minute (ΔA/min).

Calculation:

$$AST\ (U/L) = (\Delta A_{sample}/min - \Delta A_{blank}/min) \times F$$

$$F = V_t/(V_s\text{-extinction coefficient}) \times 1000$$

$$= 1746$$

V_t = total volume

V_s = volume of sample

The extinction coefficient of NADH at 340 nm is 6.3/mmol.

d) Total Bilirubin Kit (T. Bili):

Intended Use:

Used for the quantitative determination of total bilirubin (T. Bili) in blood serum.

Assay principle:

Sulfanilic acid + HCl + $NaNO_2$→Diazotized Sulfanilic acid+ NaCl.

Total bilirubin + Diazotized Sulfanilic $\xrightarrow{\text{Accelerator}}$ Acid Azobilirubin (magenta).

Procedure:

R_1 was mixed with R_2 (5:1) and used as working reagent

	Reagent black	**Reagent**	**Standard blank**	**Standard**	**Sample blank**	**Sample**
R1	2.00 ml	--	2.00 ml	--	2.00 ml	--
Working reagent	--	2.00 ml	--	2.00 ml	--	2.00 ml
0.9% NaCl	0.10 ml	0.10 ml	--	--	--	--
Standard	--	--			--	--
Sample	--	--	--	--	0.10 ml	0.10 ml

It was then mixed thoroughly and incubated for 10 minutes at 37°c temperature. The absorbance was read within 60 minutes.

Calculation:

Concentration of total bilirubin in sample (µmol/L or mg/dl).

$$C = [(A_{\text{sample}} - A_{\text{sample blank}}) - (A_{\text{reagent}} - A_{\text{reagent blank}})] \times F$$

$$F = C_{\text{stant}} / [(A_{\text{sant}} - A_{\text{sant blank}}) - (A_{\text{reagent}} - A_{\text{reagent blank}})]$$

e) Alkanline Phosphatase Assay Kit (ALP):

Principle of Reaction:

$$\rho\text{-nitrophenyl phosphate} + H_2O \xrightarrow{ALP} \rho \text{ nitrophenol} + \text{phosphate.}$$

Procedure:

One bottle of R_1 was dissolved with R_2 and used as working reagent.

	Blank	**Sample**
Working reagent	1.00 ml	1.00 ml
Distilled water	0.02 ml	--
Sample	--	0.02 ml

Then it was mixed thoroughly and incubated for 60 second at 37°C temperature. The initial absorbance and the changed absorbance for 3 minute were taken separately. The absorbance change was determined per minute (ΔA/min).

Calculation:

$$ALP\ (U/L) = (\Delta A_{\text{sample}}/\text{min} - \Delta A_{\text{blank}}/\text{min}) \times F$$

$$F = V_t/(V_s \times \text{extinction coefficient}) \times 1000$$

$$= 2757$$

V_t: total volume, V_s: sample volume

The extinction coefficient of ρ-nitrophenyl at 405 nm is 18.50.

f) Cholesterol (Enzymatic-colorimetric test):

Principle:

Cholesterol and its esters were released from lipoproteins by detergents. Cholesterol esterase hydrolyses the esters and H_2O_2 was formed in the subsequent enzymatic oxidation of cholesterol by cholesterol-oxidase according to the following equation. In the last reaction a red dye quinonimine dye formed whose intensity is proportional to the cholesterol concentration.

$$\text{Chol. Esters} + H_2O \xrightarrow{\text{CHE}} \text{Cholest.} + \text{fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{CHOD}} \text{4-Cholestenon} + H_2O_2$$

$$2H_2O_2 + \text{4-AP} + \text{Phenol} \xrightarrow{\text{POD}} \text{Quinonimine} + 4H_2O$$

Procedure:

1. At temperature 37°C, the wavelength was adjusting 505nm.
2. The instrument was adjusted to zero with distilled water.
3. Pipette into a cuvette:

	Blank	**Standard**	**Sample**
Standard	--	10 µl	--
Sample	--	--	10 µl
Working reagent	1 ml	1 ml	1 ml

Then it was mixed and incubated 5 min at 37°C.

The absorbance (Abs) of standard and sample against blank were measured. The color was stable at least 60 min.

Calculation:

Cholesterol conc. (mg/dl)

= (Abs Sample / Abs Stand) × 200 (stand. Conc.)

Conversion factor: mg/dl × 0, 0258 = mmol/l.

g) Serum glucose Assay:

Method: Enzymatic end point method

Principle:

Glucose on boiling with alkaline copper solution reduces copper from the cupric to the cuprous state (cuprous oxide). The cuprous oxide so formed reduced phosphomolybdic acid to blue colored molybdenum blue, which is measured colorimetrically. The intensity of the blue color is proportional to glucose concentration.

Procedure:

1) 3 test tubes were taken in the rack and marked serially T, S and B
2) Then taken working reagent 1.0 ml in each test tube
3) Added 10µl serum and 10µl standard in T. & S. marked tubes.
4) Then added 10µl distilled water in B. marked tube by micropipette
5) Mixed and wait for 10 minutes at room temperature.
6) Absorbance of the sample against the reagent blank was measured

Calculation:

$$\frac{\text{OD of sample}}{\text{OD of standard}} \times \text{Concentration of standard}$$

5.1.5.7. Analysis of Data:

After estimation of serum biochemical parameter the data were statistically analyzed by using t-test (Un-paired student's t-test) to determine the level of significance. A *P* value of ≤0.01, ≤0.05 were considered to represent a statistically significant difference between the results of the two-group compared.

PART-B: RESULTS AND DISCUSSIONS

5.2. Effect of Oyster mushroom powder and extracts (Methanol and ethyl acetate) on different biochemical Parameters of Blood in normal rats:

The rats of Gr-II (Normal control) showed no signs of tremor, convulsion and reflex abnormalities. Muscular numbness of the hind and fore legs, salivation or diarrhoea was not observed. The body weight of all the rats was recorded during the treatment and data were compared. No significant changes in body weights of all the rats were observed. Small changes in body weights were due to normal body weight gain for 30 days experiment. Serum glucose levels were measured in normal and experimental rats at 1st day and at the end of 7, 15 and 30 days of treatment (table 5.2, figure-5.1). Other biochemical parameters of blood such as SGPT, SGOT, SALP, Bilirubin, Creatinine, Urea, (table 5.4, figure 5.3) Cholesterol, Triglyceride, LDL and HDL cholesterol of normal rats were determined at 1st day and at the end of 30 days of treatment (table 5.3, figure 5.2).

Streptozotocin-treated diabetic control rats showed significant increase in the level of blood glucose as compared to normal rats. Following oral administration of mushroom powder (50mg/kg.b.wt), methanol extract (200mg/kg.b.wt.), ethyl acetate (200mg/kg.b.wt.) and glipzide (0.6mg/kg.b.wt.), blood glucose levels reverted back to near normal level. So oral administration of mushroom powder and extracts showed significant ($p<0.01$) effect on serum glucose level compared to diabetic control. Table-5.3 shows the levels of serum cholesterol in normal and experimental rats. Diabetic control rats showed significant

increase in the level of blood cholesterol, triglyceride and LDL-cholesterol level as compared to normal rats. Following oral administration of mushroom powder, extracts and glipzide, there were significant ($p<0.01$) decrease in the Cholesterol, Triglyceride, LDL-cholesterol but increase HDL-cholesterol level in experimental rats compared to diabetic control table-5.3.

Table-5.2: Effect of Oyster mushroom *(Pleurotus ostreatus)* powder and extracts on serum glucose level in Streptozocin induced long evan rats.

Groups	Serum glucose level (mmol/L)			
	1st day	**7th day**	**15th day**	**30th day**
Gr-I (Normal)	5.21 ±1.11	5.08 ±1.21	5.72±1.12	5.4±1.12
Gr-II (Normal Control-50gm\kg.b.wt)	5.71±1.12	5.36±1.12	5.63±1.21	5.6±1.12
Gr-III (Diabetic Control)	5.44±1.11	11.73±1.41^{t}	16.91±1.82^{t}	21.4±2.11^{t}
Gr-IV (Powder 50gm/kg.b.wt)	5.64±1.71	11.72±1.61*	10.73±1.51*	9.8±1.71*
Gr-V (Methanol extract 200mg/kg.b.wt)	5.31±1.12	10.41±2.12**	10.05±1.41**	9.2±1.32**
Gr-VI (E. Acetate extract 200mg/kg.b.wt)	5.46±1.11	12.09±1.21**	10.15±1.93**	8.0±1.31**
Gr-VIII Standard (gliplizide 0.6mg/kg.b.wt)	5.77±1.31	12.53±1.72**	8.02±1.44**	6.1±1.13**

Data are the mean± SD for six rats in each group; Where significant Values are **$p<0.01$ and *$p<0.05$ compared to Diabetic Control (Gr-III), $^{t}p<0.01$ Compared to normal (Gr-I) (table-5.2).

Table-5.3: Effect of oyster mushroom *(Pleurotus ostreatus)* powder and extracts on total Cholesterol, TG, HDL-Chol., LDL-Chol. level in Streptozocin induced long evan rats.

Groups	T.Chol. (mg/dl)	TG (mg/dl)	HDL-Chol. .(mg/dl)	LDL-Chol. (mg/dl)
Gr-I (Normal)	94.51 ±2.54	36.26±3.21	52.18 ± 1.21	119.12±3.15
Gr-II (Normal Control 50gm/kg. b. wt.)	93.92±2.4	35.81±2.12	52.7 1± 2.10	118.81±3.19
Gr-III (Diabetic Control)	126.23±4.12^{t}	53.12±3.41^{t}	39.10 ± 1.81^{t}	130.62±3.35^{t}
Gr-IV (Mushroom powder100gm/kg.b.wt)	119.44±2.8*	50.23±2.61*	50.21 ± 2.31*	128.72±3.82*
Gr-V (Methanol extract 200mg/kg.b.wt)	111.25±3.14**	47.34±2.12**	49.75 ± 1.73**	126.41±3.63**
Gr-VI (E. Acetate extract 200mg/kg.b.wt)	107.25±2.11**	44.15±3.21**	48.80 ± 2.25**	124.81±3.13**
Gr-VIII Standard (gliplizide 0.6mg/kg.b.wt)	103.18±4.31**	39.26±3.72**	48.24 ± 2.63**	121.12±3.45**

Data are the mean± SD for six rats in each group; where significant Values are **p<0.01 and *p<0.05 compared to Diabetic Control (Gr-III). ^{t}p<0.01 Compared to normal (Gr-I) (table-5.3).

Table-5.4: Effect of oyster mushroom *(Pleurotus ostreatus)* powder and some extracts on SGPT, SGOT, SALP, SUrea, SCreatinine level in Streptozocin induced long evan rats.

Groups	SGPT (U/L)	SGOT (U/L)	SALP (U/L)	Blood Urea (mg/dl)	SCreatinine (mg/dl)
Gr-I (Normal)	46.1±1.72	67.12±2.21	150.15±3.22	24.19±2.22	.30±0.05
Gr-II (Normal Control)	47.13±2.41	67.21±2.12	149.17±2.18	23.12±2.12	0.30±0.06
Gr-III (Diabetic Control)	52.12±2.12^{t}	70.13±2.41^{t}	154.66±2.49^{t}	30.14±2.29^{t}	0.41±0.05^{t}
Gr-IV (Powder100gm/kg.b. wt)	51.21±2.81*	69.12±2.61*	148.11±3.61 *	28.13±2.21*	0.37±0.07*
Gr-V (Methanol 200mg/kg.b.wt)	50.31±2.14**	67.13±2.12**	148.12±1.12 **	27.12±1.62**	0.33±0.08**
Gr-VI (E.Acetate 200mg/kg.b.wt)	48.12±2.11**	66.14±2.21**	46.15±3.21* *	26.16±2.24**	0.32±0.06**
Gr-VIII Standard (gliplizide 0.6mg/kg.b.wt)	45.12±2.31**	66.21±2.72**	145.23±2.12 **	24.23±2.31**	0.32±0.08**

Data are the mean± SD for six rats in each group; Where significant Values are **p<0.01 and *p<0.05 compared to Diabetic Control (Gr-III). ^{t}p<0.01 Compared to normal (Gr-I) (table-5.4).

Table-5.5: Effect of mushroom powder and extracts on body weight in Streptozotocin-induced diabetic rats.

Groups	Body weight (gm/day)	
	Initial	**Final**
Normal (Gr-I)	110.80±5.6	144.30±8.3
Normal Control (Gr-II)	112.80+3.20	145.10+4.20
Diabetic control (Gr-III)	140.50±5.2	125±6.5**
Diabetic + glipizide (0.6mg/kg.b.wt.) (Gr-VII)	155.30±4.7	150±9.2**
Diabetic + Mushroom powder (100gm/kg.b.wt.) Gr-IV	156.70±3.4	150±8.6**
Diabetic + Methanol extract (200mg/kg.b.wt.) Gr-V	158.40±5.7	154±7.2**
Diabetic+EA extract (200 mg/kg.b.wt) Gr-VI	155.30+3.50	150.0+4.30

Table-5.4 shows the levels of SGPT, SGOT, SALP, SBilirubin, Urea, SCreatinine in normal and experimental rats. Streptozotocin-treated diabetic control rats showed increase in the level of SGPT, SGOT and SALP as compared to normal rats. Following oral administration of mushroom powder (50mg/kg.b.wt.), extracts(200mg/kg.b.wt.) and glipzide (0.6mg/kg.b.wt.), SGPT, SGOT and SALP levels reverted back to near normal level however SBilirubin, Urea and SCreatinine were changed but not significant. So oral administration of mushroom powder (50gm/kg.b.wt) and extracts (200mg/kg.b.wt.) showed significant ($p<0.01$) effect on SGPT, SGOT and SALP level compared to diabetic control (table-5.4).

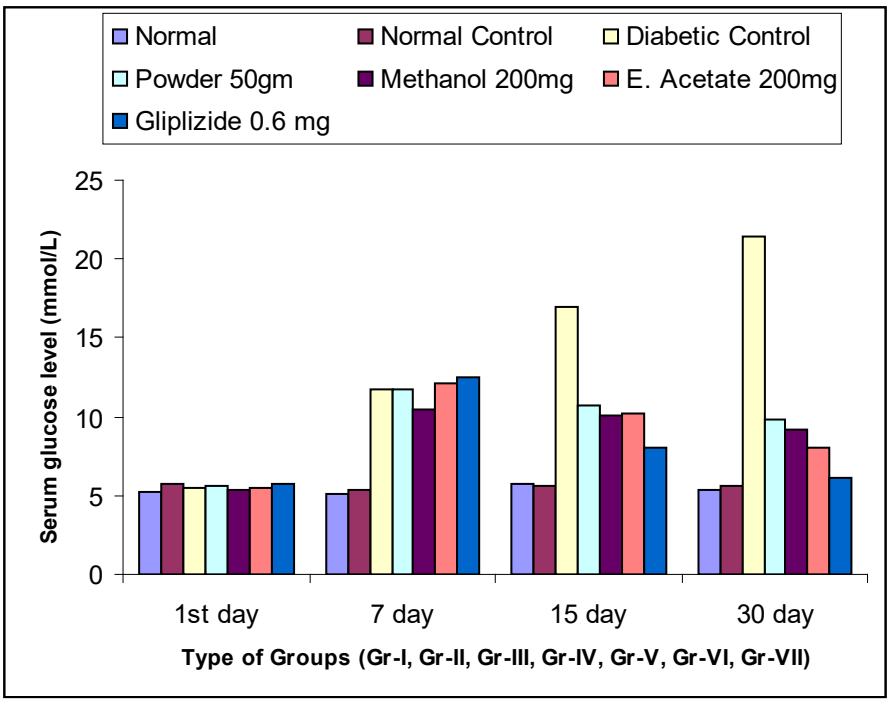


Figure-5.1: Effect of Mushroom powder and extracts (Methanol and Ethyl Acetate) on serum glucose level in diabetic rats.

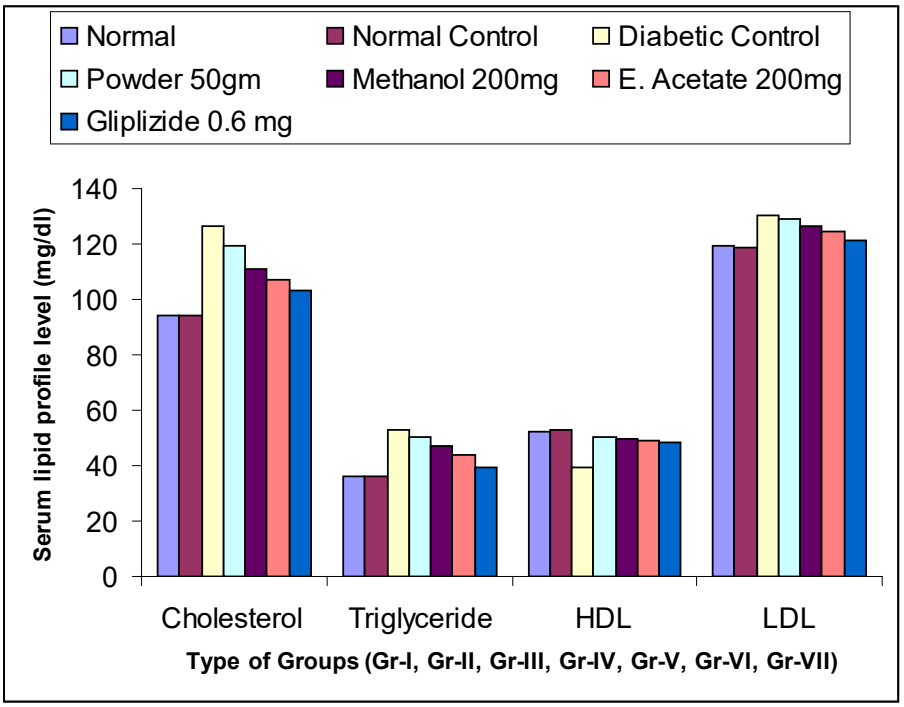


Figure-5.2: Effect of mushroom powder and extracts (Methanol and Ethyl Acetate) on serum glucose level in diabetic rats.

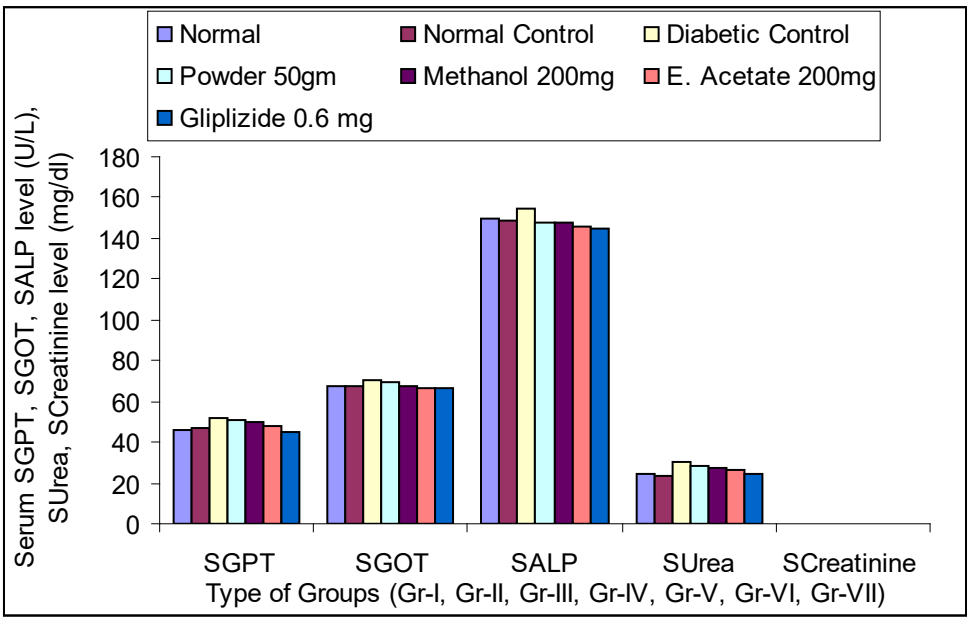


Figure-5.3: Effect of Mushroom powder and extracts (methanol and ethyl acetate) on serum SGPT, SGOT, SALP, SUrea, SCreatinine in diabetic rats.

5.3 Discussion:

The aim of this study was to evaluate the hypoglycemic and hypolipidimic effect of mushroom powder and extracts (Methanol and Ethyl acetate) in normal and Streptozotocin-induced diabetic rats. Diabetes mellitus causes a disturbance in the uptake of glucose as well as glucose metabolism. Streptozotocin is a compound commonly used for the induction of type-1 diabetes in experimental rats[83]. The use of a lower dose of STZ (60 mg/kg) produced an incomplete destruction of pancreatic β-cells even though the rats become permanently diabetic[84]. After treatment with a low dose of STZ there should be many surviving β-cells and regeneration is also possible[85]. In the present study, the results show that oral administration of mushroom powder, methanol and ethyl acetate extracts significantly ($p<0.01$) decreases the fasting blood glucose level in

the normal rats as compared to controls. However, the reduction in the blood glucose level is less than that brought about by the standard drug, Glipizide.

Lipids play a vital role in the pathogenesis of diabetes mellitus. In STZ-induced diabetes, the increase in blood glucose levels is usually accompanied by an increase in plasma cholesterol, triglycerides, LDL and decreases in HDL[86]. The increased levels of serum lipids in diabetes represent a risk factor for coronary heart disease[87]. Under normal circumstances, insulin activates lipoprotein lipase and hydrolyzes triglycerides. Insulin increases uptake of fatty acids into adipose tissue and increases triglyceride synthesis. Moreover, insulin inhibits lipolysis. In insulin-deficient diabetes, the concentration of serum free fatty acids is elevated, as a result of free fatty acid outflow from fat depots, where the balance of the free fatty acids esterification-triglyceride lipolysis cycle is displaced in favor of lipolysis[88]. Thus an excess fatty acid in the plasma produced by the STZ-induced diabetes promotes the conversion of excess fatty acids into phospholipids and cholesterol in the liver. These two substances, along with excess triglycerides formed in the liver may be discharged into the blood in the form of lipoproteins[89]. HDL is an antiatherogenic lipoprotein. It transports cholesterol from peripheral tissues into the liver and thereby acts as a protection factor against coronary heart disease. Oral administration of Mushroom powder and extracts lowers serum lipids and also increases the serum HDL-cholesterol level in diabetic rats.

The liver is an important insulin-dependent tissue, which plays a pivotal role in glucose and lipid homeostasis and is severely affected

during diabetes[90]. The increase in the activities of SGPT, SGOT and ALP indicated that diabetes might be induced due to liver dysfunction[91]. Ohaeri also found that liver was necrotized in STZ-induced diabetic rats. Therefore, increase in the activities of SGPT, SGOT and SALP in diabetic control, may be mainly due to the leakage of these enzymes from the cytosol of hepatic cells into the blood stream[92], which gives an indication of the hepatotoxic effect of STZ. On the contrary in our present study the treatment of the diabetic rats with mushroom powder, methanol and ethayl acetate extract caused reduction in the activity of these enzymes in serum compared to the mean values of diabetic group and consequently might alleviate liver damage caused by STZ-induced diabetes. When STZ administered intraperitoneallly, Plasma levels of streptozotocin rapidly decrease within 15 minutes and concentrate in the liver and kidneys. Twenty percent of the drug is metabolized and/or excreted by the kidneys[93]. The changes in blood urea and serum creatinine observed in the present study could be attributed to the functional and/or morphological changes in the kidneys[94]. Kedar and chakrabarti[142] had reported elevated level of blood sugar to 18.0 mmol/L associated with glycolysis, ureamia, hypercholesterolemia, hypertriglyceridemia and loss of body weight in rabbits by a single intravenous injection of streptozotocin (65 mg/kg). Further, a significant increase of total protein excreted, albuminuria, glycosuria and urinary urea levels indicating impaired renal function have been reported[95]. The administration of mushroom powder, methanol and ethyl acetate extracts significantly ($p<0.05$) reduces the urea and creatinine levels in diabetic rats compared to diabetic control.

Our finding shows that oral administration of mushroom powder, methanol and ethyl acetate extracts produces significant hypoglycemic and hyplolipidemic effect which lowers glucose level as well as total cholesterol and TG, and at the same time increases HDL-cholesterol to near normal range in STZ-induced diabetic rats. This investigation reveals that mushroom powder, methanol and ethyl acetate extracts have potent antidiabetic and hypolipidemic effects in normal and STZ-induced diabetic rats. Further studies are needed to identify the chemical constituent of Oyster mushroom *(Pleurotus ostreatus)* responsible for these activities.

CHAPTER-SIX

HEPATOPROTECTIVE ACTIVITY

MATERIALS AND METHODS

6.1. Materials:

6.1.1. Chemicals:

1. Total bilirubin kit.
2. ALT/GPT reagent kit.
3. ALP reagent kit.
4. AST/GOT reagent kit.

6.1.2. Working Instruments:

1. Bio-analyzer (Model: Microlab 200).
2. Eppendorf centrifuge 5415C.
3. Refrigerator.
4. Electric Balance.
5. Micro pipette.
6. Water bath.

6.2. Experiment of Hepatoprotective activity of Oyster mushroom powder and extracts (Methanol and Ethyl Acetate):

In the present study we will observe the hepatoprotective effect of mushroom powder and two extracts (Methanol and Ethyl acetate) in Carbon Tetrachloride (CCl_4) induced long evan rats.

6.2.1. Test Animal:

Long Evan rats were selected as experimental animal to carry out this study. Rats were collected from the Animal Resource Division of ICDDR'B Mohakhali, Dhaka, weighing 100-180 gm.

6.2.2. Methods:

6.2.2.1. Maintenance of Animal:

Forty two (42) rats were randomly divided into seven groups. Each rat was numbered with a permanent marker for experimental purpose, weighed and recorded; seven cages (containing 6 rats) were kept in the departmental animal house. The animals were fed on standard laboratory diet with water and kept at room temperature. Rats were acclimatized to the laboratory conditions for one week before experimental work was undertaken.

6.2.2.2. Induction of Hepatic injury:

Hepatic injury was induced by a single intraperitoneal injection with a dose of 0.5 ml/kg. b. wt. of Carbon Tetrachloride (CCl_4) to the experimental rats. Silymarin, a standard drug was used as reference.

Table-6.1: Grouping of experimental rats.

Group	No of Rats	Average bodyweight (gm)	Age (week)	Dose mg/kg bodyweight
Gr-I (Normal)	6	110.80	6-7	Normal diet
Gr-II (Normal control)	6	112.20	6-7	50 gm/kg.b.wt of mushroom powder
Gr-III (Diabetic control)	6	114.50	6-7	Normal Diet
Gr-IV (Experimental powder)	6	107.30	6-7	50 gm/kg.b.wt of mushroom powder
Gr-V (Experimental Extract)	6	113.20	6-7	Methanol extract (200mg/ kg.b.wt)
Gr-VI (Experimental Extract)	6	109.30	6-7	Ethayl acetate extract (200mg/kg.b.wt)
Gr-VII (Standard)	6	111.60	6-7	Silymarin (100mg/kg.b. wt)

6.2.2.3. Preparation of standard drug solution for oral administration:

About 140mg of Silymarin (Trade name Silybin, contain Silymarin BP 100mg/tablet; Square pharmaceuticals Ltd.) was suspended uniformly in 5ml distilled water and mixed well with a vortex mixture. The drug was not completely dissolved but dispersed in water. This dispersed drug was fed orally daily once with the help of a dropper to the experimental rats at equal dose.

6.2.2.4. Preparation of Oyster mushroom powder and extracts (methanol and ethyl acetate) for oral administration to long evan rats:

50gm of mushroom powder, 950gm of normal diet and 2 lits distilled water were uniformly mixed, made small pill (about 6 gms) and dried in the sun. 200mg of methanol and 200mg of ethyl acetate extracts were individually dissolved in 5ml of distilled water. Though these extracts were not completely dissolved but dispersed in water. After

shaking these dispersed extracts at once the rats were orally administered daily once with the help of individual dropper.

6.2.2.5. Collection of blood for assessment of serum enzymes:

After treatment of 10 consecutive 10 days, CCl_4 (0.5ml/kg. b. wt.) was intraperitonially injected on 11th day. At 24 hrs post–CCl_4 administration experimental rats were anaesthetized by putting each one in a glass jar containing ether-soaked cotton wool for about 5mins. When the heart rhythm was completely stopped, blood was then collected from retroorbital sinus and allowed to coagulate for 30mins followed by centrifugation at 2500 rpm for 15mins. Seperated serum was used for estimating serum bio-chemical parameters.

6.2.2.6. Effects of mushroom powder and extracts (methanol and ethyl acetate) on serum enzyme parameters after Carbon Tetrachloride (CCl_4) induced hepatotoxicity in long evan rats:

The effects of mushroom powder and extracts (methanol and ethyl acetate) on serum enzymes were studied and their results are given in (table-6.1). It is well known that CCl_4 is used as a hepatotoxic agent on various animals. Administration of CCl_4 to rats produced hepatotoxicity showed by significant increase ($^{t}p<0.01$) in the serum levels of SGPT, SGOT, SALP and SBilirubin in comparison to normal group (Gr-I). Elevated levels of these enzymes are indicative of cellular leakage and loss of functional integritry of cell membrane in liver. Oral administration of mushroom powder and extracts showed a significant decrease ($^{**}p<0.01$) in the serum enzymes SGPT, SGOT, SALP and SBilirubin with compared to CCl_4 treated control group (Gr-III). Hepatoprotective

standard drug silymarin (100mg/Kg. b. wt) retained the serum enzymes about normal level compared to CCl_4 induced hepatic injured rats.

Table-6.2: Effect of Oyster mushroom *(Pleurotus ostreatus)* powder and extracts (methanol, ethyl acetate) on SGPT, SGOT, SALP, SBilirubin level in Carbon Tetrachloride (CCl_4) induced hepatic injury in long evan rats.

Groups	Type of Groups/Dose	SGPT (U/L)	SGOT (U/L)	SALP (U/L)	SBilirubin (mg/dl)
Gr-I	(Normal)	46.1±1.72	67.12±2.21	150.15±3.22	0.35±0.08
Gr-II	(Normal Control)	47.13±2.41	67.21±2.12	149.17±2.18	0.34±0.07
Gr-III	(CCl_4 Treated-0.5mL/kg.b.wt)	122.12±2.12^t	140.13±2.41^t	298.66±2.49^t	1.35±0.09^t
Gr-IV	(Powder 50gm/kg.b.wt)	101.21±2.81*	120.12±2.61*	272.11±3.61*	1.21±0.08*
Gr-V	(Methanol extract 200mg/kg.b.wt)	88.31±2.14**	111.13±2.12**	252.12±1.12**	1.12±0.07**
Gr-VI	(E. Acetate extract 200mg/kg.b.wt)	73.12±2.11**	102.14±2.21**	233.15±3.21**	1.02±0.06**
Gr-VIII	Standard (Silymarin 100 mg/kg.b.wt)	60.12±2.31**	87.21±2.72**	177.23±2.12**	0.82±0.04**

Data are the mean±SD for six rats in each group; where significant values are **$p<0.01$ and *$p<0.05$ compared to diabetic control (Gr-III). $^t p<0.01$ compared to normal (Gr-I).

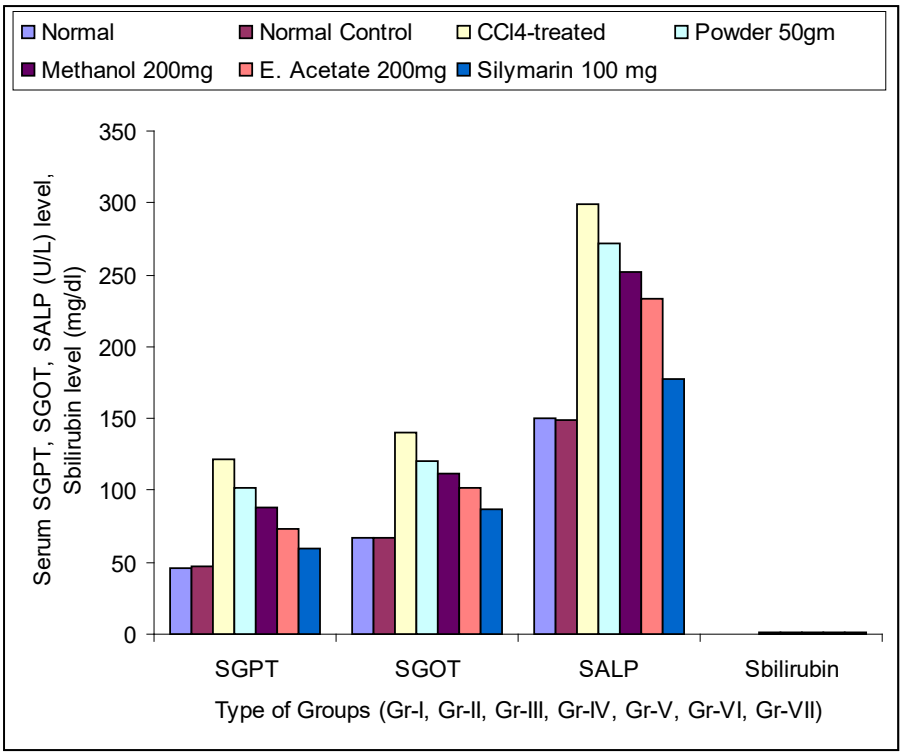


Figure-6.1: Effect of mushroom powder and extracts (methanol and ethyl acetate) on serum SGPT, SGOT, SALP, SBilirubin CCl_4 induced hepatic injured rats.

Oral administration of mushroom powder (50gm/Kg.b.wt), did not show any mortality or abnormalities of the normal control rats (Gr-II).

So, from above investigation we can say that Oyster mushroom (*Pleurotus ostreatus*) powder & extracts have hepatoprotective activity. Further studies are needed to identify the chemical constituent of Oyster mushroom (*Plearotus oystreatus*) responsible for such activity.

6.3. Discussion

The aim of the present study was to investigate the hepatoprotective activity of mushroom powder and extracts (methanol and ethyl acetate) in CCl_4-indueced rats. Liver is the vital organ of metabolim and excretion. Liver diseases have become one of the major

causes of morbidity and mortality all over the world. Many synthetic chemical compounds are responsible for hepatic injury. CCl_4 is one of them. The mechanism of CCl_4 induced liver injury is due to the lipid peroxidation caused by the free radical derviatives of CCl_4. It is metabolized in endoplasmic reticulum and mitochondria with the formation of CCl_3O by cytochrome P-450. The nascent oxygen O^-causes rise in intracellular reactive Fe^{2+} ions, aldehyde and depletion of GSH, and calcium sequestration via lipoperoxidation [Zimmerman & Hayman, 1976; Agarwal *et al*., 1983].

$$CCl_4 \rightarrow CCl_3O^- + O^-$$

CCl_4 incubation caused an abnormal reduction in the percentage viability of hepatocytes, while abnormal increase in the SGOT, SGPT, SALP and SBilirubin levels in the media. Treatment with mushroom powder and extracts significantly reversed all these abnormal changes and thus offered protection against CCl_4 induced toxicity to rat hepatocytes. Mushroom powder (100 gm/Kg.b.wt), methanol (200 mg/kg. b.wt) and ethyl acetate (200 mg/Kg.b.wt) extract decreased the viability of hepatocytes as well as cytosolic enzymes to near normal indicating that above dose of mushroom powder and extracts is effective in reverting CCl_4 induced toxicity to rat hepatocytes.

In conclusion, the present studies indicated that the powder and extracts of Oyster mushroom (*Plearotus, oystreatus*) possess hepatoprotective activity comparable to that of standard Silymarin which is known hepatoprotective agent as evidenced by the serum biochemical parameter and liver endogenous antioxidant enzymes activity.

CHAPTER-SEVEN

ISOLATION AND PURIFICATION OF BIOACTIVE COMPOUNDS

INTRODUCTION

7.1. Techniques for the isolation and purification of bioactive compounds:

The key to any successful program involving the investigation of biologically active plant constituents is the availability and choice of chromatographic techniques for the separation of pure substances. The aim is to have maximum yield with minimum effort (to reduce the time and cost of the separation procedure). Preparative separation techniques can be tedious and time consuming, especially when complex mixtures, such as, crude plant extracts have to be resolved. Over the past decade or so, several new techniques have been introduced, leading to the acceleration and simplification of different separation problems. However, there is no universal technique capable of solving every isolation problem. All methods have advantages and limitations. So, that the best results are often obtained by a combination of two or more of these.

The most important preparative separation techniques employed in the isolation and purification of plant constituents are as follows:

7.1.1. Preparative separation methods for mushroom constituents:

Solid phase chromatography:

Paper chromatography

Preparative TLC, Centrifugal TLC

Open-column chromatography

Vacuum liquid chromatography

Pressure column chromatography, flash chromatography

Low-pressure liquid chromatography (LPLC)

Medium-pressure liquid chromatography (MPLC),

High-pressure/High-performance liquid chromatography (HPLC)

Liquid chromatography:

Craig distribution

Droplet countercurrent chromatography (DCC)

Rotation locular countercurrent chromatography (RLCC)

Centrifugal partition chromatography (CPC)

According to the laboratory set up and the availability of essentials in the Department of Biochemistry & Molecular Biology, Rajshahi University and certain other neighboring laboratories there were limitations in choosing out the preparative separation methods, while the thin layer chromatography and the open column chromatography were used simultaneously in this investigation for the isolation and purification of the bioactive lichen compounds.

MATERIALS AND METHODS

7.2. Chromatographic techniques used in this investigation:

7.2.1. Chromatography on TLC plates:

Thin layer chromatography is a very convenient technique for finding the separation slurry along with its stationary phase. The mixtures of the compounds were well separated from each other and resolved by preparative thin layer chromatographic technique. This tool is considered to be one of the most helpful methods of the detection of organic compounds, which involves an adsorbent (using silica gel) as stationary phase and a solvent system as a mobile phase. Due to the differential rate of adsorption on the adsorbent, the components in a mixture migrate differentially along with the TLC plate. In other words due to the difference in mobility of the components often depend on their polarity and that of the solvents used.

To select the solvent system for the run of the open column separation was made on the preparative thin layer chromatographic plates. For the normal phase chromatography silica gel GF_{254} on Al sheets (Merck) were used. Ten mg/ml of he sample in the solvent extraction offered 100µl/spot by spotting 10µl for each of he sample extracts. The chromatograms then developed within a conventional chamber with the following solvent systems: n-hexane: E acetate 5:4, 7:3, 7:2. All chromatograms were observed under UV at 254 and 366 nm and marked with a pencil.

7.2.2. Detection of the compound on TLC by Godin revelation:

The properly developed plates were dried and viewed visually under UV light and Godin reagent spray were used and number of compounds separated.

Visual detection: The development chromatogram was examined visually to detect the presence of colored compound.

I) **UV light (254 and 366nm):** After development and drying the chromatogram was examined under UV light to detect fluorescent compound and the glowing spots, which were then marked.

II) **Godin spray reagent:** Equal volume of 1% ethanolic solution of vanillin and 3% aqueous solution of percloric acid was mixed sprayed on to the prepared chromatogram and 10% ethanolic solution of H_2SO_4 was also sprayed afterwards and allowed the plate to dry out at 100°C by using a hair dryer. Revelation was observed in different colors for different compounds.

III) **Measurement of R_f values:** The R_f values of he separated compounds were calculated on the developed chromatogram using the pre-established solvent system. The R_f values were calculated by the following formula.

$$R_f = \frac{\text{Distance traveled by the compound}}{\text{Distance traveled by the solvent}}$$

7.3. Open column chromatography:

Of the methods in the solid phase category, column chromatography is very popular and used extensively. It can include non-exchange resins, polymeric columns, gel-filtration and chromatography over silica gel or chemically modified silica gel. Open column chromatography has a high load capacity but the separation time is long and the resolution is respectively low.

The stationary phase for the open column chromatography was silica gel (60-120 and 200-400 mesh, sigma) and glass column of different size (2.5×28cm, 2.5×32cm and 3×35cm etc.) were used. Cotton pads washed with acetone, chloroform and methanol was used at the base of the gel column. A similar cotton cloud was used at the top of the column (after application of the sample and the solvent) to protect destruction of the sample layer (Plate). Selected solvent systems were used as eluents and the elution rate was 1 ml/min. Fractions were collected carefully.

7.4. Selection of extracts for fractionation:

For fractionation of the extraction with a view to isolate biologically active compounds only ethyl acetate extract was subjected to biological assay. Repetition of the same assay is required until the purification of the target compound, and thus a suitable bioassay technique was selected.

7.4.1. Selection of slurry (solvent system) for respective extract:

Aluminiun backed precoated preparative thin layer chromatographic (TLC) plates (20×20cm) with silica gel GF_{254} with 0.5mm thickness and active in the usual manner (Merck, Germany) were

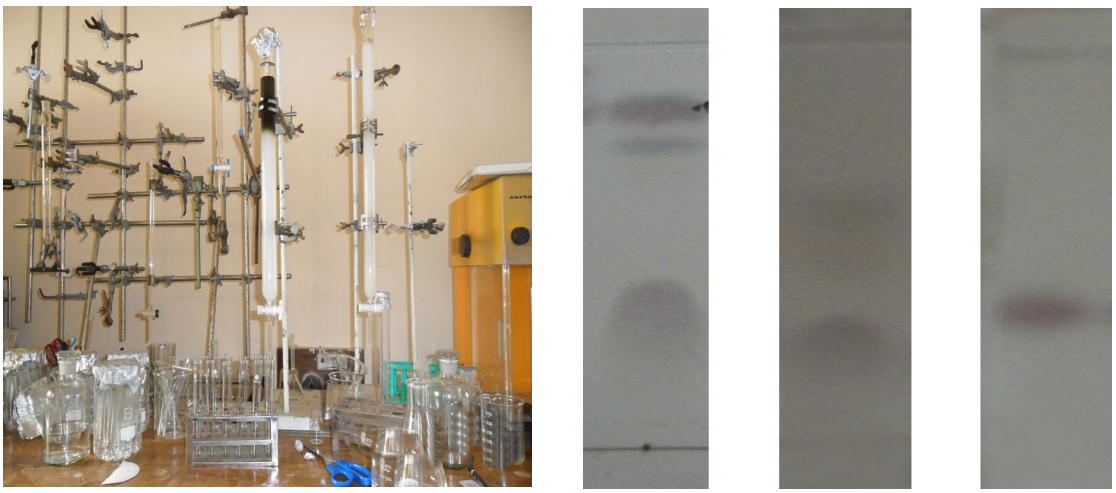
used. The sample was applied on the activated plates with the help of a gradient micropipette as a narrow band at 1cm above the lower edge of the plate to make sure that the sample was washed away when the plates were placed inside the TLC chamber with the solvent system. The plates were then developed in the usual manner.

After development, the chromatograms were air dried and observed visually under UV light (254nm) and sprayed with Godin reagent. The distinct bands were expected and by changing the solvent system with increase of either the polar or the apolar one. After having a better separation the selected solvent system (n-hexane: ethyl acetate = 7:2) was applied on the open column for isolation the compounds by fractionation. Small pieces of aluminium backed TLC plate was taken to spot the target extracts and run with a mixture of a relatively polar and relatively apolar solvent (1:1). For the better separation on the TLC with a known stationary phase the amount of both solvents were increased or decreased and applied accordingly. The combination given a better separation was selected for that extract for fractionation on the open column.

7.5. Isolation of the bioactive compound:

For the first fractionation of the ethyl acetate extract of mushroom Silica gel (60-120 mesh, Sigma) was used as the stationary phase and n-hexane: ethyl acetate (7:2) was the eluent on a glass column of 2.5×36cm for 500mg of the extract. Elution time was adjusted to yield 1ml/min. It gave 47 tubes, which were then spotted on TLC to run and reveal the compounds by reagent spray. Three fractions were made for tubes 1-14 (Fr. I), 15-32 (Fr. II) and (33-47) (Fr. III). The 2nd fraction was found to show biological activity and was subjected this fraction on selecting a

solvent system by TLC. n-hexane:ethyl acetate =5:1 was selected as solvent system and applied on a glass column of 2.5×27cm was packed with silica gel (200-400 mesh, Sigma). The elution was kept similar to that of the previous one. This fractionation yielded 42 tubes and TLC was made for all of them to get 3 fractions as Sfr. I (T/10-23), Sfr. II (T/24-32) and Sfr. III (T/33-42). Biological assay revealed of Fr (I) to contain bioactive compounds and measured that it may contain only two compounds. This fraction (FI) was then subjected on selecting the solvent system for later column. But this faction did not run though the stationary phase. Most probabely, due to the change of the chemical nature of the bioactive compounds, existed in this fraction, did not run through the solvent system.



Column running | Crude extract | After 1st column | After 2nd column

Photograph-7.1: Open column used in the experiment.

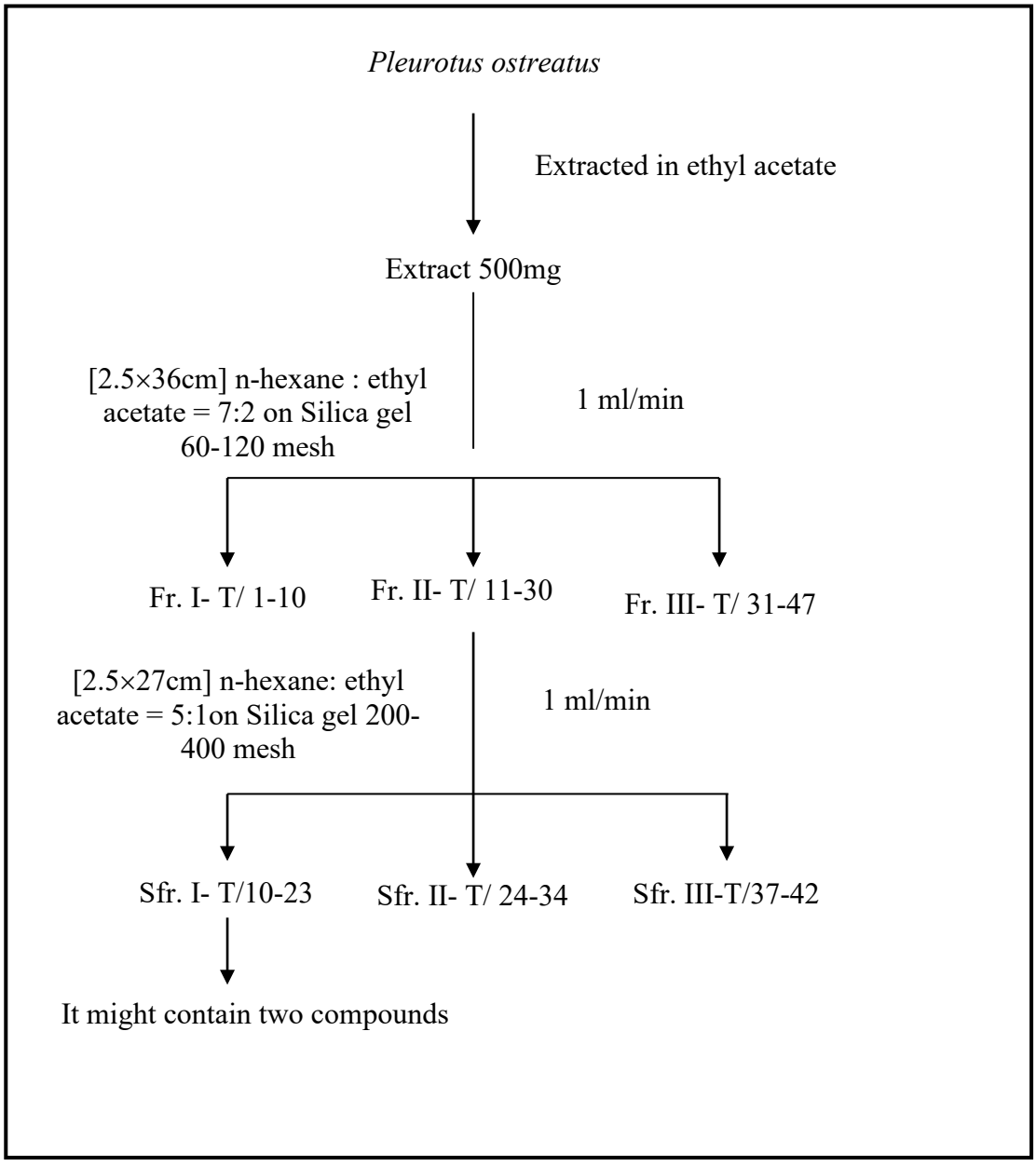


Fig. 7.1: Isolation pathway of bioactive compound.

RESULTS AND DISCUSSION

7.6. Physical remarks of the compounds:

On the basis of R_f values, color, solubility behavior and UV detection it was guessed that steroid, terpenoid type compound might be located in Oyster mushroom (*Pleurotus ostreatus*).

Table-7.1: Possible compounds present in ethyl acetate extract of *Pleurotus ostreatus.*

Extract	Solvent system	Sample code name	R_f value	Color with gordin reagent	Possible compound
Ethyl acetate	n-hexane : ethyl acetate=7:2	EA-1	0.197	Pink	Steroid
		EA-2	0.201	Violet	Terpenoid

CONCLUSION

Mushrooms have been used as food supplement from times immemorial not only for their flavor, aroma and nutritive values but also for their medicinal properties as evident from ancient literature. From nutritional analysis, we observed that *Pleurotus ostreatus* is a excellent source of protein. But lipid and carbohydrate content is not so accountable. Various nutrients content of mushroom are changed at different maturity stages.

Extracts of *Pleurotus ostreatus* were observed to inhibit two gram (+) bacteria (*Bacillus megatorium* & *Bacillus subtilis*) and three gram (-) bacteria (*Shigella dysenteriae*, *Bacillus cerus* and *E. coli*), suggest that this mushroom has a broad spectrum antibacterial activity. Similar antibacterial activity have been observed in the culture extracts of *Lrpen lacts*[78], *Agrocybe* sp.[79,80] and Juice of *L. edodes*.[81]

The methanol, ethyl acetate, chloroform, pet-either and hot water extracts of *Pleurotus ostreatus* have been thoroughly screened by residual film assay against *T. castaneum* adults yields a positive result.

The findings through insecticidal activity tests receive supports by several authors who carried out different biological assays including insecticidal activity tests. Insecticidal activities of *Theventia peruviana* seed and leaf respectively against *T. castaneum* was also found. Spice extracts against *T. castaneum* adults were shown for *Cinnamomum zeylanicum, Sygygium* aromaticism & Myristica fragrans were also shown.[103] A persual of the data achieved in this experiment clearly showed by the presence of insecticidal properties in *Pleurotus ostreatus* as well as traces of repellent potential. Lithality test against *A. Salina*

nauplii, larvicidal test against mosquito larvae; repellent activity test against *T. castaneum* adults were also carried to find out the efficacy of the test mushroom and all the extracts showed positive response against these assays.

Oyster mushroom powder (50gm/kg.b.wt.), methanol (200mg/kg.b.wt.) and ethyl acetate (200mg/kg.b.wt.) extracts reduced the glucose level by 16.0%, 21.0% and 31.1% respectively compared with diabetic control, in strepotozotocin-induced diabetic long evan rats. In the same way *Agaricus compestris*[104] and *Ganoderma lucidium*[105] mushroom also showed hypoglycemic activity in strepotozotocin-induced diabetic long evan rats.

Ganoderma applanatum[107], *Collybia confluens*[107] and *Phellinus linteus*[106] mushroom also showed hypoglycemic activity in streptozotocin-induced diabetic rats.

Mushroom powder and used extracts in this experiment also reduced the cholesterol level by 5%, 6.72% and 10% respectively compared with diabetic control, in streptozotocin-induced long evan rats. Hypocholesteromic effect of the above mushroom was also observed.

Administration of oyster mushroom powder (50gm/kg.b.wt.), methanol extract (200mg/kg.b.wt.) and ethyl acetate extract (200mg/kg.b.wt.) lowered the SGPT level by 17.2%, 27.88% and 40%; SGOT level by 14.2%, 20.7% and 27.1%; SALP level by 8.7%, 15.43% and 21.81% respectively against CCl_4-treated hepatic injured long evan rats. Some plants extracts i.e. *P. niurri; M. emerginata; E. alba, A. vera; S. indicum* and *A. marmelos*[108] showed hepatoprotective activity against

CCl_4-treated hepatic injured long evan rats. *Pleurotus Florida*[109] has also hepatoprotective activity that was previously studied.

So, from the comprehensive study on nutritional, phytochemical and antidiabetic properties, it is clear that oyster mushroom (*Pleurotus ostreatus*) can play vital roll in nutrient supplement as well as pharmaceutical endeavors.

REFERENCES

1. P.P. Joy, J. Thomas, Samuel Mathew, Baby P. Skaria, Medicinal Plants, 1998, p. 1-123.
2. A. Sofowora; Medicinal plants and traditional medicine in Africa, (1982).
3. World of Science "The World of Plants" Published by Bay Books, London 1980.
4. Biswas T. K, Mukherjee B., Plant medicines of Indian origin for wound healing activity: a review, Int J Low Extrem Wounds. 2003 Mar;2(1): 25-39.
5. Kim M-Y *et al*. Phenolic Compound Concentration and Antioxidant Activities of Edible and Medicinal Mushrooms from Korea *J. Agric. Food Chem.*, 2008, *56* (16), pp 7265–7270.
6. Wasser, S.P and A. L. Wies, 1999. Medicinal properties of substances occurring in higher basidiomycetes's mushrooms. Current perspectives (Review) Intl. J. Med. Mushrooms, 1(1):31.
7. Flora Agaricina Neerlandica 2 (1990) by C. Bas; p 100.
8. Kuo M. (2004). The Tricholomataceae. Mushroom Expert.Com (website)
9. Ayman. S. Daba Kabeil S.S. Bolros W.A and El-saadani M.A. Worlds of Agriculture Science, 4(5); 630-634, 2008.
10. D. S. Hibbett; R. G. Thorn. Nematode-Trapping in *Pleurotus tuberregium*; Mycologia, 86 (5) , 1994, pp. 696-699.

11. Chang, S.T., 1999. World production of cultivated edible and medicinal mushrooms in 1997 with empasis on Lentinus edodes (Berk) Sing. In China. Intl. J. Med. Mush., 1:291-30.

12. Change, S.T. and P.G. Miles, 1982. Introduction to mushroom science. In S.T. Change T.H Quimio (Eds). Tropical Mushrooms: Biological nature and cultivation methods. The Chinese University press. pp: 3-10.

13. Sachin L. B., Shweta N. S., Naimesh M. P., Prasad A. T. Subhash L. B. Hypoglycemic activity of aqueous extract of *Pleurotus*. in alloxan-induced diabetic mice (2006) Pharmaceutical biology vol. 44, (6), pp. 421-425

14. E. R. S. Wolff. E. Wisbeck. M. L. L. Silveira . R. M. M. Gern. M. S. L. pinho. S. A. Furlan. Antimicrobial and Antineoplastic Activity of pleurotus ostreatus. Appl Biochem Biotecnol (2008) 151:402-412.

15. Mizuno, T., H. saito, T. Nishitoba and H. Kawagishi, 1995. Antitumor active substances from mushrooms. Food Rev. Intl., 111:23-61.

16. Ginter, K. and P. Bobek, 1987. Hypolipaemic action of fungus substances (in slovak) Cs Gastroenterol. VYZ, 41:493-499.

17. Opletal L, Jahodár L, Chobot V, *et al.* (1997). "Evidence for the anti-hyperlipidaemic activity of the edible fungus Pleurotus ostreatus". *Br. J. Biomed. Sci.* 54 (4): 240.

18. Daba, A.S., 2001. Biochemical studies on the effect of oyster mushroom and its extracts in hypocholesterolemic hamster. Ph.D Thesis Ain Shames University, Egypt.

19. Ana; Gunde-Cimerman Nina; Hypocholesterolemic Activity of the Genus Pleurotus (Jacq.: Fr.) P. Kumm. (Agaricales s. I., Basidiomycetes) International Journal of Medicinal Mushrooms": December 1, 2001.

20. Suzuki, X. and S. Ohshima, 1976. Influence of shiitake on human serum cholesterol. Mushroom science 1X (part.I) Proceedings of the 9th International Scientific congress on the cultivation of edible Fungi, Tokyo, pp: 463-467.

21. Kurusawa, S., T. Sugahara and J. Hovashi, 1982. Studies on dietary fiber of mushroom and edible wild plants. Nutr Rep. Intl., 26: 167-173.

22. Buwei Yang Chao, “How to cook and eat in Chinese” first ed. 1945.

23. Zadrazil, F., 1980. Conversion of different plant waste into feed by basidiomycetes. Europ. J. Appl. Microbiol. Biotecnol, 9:243-48

24. Bas C. (1990). Tricholomataceae R.Heim ex Pouz. In: Flora Agaricina Neerlandica 2:65.

25. Periera J and Wohlgemuth R (1982) Neem (*Azadirachta indica* A. Juss.) of West African origin as a protectant of stored maize. *Z. Ang. Ent.* 24(2): 208-214.

26. Saxena R C (1983) Naturally occurring pesticides and their potential. In: *Chemistry and food supplies: The New Frontiers*. Pergamon Press, Oxford, New York, 143-161:

27. Ahmed, T.U., Checklist of mosquitoes of Bangladesh, Mosq. Syst., 19. 187-200, 1987.

28. Munakata, K., Insect feeding deterrents in plants, In: Shorey, H.H and Mckelvey, J.J.J.r, Chemical control of insect behavior, Theory and application, John Wiley and Sons, New York, 93-102, 1977.

29. Pimentel, D., An overview of integrated pest management. Department of Ecology and Systematics. Cornell University. Ithaca, Ny, (mimco). 52, 1981.

30. Sexana, R.C., Jilani, G. and Kareem, A.A., Focus on Phytochemical pesticides, In: Effects of neem on stored grain insects, Jacobson, M., (ed). CRS press, Florida, 1, 97-111, 1988.

31. Iwuala, M.O.E., Osisogu, I.U.W. and Agbakwuru, E.O.P., Dinnettia oil, a potential new insecticides, tests with adults and nymphs of *Peripbmeta americana* and *Zonocenus varicgatus. J. Econ. Ent.,* 74, 252-254, 1981.

32. Schmutterer, H., Higher plants as sources of novel pesticides. In: *Insecticides, Mechanism of Action and Resistance.* Intercept Ltd., Andover, pp. 3-15, 1992.

33. Schmutterer, H., Properties and potential of natural pesticides from neem tree, *Azadirachta indica. Ann. Rev. Entomol.,* 35: 271-297, 1990.

34. Kuusik, A., Metspalu, L. and Hiiesaar, K., *Insektitsiidide toimemehhanismide uurimine putukatel.* Tarku, 292 lk. 1995.

35. Golob, P. and Webley, D.J., The use of plants and minerals as traditional protectants of stored products, Tropical Products Institute, London, 32, 1980.

36. Talukder, F.A. and Howse, P.E., Evaluation of *Aphanamixis polystachya* as a source of repellents, antifeedants, toxicants and protectants in storage against *Tribolium castameum* (Herbst), *J. Stor. Prod. Res.,* 31, 55-61, 1995.

37. Fundamental of Biochemistry, A.C. Dev. p. 32-530.

38. Carl A. Burtis, Edward R. And Ashweed; *Tietz Fundamentals of Clinical Chemistry,* 5th edition, p. 480-481.

39. WHO (1978). *Classification of hypertension. Report of WHO Scientific Group.* Technical Report Series, 657: 87-95.

40. Lowry, O.H., N.J. Rosenbrough, A.L Farr and RJ. Rendall (1951). Protein measurement with the Folin Phenol reagent, Biol, Chem. 183: 265-275.

41. Bligh, E. G. and W.J. Dyer (1950). Total lipid extraction and purification. Can. J. Bio. Chem. Physiol. 37: 911.

42. Jayaraman, j. (1981). Laboratory Manual in Biochemistry (1st ed). Wiley Estern Ltd. New Delhi, India.

43. Loomis, W.A. and C.A. Shull (1927). Methods in plant physiology. McGraw-Hill, New York.

44. Miller, G. L. (1972). Use of dinitrosalicylic acid reagent for the determination of glucose Anal. Chem. 31: 426-428.

45. Rangama, S. (1979). Manual of analysis of Fruits and Vegetable products, Tata McGraw-Hill Publishing Company Ltd. New Delhi.

46. Bray, H. G. and W. V. Thrope (1954). Analysis of phenolic compounds of interest in metabolism. Meth . biochem. Anal. . 1027-52

47. Loomis, W. F. and C. A. Shull (1937). Methods in plant physiology. McGraw Hill, New York.

48. P. K. Talwar and Arun G. Jhingran, Indian Fishes, Vol. 2, PP-559, 560.

49. S. Ranganna, Hand Book of Analysis and Quality Control for Fruit and Vegetable Products, Second Edn. Tata McGraw Hill Publishing Co. Ltd. New Delhi. p. 25.

50. Smith, Jr. and G. M. Hieftje, Applied Spectroscopy; 1983, 37, 419-424.

51. Tohn, F.R.B. and T. White. Biochemical techniques theory and practice.

52. Burens V. (1970). Fruits Phenolies. In the biochemistry of Fruits and their products (ed. A.C. Hulme) Vol. 1 Academic Press: London and New York. p. 269-309, (1970).

53. Lehninger A.L. "Principles of Biochemistry", *School of Medicine,* The Johns Hopkins Uni. USA, 778 (1982).

54. Valley Fig Growers 2028 South Third St., Fresno, California 93702. (559) 237-3893; © 2004 Valley Fig Growers. Email: info@valleyfig.com Legal Disclaimer.

55. Anderson I. "Nutrition in health and disease", 17th (ed.). *J. B. Lippicopp Company,* Toronto, (1982).

56. Sue Rodwell Williams; Essentials of Nutrition and Diet Therapy; Fourth Edition, ISBN: 0-8016-5539-0.

57. Robert A. Anderson, MD. President, American Board of Holistic Medicine, East Wenatchee, WA; Copyright@2004 A.D.A.M., Inc.

58. Saner G. "Chromium deficiency and disease" New York: Alan R Liss. (1980).

59. Harborne, J.B., "Phytochemical Methods: A Guide to Modern Technique of Plant Analysis", Reprint, Chapman and Hall Ltd., London, 1976.

60. Touchstone, J.C. and Dobbins, F.M., "Practice of Thin Layer Chromatography", 1st ed. John Willey and Sons Co. Ltd., 1978.

61. Bobbilt, J.M., "Thin layer Chromatography", Chapman and Hall, Ltd., London, 94, 1963.

62. Stahl, E., "Thin Layer Chromatography", Springer International Student Edition, Toppan Company Limited, Tokyo, Japan, 860, 1969.

63. Touchstone, J.C. and Dobbins, F.M., "Practice of Thin Layer Chromatography", 1st Print, John Willey and sons Co. Ltd., New York, 170, 1977.

64. McLaughlin JL, Chang C-J, Smith DL, Bench top bioassays for the discovery of bioactive natural products: An update. In: Atta-ur-Rahman, ed. Studies in Natural Products Chemistry. Amsterdam: Elsevier, 1991, 9: 388-409.

65. Mikolajczak KL, McLaughlin JL, Rupprecht JK. Control of pests with annonaceous acetogenins (divisional patent on asimcin). U.S> Patent No. 4, 855, 319 issued August 8, 1989.

66. Finney, D.J., *Probit Analysis: a statistical treatment of sigmoid response curve.* Cambridge University Press, London, pp. 333, 1947.

67. Busvine, J.R., *A critical review of the techniques for testing insecticides:* Commonwealth Agricultural Buereux: London, pp. 345, 1971.

68. Vander, B.D.A. and Vlietnck, Screening methods for antibacterial and antiviral agents from higher plants, In Assay for Bioactivity, Hostiettman, K. (Ed.). Academic Press, London, 47-69, 1991.

69. Park, T and Frank, M.B. The fecundity and development of the flour beetle Tribolium Castaneum and Tribolium confusum at three constant temperatures. Ecology, 29: 368-375 (1948).

70. Park, T. Beetles, competition, and population. Science, 138: 1369-1375 (1962).

71. Zyromska-Rudzka, H. Abundance and emigrations of in the laboratory model Ekol. Pol. A, 14: 491-518. (1966).

72. Khan, A.R, and Selman, B.J. Some technique for minimizing the var. 93: 36-37 (1981).

73. Good, N. E. The flour beetle of the genus. Tech Bull. U.S. Dept. Agric, 498:1-57 (1936).

74. Good, N.E. Biology of the flour beetles *Tribolium confusum* Duv., and *T. ferruginaeum* Fab. *J. Agric. Res*. 46: 327-334. (1933).

75. Park, T. Studies in population physiology. IV. Some physiological effects of conditioned flour upon *Tribolium confusum* Duv. And its population. *Physiol. Zool.,* 8: 91-115. (1935).

76. Abbott, W.S. A method of computing the effectiveness of an insecticide, *J. Econ. Entomol.* 18: 265-267. (1925).

77. Busvine, J.R. A critical review of the techniques for testing insecticides: Commonwealth Agricultural Buereux: London. 345 5.03 5.786654.

78. Rosa LH, Machado KMG, Jacob CC, Capelkari M, Rosa CA, Zani CL. (2003). Screening of Brazillian basidiomycetes for antimicrobial activity. *Mem. Inst. Oswaldo. Cruz*, 98: 1-19.

79. Kavanagh F, Hervey A, Robbins WJ (1950). Antibiotic substances from basidiomycetes. VI. *Agrocybe dura.* Proc. Natl. Acad. Sci. USA 36: 102-106.

80. Mavoungou H, Porte M, Oddoux L (1987). Active antitumoraled des myceliums d'Agrocybe dura, Mycoacia uda et Phanerochaete laevis. Ann. Pharmaceutic. Francaises, 45: 71-77.

81. Kuznetsov OIU, Mil'kova EV, Sosnina AE, Sotnikova NIU (2005). Antimicrobial action of *Lentinus edodes* juice on human microflora Zh Mikrobiol. Epidemiol. Immunobiol. 1:80-82.

82. Sarkar, S., Pranava, M., Marita, R. A., 1996. Demonstration of the hypoglycemic action of *Momordica charantia* in a validated animal model of diabetics. Pharmacol. Res. 33(1), 1-4.

83. Tomlinson KC, Gardiner SM, Hebden RA, Bennett T. Functional consequences of streptozotocin-induced diabetes mellitus, with particular reference to the cardiovascular system. *Pharmacol. Rev.* 1992; 44: 103-50.

84. Aybar, M., Sanchez Riera, A. N., Grau, A., Sanchez, S. S., 2002. Hypoglycemic effect of the water extract of *Smallanthus*

soncifolius (yacon) leaves in normal and diabetic rats. *J. Ethnopharmacol.* 74:125-132.

85. Gomes, A., Vedasiromoni, J. R., Das, M., Sharma, R. M., Ganguly, D. K., 2001. Anti-hyperglycaemic effect of black tea (*Camellia sinensis*) in rat. *J. Enthnopharmacol*. 27:243-275.

86. Mitra SK, Gopumadhavan S, Muralidhar TS, Anturlikar SD, Sujatha MB. Effect of D-400, a herbomineral preparation on lipid profile, glycated hemoglobin and glucose tolerance in streptozotocin induced diabetes in rats. *Indian J. Exp. Biol.* 1995; 33: 798-800.

87. Al-Shamaony, L., Al-khazrajoi, S. M., Twaij, H. A. A., 1994. Hypoglycaemic effect of *Artemisia herba alba.* II. Effect of a valuable extract on some blood parameters in diabetic animals. J. Ethnopharmacol. 43: 167-171.

88. Shirwaikar, A., Rajendran, K., Kumar, C. D., Bodla, R., 2004. Antidiabetic activity of aqueous leaf extract of *Annona squamosa* in streptozotocin-nicotinamide type 2 diabetic rats. J. Ethnopharmacol. 91:171-175.

89. Bopanna KN, Kannan J, Sushma G, Balaraman R, Rathod SP. Antidiabetic and antihyperlipidemic effects of neem seed kernal powder on alloxan diabetic rabbits. Indian J. Pharmacol. 1997; 29:162-7.

90. Seifter S, England S. Energy metabolism. In: Arias I, Popper H, Schacter D et al. (eds). *The Liver: Biology and Pathophysiology.* Raune Press, New York. 1982; 219-49.

91. Ohaeri, O. C., 2001. Effect of garlic oil on the levels of various enzyme in the serum and tissue of streptozotocin diabetic rats. Biosci. Repord. 21: 19-24.

92. Narvarro, C. M., Montilla, P. M., Martin, A., Jimenez, J., Utrilla, P. M., 1993. Free radicals scavenger and antihepatotoxic activity of Rosmarinus. Plant Med. 59:312-314.

93. Sicor Pharmaceuticals, 2003. Material Safety Dats Sheet. Sicor Pharmaceuticals Inc. Irvine CA.

94. Alderson, N. L., M. E. Chachick, N. Frizzell, P. Canning, T. O. Metz and A. S. Januszewski, 2004. Effect of antioxidants and ACE inhibition on chemical modification of proteins and progression of nephropathy in streptozotocin diabetic rat. Diabetologia, 47: 1385-1395.

95. Kedar, P. and C. H. Chakrabarti, 1983. Effects of Jambolan seed treatment on blood sugar, lipids and urea in streptozotocin induced diabetes in rabbits. Indian J. Pharmacol., 27:135-40.

96. Tripathi, A. K., Prajapati, V., Aggarwal, K. K. and Kumer, S. 2001. Toxicity, feeding, deterrence and effect of activity of 1, 8-Cineole from *Artemisia annua* on progeny production of *Tribolium castaneum* (Coleoptera: Tenebrionidae). *J. Economic Entomology* 94:979-983.

97. Metcalf, C. L. and Flint, W. P. 1962. *Destructive and useful insects.* McGraw-Hill Publishing, New York, 1087pp.

98. Purthi, H. S. and Singh, M. 1950. Pest of stored grains and their control. *Indian J. Agric. Sci.* 18:1-88.

99. Via, S. 1999. Cannabolism facilitates the use of a novel environment in the flour beetle, *Tribolium castaneum*. *Heredity.,* 82:267-275.

100. Weston, P. A. and Rattingourd, P. L. 2000. Weston, P. A. and Rattingourd, P. L. 2000. Progeny production by *Tribolium castaneum* (Coleoptera: Tenebrionidae) and *Oryzaephilus surinamensis* (Coleoptera: Silvanidae) on maize previously infested by *Sitotroga cerealla* (Lepidoptera: Gelechiidae). *J. Ecnomic Entomology*., 93:533-536.

101. Bousquet, Y. 1990. *Beetles associated with stored products in Canada*. Canadian Government Publishing Centre, Ottawa. pp. 189-192.

102. Talukder, F.A. and Howse, P.E., Evaluation of *Aphanamixis polystachya* as a source of repellents, antifeedants, toxicants and protectants in storage against *Tribolium castameum* (Herbst), *J. Stor. Prod. Res.,* 31, 55-61, 1995.

103. Islam, H., Farhana, K. and Islam, N. 2004. Screening of species for biological activity against *Tribolium castaneum* (Herbst) adults. *Univ. J. Zool. Rajshahi Univ.* 23:65-68.

104. Gray AM, Flatt PR. 1998. Insulin-releasing and insulin-like activity of *Agaricus compestris* (mushroom). *J Endocrinol* 157: 259-266.

105. Hikino H, Ishiyama M, Suzuki Y, Konno C. 1989. Mechanisms of hypoglycemic activity of ganoderan B: a glycan of *Gonoderma Iucidum* fruit bodies. *Planta Med* 55: 423-428.

106. Kim DH, Yang BK, Jeong SC *et al*. 2001a. Production of a hypoglycemic, extracellular polysaccharide from the sub-merged culture of the mushroom, *Phellinus linteus. Biotechnol Lett* 23: 513-517.

107. Byung *et al*. (2007). Phytotherapy Research, Phytother, Res. 21, 1066-1069.

108. Simon *et al.* (2010). Journal of Herbal Medicine and Toxicology 4(2): 101-106.

109. Sumy *et al* (2010). Bangladesh Soc Physiol. December, 5(2): 46-52.

APPENDICES TABLE

Appendix Table-I: Dose-mortality effect of methanol extracts of *Pleurotus ostreatus* against *T. castaneum* after 12h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1.020	2.009	30	20	66.667	67	5.44	5.589	5.416	17.43	5.575
0.510	1.708	30	16	53.333	53	5.08	5.062	5.075	19.11	5.046
0.250	1.398	30	13	43.333	43	4.82	4.520	4.824	17.43	4.502
0.130	1.114	30	4	13.333	13	3.87	4.023	3.873	13.17	4.003
0.060	0.778	30	1	3.333	3	3.12	3.435	3.180	7.14	3.414

Y =2.046704+1.756587X

CHI-SQUARED IS 2.873566 WITH 3 DEGREES OF FREEDOM

NO SIG HETEROGENEITY

LOG LD-50 IS 1.68127

LD-50 IS.4800317

95% CONF LIMITS ARE.3466482 TO.6647386

Appendix Table-II: Dose-mortality effect of methanol extracts of *Pleurotus ostreatus* against *T. castaneum* after 24h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
0.250	1.398	30	27	90.000	90	6.28	6.083	6.210	13.17	5.999
0.130	1.114	30	10	33.333	33	4.56	4.924	4.565	19.02	4.835
0.060	0.778	30	3	10.000	10	3.72	3.553	3.750	8.07	3.459

Y =.2691331+4.09867X

CHI-SQUARED IS 2.657753 WITH 1 DEGREES OF FREEDOM

NO SIG HETEROGENEITY

LOG LD-50 IS 1.154245

LD-50 IS.1426411

95% CONF LIMITS ARE.1198699 TO.169738

Appendix Table-III: Dose-mortality effect of methanol extracts of *Pleurotus ostreatus* against *T. castaneum* after 36h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
0.250	1.398	30	28	93.333	93	6.48	6.467	6.491	9.06	6.463
0.130	1.114	30	19	63.333	63	5.33	5.354	5.318	18.48	5.344
0.060	0.778	30	5	16.667	17	4.05	4.039	4.037	13.17	4.021

Y =.9542813+3.940344X
CHI-SQUARED IS.022995 WITH 1 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 1.026742
LD-50 IS.1063512
95% CONF LIMITS ARE.0886122 TO .1276413

Appendix Table-IV: Dose-mortality effect of methanol extracts of *Pleurotus ostreatus* against *T. castaneum* after 48h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
0.250	1.398	30	29	96.667	97	6.88	6.552	6.759	8.07	6.544
0.130	1.114	30	21	70.000	70	5.52	5.612	5.520	16.74	5.608
0.060	0.778	30	8	26.667	27	4.39	4.501	4.376	17.43	4.502
0.030	0.477	30	3	10.000	10	3.72	3.505	3.750	8.07	3.510

Y = 1.938714+3.294042X
CHI-SQUARED IS1.244274 WITH 2 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS.9293405
LD-50 IS 8.498466E-02
95% CONF LIMITS ARE 7.004655E-02 TO.1031084

Appendix Table-V: Dose-mortality effect of ethyl acetate extracts of *Pleurotus ostreatus* against *T. castaneum* after 12h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
4.580	0.661	30	12	40.000	40	4.75	4.748	4.740	18.48	4.727
4.070	0.610	30	9	30.000	30	4.48	4.581	4.460	17.43	4.568
3.560	0.551	30	9	30.000	30	4.48	4.392	4.490	15.96	4.389
3.060	0.486	30	6	20.000	20	4.16	4.178	4.170	14.13	4.186
2.580	0.412	30	5	16.667	17	4.05	3.936	4.062	12.15	3.957
2.040	0.310	30	2	6.667	7	3.52	3.604	3.529	9.06	3.642

Y =2.685356+3.089111X
CHI-SQUARED IS.6246195 WITH 4 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS.7492914
LD-50 IS 5.614245
95% CONF LIMITS ARE 3.974293 TO 7.930907

Appendix Table-VI: Dose-mortality effect of ethyl acetate extracts of *Pleurotus ostreatus* against *T. castaneum* after 24h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
4.580	0.661	30	23	76.667	77	5.74	5.630	5.730	16.74	5.619
4.070	0.610	30	19	63.333	63	5.33	5.401	5.321	18.03	5.392
3.560	0.551	30	15	50.000	50	5.00	5.142	4.990	19.02	5.133
3.060	0.486	30	14	46.667	47	4.92	4.848	4.942	18.81	4.841
2.580	0.412	30	10	33.333	33	4.56	4.517	4.544	17.43	4.512
2.040	0.310	30	5	16.667	17	4.05	4.062	4.037	13.17	4.060

Y =2.684786+ 4.440258X
CHI-SQUARED IS.8998776 WITH 4 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS.5214143
LD-50 IS 3.322112
95% CONF LIMITS ARE 3.004951 TO 3.672748

Appendix Table-VII: Dose-mortality effect of ethyl acetate extracts of *Pleurotus ostreatus* against *T. castaneum* after 36h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
4.580	0.661	30	29	96.667	97	6.88	6.544	6.759	8.07	6.528
4.070	0.610	30	28	93.333	93	6.48	6.244	6.383	11.10	6.231
3.560	0.551	30	25	83.333	83	5.95	5.904	5.984	14.13	5.894
3.060	0.486	30	17	56.667	57	5.18	5.519	5.136	17.43	5.513
2.580	0.412	30	14	46.667	47	4.92	5.085	4.925	19.11	5.084
2.040	0.310	30	12	40.000	40	4.75	4.488	4.780	16.74	4.493

Y = 2.699999 + 5.792284X
CHI-SQUARED IS 5.143326 WITH 4 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS .3970803
LD-50 IS 2.495056
95% CONF LIMITS ARE 2.250366 TO 2.766352

Appendix Table-VIII: Dose-mortality effect of ethyl acetate extracts of *Pleurotus ostreatus* against *T. castaneum* after 48h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
3.560	0.551	30	26	86.667	87	6.13	5.874	6.038	15.09	5.776
3.060	0.486	30	19	63.333	63	5.33	5.590	5.304	17.43	5.532
2.550	0.407	30	16	53.333	53	5.08	5.248	5.098	18.81	5.238
2.040	0.310	30	15	50.000	50	5.00	4.829	5.020	18.81	4.879

Y = 3.729276 + 3.711713X
CHI-SQUARED IS 2.688522 WITH 2 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS .3423552
LD-50 IS 2.199658
95% CONF LIMITS ARE 1.791917 TO 2.700178

Appendix Table-IX: Dose-mortality effect of chloroform extracts of *Pleurotus ostreatus* against *T. castaneum* after 12h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1.530	1.185	30	25	83.333	83	5.95	5.470	5.861	18.03	5.468
1.020	1.009	30	11	36.667	37	4.67	5.032	4.675	19.11	5.030
0.510	0.708	30	5	16.667	17	4.05	4.285	4.048	15.09	4.280
0.250	0.398	30	3	10.000	10	3.72	3.516	3.750	8.07	3.510
0.130	0.114	30	1	3.333	3	3.12	2.810	3.256	2.76	2.803

Y = 2.519185 + 2.48893X
CHI-SQUARED IS 7.036492 WITH 3 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS .9967398
LD-50 IS .9925211
95% CONF LIMITS ARE .7751471 TO 1.270853

Appendix Table-X: Dose-mortality effect of chloroform extracts of *Pleurotus ostreatus* against *T. castaneum* after 24h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1.020	1.009	30	17	56.667	55	5.13	5.061	5.125	19.11	5.076
0.510	0.708	30	8	26.667	24	4.29	4.397	4.298	15.96	4.394
0.250	0.398	30	4	13.333	10	3.72	3.714	3.720	10.08	3.693
0.130	0.114	30	2	6.667	3	3.12	3.088	3.135	3.93	3.050

Y = 2.792087 + 2.264505 X
CHI-SQUARED IS .2296562 WITH 2 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS .9750092
LD-50 IS .9440809
95% CONF LIMITS ARE .6430875 TO 1.385953

Appendix Table-XI: Dose-mortality effect of chloroform extracts of *Pleurotus ostreatus* against *T. castaneum* after 36h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1.020	1.009	30	20	66.667	66	5.41	5.294	5.436	18.81	5.324
0.510	0.708	30	10	33.333	31	4.50	4.678	4.497	18.03	4.680
0.250	0.398	30	6	20.000	17	4.05	4.045	4.037	13.17	4.017
0.130	0.114	30	3	10.000	7	3.52	3.464	3.540	7.14	3.409

Y = 3.16562 + 2.140059X
CHI-SQUARED IS .9654159 WITH 2 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS .8571632
LD-50 IS .7197195
95% CONF LIMITS ARE .5167968 TO 1.002321

Appendix Table-XII: Dose-mortality effect of chloroform extracts of *Pleurotus ostreatus* against *T. castaneum* after 48h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1.020	2.009	30	25	83.333	83	5.95	5.817	5.902	15.09	5.786
0.510	1.708	30	16	53.333	52	5.05	5.147	5.040	19.02	5.116
0.250	1.398	30	8	26.667	24	4.29	4.459	4.300	16.74	4.426
0.130	1.114	30	5	16.667	14	3.92	3.827	3.924	11.10	3.794
0.060	0.778	30	2	6.667	3	3.12	3.080	3.135	3.93	3.046

Y = 1.313677 + 2.226458X
CHI-SQUARED IS .7978592 WITH 3 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 1.65569
LD-50 IS .4525743
95% CONF LIMITS ARE .3482986 TO .5880687

Appendix Table-XIII: Dose-mortality effect of pet-ether extracts of *Pleurotus ostreatus* against *T. castaneum* after 12h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
4.075	0.610	30	22	73.333	73	5.61	5.381	5.578	18.48	5.379
3.565	0.552	30	12	40.000	40	4.75	4.880	4.760	18.81	4.880
3.056	0.485	30	5	16.667	17	4.05	4.303	4.074	15.96	4.306
2.547	0.406	30	3	10.000	10	3.72	3.621	3.730	9.06	3.626
2.037	0.309	30	1	3.333	3	3.12	2.784	3.379	2.28	2.793

Y = .1409173 + 8.584647X
CHI-SQUARED IS 2.742212 WITH 3 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS .5660201
LD-50 IS 3.681461
95% CONF LIMITS ARE 3.417619 TO 3.965671

Appendix Table-XIV: Dose-mortality effect of pet-ether extracts of *Pleurotus ostreatus* against *T. castaneum* after 24h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
4.075	0.610	30	26	86.667	87	6.13	5.770	6.054	15.96	5.770
3.565	0.552	30	18	60.000	60	5.25	5.331	5.240	18.48	5.329
3.056	0.485	30	11	36.667	37	4.67	4.825	4.682	18.81	4.822
2.547	0.406	30	5	16.667	17	4.05	4.227	4.048	15.09	4.222
2.037	0.309	30	2	6.667	7	3.52	3.493	3.540	7.14	3.486
1.528	0.184	30	1	3.333	3	3.12	2.548	3.860	1.50	2.539

Y = 1.142797 + 7.583476X
CHI-SQUARED IS 4.900364 WITH 4 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS .5086326
LD-50 IS 3.225764
95% CONF LIMITS ARE 3.010537 TO 3.456377

Appendix Table-XV: Dose-mortality effect of pet-ether extracts of *Pleurotus ostreatus* against *T. castaneum* after 36h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
3.565	0.552	30	19	63.333	63	5.33	5.196	5.315	19.02	5.193
3.056	0.485	30	12	40.000	40	4.75	4.774	4.740	18.48	4.772
2.547	0.406	30	5	16.667	17	4.05	4.275	4.048	15.09	4.273
2.037	0.309	30	3	10.000	10	3.72	3.664	3.730	9.06	3.662
1.528	0.184	30	1	3.333	3	3.12	2.877	3.256	2.76	2.876

Y = 1.716099 + 6.298147X
CHI-SQUARED IS 1.508343 WITH 3 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS .5214075
LD-50 IS 3.32206
95% CONF LIMITS ARE 2.973128 TO 3.711943

Appendix Table-XVI: Dose-mortality effect of pet-ether extracts of *Pleurotus ostreatus* against *T. castaneum* after 48h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
3.565	0.552	30	26	86.667	87	6.13	5.877	6.038	15.09	5.859
3.056	0.485	30	19	63.333	63	5.33	5.401	5.321	18.03	5.368
2.547	0.406	30	11	36.667	37	4.67	4.838	4.682	18.81	4.787
2.037	0.309	30	4	13.333	13	3.87	4.147	3.904	14.13	4.075
1.528	0.184	30	2	6.667	7	3.52	3.258	3.629	5.40	3.159

Y = 1.808097 + 7.33735X
CHI-SQUARED IS 2.34021 WITH 3 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS .4350212
LD-50 IS 2.722834
95% CONF LIMITS ARE 2.530149 TO 2.930195

Appendix Table-XVII: Dose-mortality effect of hot water extracts of *Pleurotus ostreatus* against *T. castaneum* after 12h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
4.075	0.610	30	20	66.667	67	5.44	5.284	5.462	18.81	5.285
3.565	0.552	30	11	36.667	37	4.67	4.787	4.662	18.48	4.786
3.056	0.485	30	5	16.667	17	4.05	4.214	4.048	15.09	4.212
2.547	0.406	30	2	6.667	7	3.52	3.537	3.519	8.07	3.533
2.037	0.309	30	1	3.333	3	3.12	2.707	3.379	2.28	2.700

Y = 4.884529E-02 + 8.581378X
CHI-SQUARED IS 2.335819 WITH 3 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS .576965
LD-50 IS 3.775418
95% CONF LIMITS ARE 3.488467 TO 4.085971

Appendix Table-XVIII: Dose-mortality effect of hot water extracts of *Pleurotus ostreatus* against *T. castaneum* after 24h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
4.075	0.610	30	23	76.667	77	5.74	5.508	5.696	17.43	5.502
3.565	0.552	30	16	53.333	53	5.08	5.124	5.065	19.02	5.121
3.056	0.485	30	10	33.333	33	4.56	4.682	4.551	18.03	4.682
2.547	0.406	30	5	16.667	17	4.05	4.159	4.056	14.13	4.163
2.037	0.309	30	2	6.667	7	3.52	3.518	3.519	8.07	3.526
1.528	0.184	30	1	3.333	3	3.12	2.692	3.568	1.86	2.707

Y = 1.49928 + 6.56003X
CHI-SQUARED IS 2.565769 WITH 4 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS .533644
LD-50 IS 3.416992
95% CONF LIMITS ARE 3.141078 TO 3.717143

Appendix Table-XIX: Dose-mortality effect of hot water extracts of *Pleurotus ostreatus* against *T. castaneum* after 36h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
4.075	0.610	30	28	93.333	93	6.48	5.908	6.364	14.13	5.906
3.565	0.552	30	20	66.667	67	5.44	5.519	5.416	17.43	5.519
3.056	0.485	30	13	43.333	43	4.82	5.070	4.825	19.11	5.072
2.547	0.406	30	8	26.667	27	4.39	4.539	4.376	17.43	4.543
2.037	0.309	30	4	13.333	13	3.87	3.889	3.873	11.10	3.895
1.528	0.184	30	2	6.667	7	3.52	3.051	3.875	3.93	3.061

Y = 1.831614 + 6.678579X
CHI-SQUARED IS 7.401268 WITH 4 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS .4744103
LD-50 IS 2.981332
95% CONF LIMITS ARE 2.76781 TO 3.211325

Appendix Table-XX: Dose-mortality effect of hot water extracts of *Pleurotus ostreatus* against *T. castaneum* after 48h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
3.565	0.552	30	24	80.000	80	5.85	5.681	5.820	16.74	5.688
3.056	0.485	30	17	56.667	57	5.18	5.264	5.202	18.81	5.259
2.547	0.406	30	11	36.667	37	4.67	4.771	4.662	18.48	4.751
2.037	0.309	30	5	16.667	17	4.05	4.166	4.056	14.13	4.129
1.528	0.184	30	2	6.667	7	3.52	3.388	3.572	6.24	3.328

Y = 2.146533 + 6.414751X
CHI-SQUARED IS .9467926 WITH 3 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS .4448291
LD-50 IS 2.785025
95% CONF LIMITS ARE 2.562344 TO 3.027057

Appendix Table-XXI: Dose-mortality effect of methanol extract of *Pleurotus ostreatus* against mosquito larvae after 12h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1500.000	3.176	30	4	13.333	13	3.87	3.988	3.878	12.15	3.976
1200.000	3.079	30	4	13.333	13	3.87	3.709	3.894	10.08	3.721
1000.000	3.000	30	2	6.667	7	3.52	3.481	3.540	7.14	3.513
800.000	2.903	30	1	3.333	3	3.12	3.202	3.121	5.40	3.258

Y = -4.372944 + 2.6287X
CHI-SQUARED IS .5245323 WITH 2 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 3.565593
LD-50 IS 3677.837
95% CONF LIMITS ARE 798.8976 TO 16931.45

Appendix Table-XXII: Dose-mortality effect of methanol extract of *Pleurotus ostreatus* against mosquito larvae after 18h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1500.000	3.176	30	11	36.667	37	4.67	4.645	4.659	18.03	4.683
1200.000	3.079	30	9	30.000	30	4.48	4.298	4.490	15.09	4.315
1000.000	3.000	30	4	13.333	13	3.87	4.014	3.873	13.17	4.014
800.000	2.903	30	2	6.667	7	3.52	3.667	3.529	9.06	3.647
600.000	2.778	30	1	3.333	3	3.12	3.220	3.121	5.40	3.172
500.000	2.699	30	1	3.333	3	3.12	2.936	3.172	3.30	2.872

Y = -7.372587 + 3.795699X
CHI-SQUARED IS 1.173012 WITH 4 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 3.259633
LD-50 IS 1818.165
95% CONF LIMITS ARE 1357.219 TO 2435.657

Appendix Table-XXIII: Dose-mortality effect of methanol extract of *Pleurotus ostreatus* against mosquito larvae after 24h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1500.000	3.176	30	18	60.000	60	5.25	5.280	5.280	18.81	5.308
1200.000	3.079	30	14	46.667	47	4.92	4.895	4.942	18.81	4.917
1000.000	3.000	30	11	36.667	37	4.67	4.581	4.656	17.43	4.598
800.000	2.903	30	6	20.000	20	4.16	4.196	4.170	14.13	4.208
600.000	2.778	30	2	6.667	7	3.52	3.701	3.546	10.08	3.704
500.000	2.699	30	2	6.667	7	3.52	3.386	3.572	6.24	3.385

Y = -7.490371 + 4.029519X
CHI-SQUARED IS .572939 WITH 4 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 3.099718
LD-50 IS 1258.106
95% CONF LIMITS ARE 1083.315 TO 1461.099

Appendix Table-XXIV: Dose-mortality effect of methanol extract of *Pleurotus ostreatus* against mosquito larvae after 30h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1500.000	3.176	30	25	83.333	83	5.95	5.959	5.984	14.13	5.981
1200.000	3.079	30	21	70.000	70	5.52	5.568	5.500	17.43	5.583
1000.000	3.000	30	20	66.667	67	5.44	5.248	5.462	18.81	5.258
800.000	2.903	30	11	36.667	37	4.67	4.857	4.682	18.81	4.860
600.000	2.778	30	8	26.667	27	4.39	4.354	4.394	15.96	4.348
500.000	2.699	30	5	16.667	17	4.05	4.034	4.037	13.17	4.023

Y = -7.051232 + 4.103159X
CHI-SQUARED IS 1.537937 WITH 4 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 2.937062
LD-50 IS 865.0916
95% CONF LIMITS ARE 774.1616 TO 966.7013

Appendix Table-XXV: Dose-mortality effect of ethyl acetate extract of *Pleurotus ostreatus* against mosquito larvae after 12h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1000.000	3.000	30	4	13.333	13	3.87	3.936	3.878	12.15	3.926
800.000	2.903	30	3	10.000	10	3.72	3.602	3.730	9.06	3.615
600.000	2.778	30	1	3.333	3	3.12	3.172	3.116	4.62	3.214

Y = -5.701108 + 3.209142X
CHI-SQUARED IS .1920961 WITH 1 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 3.33457
LD-50 IS 2160.578
95% CONF LIMITS ARE 527.0722 TO 8856.671

Appendix Table-XXVI: Dose-mortality effect of ethyl acetate extract of *Pleurotus ostreatus* against mosquito larvae after 18h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1000.000	3.000	30	8	26.667	27	4.39	4.352	4.394	15.96	4.371
800.000	2.903	30	6	20.000	20	4.16	4.133	4.170	14.13	4.144
600.000	2.778	30	3	10.000	10	3.72	3.851	3.720	11.10	3.851
400.000	2.602	30	2	6.667	7	3.52	3.453	3.540	7.14	3.439

Y = -2.657347 + 2.342816X
CHI-SQUARED IS .282269 WITH 2 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 3.268438
LD-50 IS 1855.401
95% CONF LIMITS ARE 772.7275 TO 4455.01

Appendix Table-XXVII: Dose-mortality effect of ethyl acetate extract of *Pleurotus ostreatus* against mosquito larvae after 24h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1000.000	3.000	30	14	46.667	47	4.92	4.964	4.915	19.02	4.959
800.000	2.903	30	11	36.667	37	4.67	4.744	4.662	18.48	4.737
600.000	2.778	30	9	30.000	30	4.48	4.461	4.480	16.74	4.451
400.000	2.602	30	8	26.667	27	4.39	4.061	4.447	13.17	4.048
300.000	2.477	30	2	6.667	7	3.52	3.778	3.546	10.08	3.762
200.000	2.301	30	1	3.333	3	3.12	3.378	3.148	6.24	3.359

Y = -1.907974 + 2.289075X
CHI-SQUARED IS 2.999272 WITH 4 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 3.017802
LD-50 IS 1041.842
95% CONF LIMITS ARE 743.3703 TO 1460.153

Appendix Table-XXVIII: Dose-mortality effect of ethyl acetate extract of *Pleurotus ostreatus* against mosquito larvae after 30h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
%1000.000	3.000	30	17	56.667	57	5.18	5.310	5.162	18.48	5.313
800.000	2.903	30	15	50.000	50	5.00	5.064	5.000	19.11	5.066
600.000	2.778	30	14	46.667	47	4.92	4.747	4.922	18.48	4.747
400.000	2.602	30	10	33.333	33	4.56	4.301	4.586	15.96	4.297
300.000	2.477	30	4	13.333	13	3.87	3.984	3.878	12.15	3.978
200.000	2.301	30	1	3.333	3	3.12	3.538	3.211	8.07	3.529

Y = -2.347029 + 2.553522X
CHI-SQUARED IS 3.339396 WITH 4 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 2.877214
LD-50 IS 753.7266
95% CONF LIMITS ARE 605.9258 TO 937.5799

Appendix Table-XXIX: Dose-mortality effect of chloroform extract of *Pleurotus ostreatus* against mosquito larvae after 6h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
600.000	2.778	30	3	10.000	10	3.72	3.746	3.720	10.08	3.743
500.000	2.699	30	2	6.667	7	3.52	3.474	3.540	7.14	3.480
400.000	2.602	30	1	3.333	3	3.12	3.141	3.116	4.62	3.158

Y = -5.497559 + 3.32633X
CHI-SQUARED IS 3.923726E-02 WITH 1 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 3.155898
LD-50 IS 1431.852
95% CONF LIMITS ARE 213.0851 TO 9621.512

Appendix Table-XXX: Dose-mortality effect of chloroform extract of *Pleurotus ostreatus* against mosquito larvae after 12h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
600.000	2.778	30	7	23.333	23	4.26	4.313	4.266	15.96	4.304
500.000	2.699	30	6	20.000	20	4.16	4.127	4.170	14.13	4.126
400.000	2.602	30	4	13.333	13	3.87	3.898	3.873	11.10	3.909
300.000	2.477	30	3	10.000	10	3.72	3.604	3.730	9.06	3.628
200.000	2.301	30	1	3.333	3	3.12	3.189	3.116	4.62	3.233

Y = -1.934287 + 2.24552X
CHI-SQUARED IS .2209168 WITH 3 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 3.088054
LD-50 IS 1224.768
95% CONF LIMITS ARE 497.5709 TO 3014.761

Appendix Table-XXXI: Dose-mortality effect of chloroform extract of *Pleurotus ostreatus* against mosquito larvae after 18h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
600.000	2.778	30	10	33.333	33	4.56	4.657	4.551	18.03	4.634
500.000	2.699	30	9	30.000	30	4.48	4.482	4.480	16.74	4.470
400.000	2.602	30	8	26.667	27	4.39	4.268	4.388	15.09	4.270
300.000	2.477	30	5	16.667	17	4.05	3.991	4.062	12.15	4.011
200.000	2.301	30	2	6.667	7	3.52	3.602	3.529	9.06	3.647

Y = -1.108859 + 2.067073X
CHI-SQUARED IS .4943261 WITH 3 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 2.955319
LD-50 IS 902.2329
95% CONF LIMITS ARE 480.3162 TO 1694.765

Appendix Table-XXXII: Dose-mortality effect of chloroform extract of *Pleurotus ostreatus* against mosquito larvae after 24h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
600.000	2.778	30	14	46.667	47	4.92	5.002	4.925	19.11	4.974
500.000	2.699	30	12	40.000	40	4.75	4.822	4.760	18.81	4.807
400.000	2.602	30	11	36.667	37	4.67	4.602	4.659	18.03	4.603
300.000	2.477	30	8	26.667	27	4.39	4.319	4.394	15.96	4.340
200.000	2.301	30	5	16.667	17	4.05	3.919	4.062	12.15	3.969
100.000	2.000	30	1	3.333	3	3.12	3.236	3.121	5.40	3.336

Y = -.8759308 + 2.10575X
CHI-SQUARED IS .5430508 WITH 4 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 2.790423
LD-50 IS 617.1951
95% CONF LIMITS ARE 440.8318 TO 864.1154

Appendix Table-XXXIII: Dose-mortality effect of chloroform extract of *Pleurotus ostreatus* against mosquito larvae after 30h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
600.000	2.778	30	20	66.667	67	5.44	5.319	5.422	18.48	5.284
500.000	2.699	30	15	50.000	50	5.00	5.148	4.990	19.02	5.124
400.000	2.602	30	13	43.333	43	4.82	4.939	4.815	19.02	4.928
300.000	2.477	30	11	36.667	37	4.67	4.670	4.659	18.03	4.676
200.000	2.301	30	10	33.333	33	4.56	4.291	4.592	15.09	4.320
100.000	2.000	30	2	6.667	7	3.52	3.643	3.529	9.06	3.712

Y = -.3296366 + 2.020651X
CHI-SQUARED IS 2.361324 WITH 4 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 2.637584
LD-50 IS 434.0937
95% CONF LIMITS ARE 338.5428 TO 556.6131

Appendix Table-XXXIV: Dose-mortality effect of pet-ether extract of *Pleurotus ostreatus* against mosquito larvae after 6h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
800.000	2.903	30	2	6.667	7	3.52	3.584	3.519	8.07	3.581
600.000	2.778	30	2	6.667	7	3.52	3.410	3.540	7.14	3.420
400.000	2.602	30	1	3.333	3	3.12	3.166	3.116	4.62	3.193

Y = -.1623535 + 1.28949 X
CHI-SQUARED IS .1612548 WITH 1 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 4.003407
LD-50 IS 10078.75
95% CONF LIMITS ARE 2.592201 TO 3.918725E+07

Appendix Table-XXXV: Dose-mortality effect of pet-ether extract of *Pleurotus ostreatus* against mosquito larvae after 12h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
800.000	2.903	30	8	26.667	27	4.39	4.315	4.394	15.96	4.338
600.000	2.778	30	5	16.667	17	4.05	4.060	4.037	13.17	4.072
400.000	2.602	30	2	6.667	7	3.52	3.699	3.529	9.06	3.697
300.000	2.477	30	2	6.667	7	3.52	3.443	3.540	7.14	3.431
200.000	2.301	30	1	3.333	3	3.12	3.083	3.135	3.93	3.057

Y = -1.8395 + 2.12783X
CHI-SQUARED IS .431263 WITH 3 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 3.214317
LD-50 IS 1638.01
95% CONF LIMITS ARE 693.3436 TO 3869.769

Appendix Table-XXXVI: Dose-mortality effect of pet-ether extract of *Pleurotus ostreatus* against mosquito larvae after 18h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
800.000	2.903	30	12	40.000	40	4.75	4.770	4.740	18.48	4.733
600.000	2.778	30	8	26.667	27	4.39	4.455	4.390	16.74	4.441
400.000	2.602	30	5	16.667	17	4.05	4.010	4.037	13.17	4.030
300.000	2.477	30	4	13.333	13	3.87	3.695	3.931	9.06	3.738
200.000	2.301	30	1	3.333	3	3.12	3.250	3.121	5.40	3.326

Y = -2.05236 + 2.337455X
CHI-SQUARED IS .6110211 WITH 3 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 3.01711
LD-50 IS 1040.185
95% CONF LIMITS ARE 644.496 TO 1678.806

Appendix Table-XXXVII: Dose-mortality effect of pet-ether extract of *Pleurotus ostreatus* against mosquito larvae after 24h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
800.000	2.903	30	14	46.667	47	4.92	4.949	4.915	19.02	4.932
600.000	2.778	30	11	36.667	37	4.67	4.699	4.659	18.03	4.689
400.000	2.602	30	8	26.667	27	4.39	4.347	4.394	15.96	4.346
300.000	2.477	30	6	20.000	20	4.16	4.097	4.160	13.17	4.103
200.000	2.301	30	3	10.000	10	3.72	3.745	3.720	10.08	3.760
100.000	2.000	30	1	3.333	3	3.12	3.143	3.116	4.62	3.174

Y = -.7177735 + 1.946031 X

CHI-SQUARED IS .1328716 WITH 4 DEGREES OF FREEDOM

NO SIG HETEROGENEITY

LOG LD-50 IS 2.938172

LD-50 IS 867.3052

95% CONF LIMITS ARE 571.2132 TO 1316.879

Appendix Table-XXXVIII: Dose-mortality effect of pet-ether extract of *Pleurotus ostreatus* against mosquito larvae after 30h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
800.000	2.903	30	15	50.000	48	4.95	5.004	4.950	19.11	5.005
600.000	2.778	30	13	43.333	41	4.77	4.777	4.766	18.48	4.777
400.000	2.602	30	9	30.000	28	4.42	4.456	4.420	16.74	4.456
300.000	2.477	30	9	30.000	28	4.42	4.228	4.422	15.09	4.228
200.000	2.301	30	5	16.667	14	3.92	3.907	3.924	12.15	3.906
100.000	2.000	30	2	6.667	3	3.12	3.358	3.148	6.24	3.357

Y = -.2935572 + 1.82516 X

CHI-SQUARED IS .9279785 WITH 4 DEGREES OF FREEDOM

NO SIG HETEROGENEITY

LOG LD-50 IS 2.900325

LD-50 IS 794.9219

95% CONF LIMITS ARE 530.2905 TO 1191.613

Appendix Table-XXXIX: Dose-mortality effect of hot water extract of *Pleurotus ostreatus* against mosquito larvae after 12h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
%2000.000	3.301	30	7	23.333	23	4.26	4.056	4.283	13.17	4.086
%1800.000	3.255	30	4	13.333	13	3.87	3.984	3.878	12.15	4.008
%1500.000	3.176	30	3	10.000	10	3.72	3.859	3.720	11.10	3.872
%1200.000	3.079	30	3	10.000	10	3.72	3.705	3.720	10.08	3.707
%1000.000	3.000	30	2	6.667	7	3.52	3.580	3.519	8.07	3.571
800.000	2.903	30	2	6.667	7	3.52	3.426	3.540	7.14	3.406

Y = -1.553868 + 1.708464X
CHI-SQUARED IS 1.126378 WITH 4 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 3.836117
LD-50 IS 6856.736
95% CONF LIMITS ARE 1181.732 TO 39784.63

Appendix Table-XXXX: Dose-mortality effect of hot water extract of *Pleurotus ostreatus* against mosquito larvae after 18h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
%2000.000	3.301	30	9	30.000	30	4.48	4.436	4.480	16.74	4.440
%1800.000	3.255	30	8	26.667	27	4.39	4.385	4.394	15.96	4.388
%1500.000	3.176	30	7	23.333	23	4.26	4.296	4.252	15.09	4.297
%1200.000	3.079	30	6	20.000	20	4.16	4.187	4.170	14.13	4.187
%1000.000	3.000	30	5	16.667	17	4.05	4.098	4.037	13.17	4.096
800.000	2.903	30	5	16.667	17	4.05	3.988	4.062	12.15	3.986

Y = .6739049 + 1.140834X
CHI-SQUARED IS .1797578 WITH 4 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 3.792045
LD-50 IS 6195.057
95% CONF LIMITS ARE 784.3489 TO 48930.7

Appendix Table-XXXXI: Dose-mortality effect of hot water extract of *Pleurotus ostreatus* against mosquito larvae after 24h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
%2000.000	3.301	30	10	33.333	31	4.50	4.509	4.488	17.43	4.504
%1800.000	3.255	30	9	30.000	28	4.42	4.459	4.420	16.74	4.455
%1500.000	3.176	30	9	30.000	28	4.42	4.373	4.426	15.96	4.371
%1200.000	3.079	30	8	26.667	24	4.29	4.268	4.286	15.09	4.269
%1000.000	3.000	30	7	23.333	21	4.19	4.183	4.208	14.13	4.185
800.000	2.903	30	6	20.000	17	4.05	4.078	4.037	13.17	4.082

Y = 1.009548 + 1.058481X
CHI-SQUARED IS .1115286 WITH 4 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 3.76998
LD-50 IS 5888.162
95% CONF LIMITS ARE 724.1831 TO 47875.28

Appendix Table-XXXXII: Dose-mortality effect of hot water extract of *Pleurotus ostreatus* against mosquito larvae after 30h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
%2000.000	3.301	30	14	46.667	45	4.87	4.865	4.890	18.81	4.871
%1800.000	3.255	30	13	43.333	41	4.77	4.794	4.766	18.48	4.799
%1500.000	3.176	30	12	40.000	38	4.69	4.672	4.686	18.03	4.673
%1200.000	3.079	30	11	36.667	34	4.59	4.522	4.572	17.43	4.520
%1000.000	3.000	30	8	26.667	24	4.29	4.399	4.298	15.96	4.395
800.000	2.903	30	8	26.667	24	4.29	4.249	4.286	15.09	4.242

Y = -.3489084 + 1.581325X
CHI-SQUARED IS .2552505 WITH 4 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 3.382549
LD-50 IS 2412.954
95% CONF LIMITS ARE 1343.762 TO 4332.87

Appendix Table-XXXXIII: Dose-mortality effect of methanol extract of *Pleurotus ostreatus* against *A. salina* nauplii after 12h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1200.000	3.079	30	4	13.333	13	3.87	3.965	3.878	12.15	3.962
1000.000	3.000	30	4	13.333	13	3.87	3.754	3.894	10.08	3.764
800.000	2.903	30	2	6.667	7	3.52	3.497	3.540	7.14	3.521
600.000	2.778	30	1	3.333	3	3.12	3.164	3.116	4.62	3.208

Y = -3.753568 + 2.505822X
CHI-SQUARED IS .298595 WITH 2 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 3.493293
LD-50 IS 3113.815
95% CONF LIMITS ARE 634.2608 TO 15286.84

Appendix Table-XXXXIV: Dose-mortality effect of methanol extract of *Pleurotus ostreatus* against *A. salina* nauplii after 18h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1200.000	3.079	30	10	33.333	33	4.56	4.524	4.544	17.43	4.510
1000.000	3.000	30	8	26.667	27	4.39	4.347	4.394	15.96	4.337
800.000	2.903	30	5	16.667	17	4.05	4.131	4.056	14.13	4.125
600.000	2.778	30	4	13.333	13	3.87	3.852	3.873	11.10	3.852
400.000	2.602	30	1	3.333	3	3.12	3.459	3.180	7.14	3.467
200.000	2.301	30	1	3.333	3	3.12	2.787	3.379	2.28	2.810

Y = -2.218423 + 2.185163X
CHI-SQUARED IS 1.473262 WITH 4 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 3.303379
LD-50 IS 2010.848
95% CONF LIMITS ARE 1102.036 TO 3669.131

Appendix Table-XXXXV: Dose-mortality effect of methanol extract of *Pleurotus ostreatus* against *A. salina* nauplii after 24h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1200.000	3.079	30	17	56.667	57	5.18	5.180	5.165	19.02	5.197
1000.000	3.000	30	16	53.333	53	5.08	4.993	5.065	19.02	5.003
800.000	2.903	30	13	43.333	43	4.82	4.764	4.818	18.48	4.765
600.000	2.778	30	9	30.000	30	4.48	4.469	4.480	16.74	4.459
400.000	2.602	30	3	10.000	10	3.72	4.053	3.750	13.17	4.027
200.000	2.301	30	2	6.667	7	3.52	3.342	3.572	6.24	3.289

Y = -2.352481 + 2.451863X

CHI-SQUARED IS 1.66316 WITH 4 DEGREES OF FREEDOM

NO SIG HETEROGENEITY

LOG LD-50 IS 2.998733

LD-50 IS 997.0863

95% CONF LIMITS ARE 791.3148 TO 1256.366

Appendix Table-XXXXVI: Dose-mortality effect of methanol extract of *Pleurotus ostreatus* against *A. salina* nauplii after 30h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1200.000	3.079	30	24	80.000	80	5.85	5.759	5.830	15.96	5.763
1000.000	3.000	30	21	70.000	70	5.52	5.549	5.500	17.43	5.549
800.000	2.903	30	20	66.667	67	5.44	5.293	5.462	18.81	5.286
600.000	2.778	30	13	43.333	43	4.82	4.962	4.815	19.02	4.948
400.000	2.602	30	7	23.333	23	4.26	4.497	4.270	16.74	4.472
200.000	2.301	30	4	13.333	13	3.87	3.700	3.894	10.08	3.657

Y = -2.571088 + 2.706638X

CHI-SQUARED IS 2.277962 WITH 4 DEGREES OF FREEDOM

NO SIG HETEROGENEITY

LOG LD-50 IS 2.79723

LD-50 IS 626.9455

95% CONF LIMITS ARE 529.6133 TO 742.1654

Appendix Table-XXXXVII: Dose-mortality effect of ethyl acetate extract of *Pleurotus ostreatus* against *A. salina* nauplii after 12h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1000.000	3.000	30	4	13.333	13	3.87	3.895	3.873	11.10	3.892
800.000	2.903	30	3	10.000	10	3.72	3.712	3.720	10.08	3.715
600.000	2.778	30	2	6.667	7	3.52	3.477	3.540	7.14	3.487
400.000	2.602	30	1	3.333	3	3.12	3.146	3.116	4.62	3.165

Y = -1.588686 + 1.826852X

CHI-SQUARED IS 3.568411E-02 WITH 2 DEGREES OF FREEDOM

NO SIG HETEROGENEITY

LOG LD-50 IS 3.60658

LD-50 IS 4041.849

95% CONF LIMITS ARE 359.787 TO 45406.1

Appendix Table-XXXXVIII: Dose-mortality effect of ethyl acetate extract of *Pleurotus ostreatus* against *A. salina* nauplii after 18h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1000.000	3.000	30	9	30.000	30	4.48	4.433	4.480	16.74	4.425
800.000	2.903	30	6	20.000	20	4.16	4.252	4.150	15.09	4.247
600.000	2.778	30	5	16.667	17	4.05	4.020	4.037	13.17	4.016
400.000	2.602	30	3	10.000	10	3.72	3.692	3.730	9.06	3.691
200.000	2.301	30	1	3.333	3	3.12	3.132	3.116	4.62	3.135

Y = -1.113092 + 1.846212X

CHI-SQUARED IS .2115779 WITH 3 DEGREES OF FREEDOM

NO SIG HETEROGENEITY

LOG LD-50 IS 3.311154

LD-50 IS 2047.168

95% CONF LIMITS ARE 862.4816 TO 4859.113

Appendix Table-XXXXIX: Dose-mortality effect of ethyl acetate extract of *Pleurotus ostreatus* against *A. salina* nauplii after 24h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1000.000	3.000	30	13	43.333	43	4.82	4.759	4.818	18.48	4.755
800.000	2.903	30	10	33.333	33	4.56	4.602	4.551	18.03	4.600
600.000	2.778	30	8	26.667	27	4.39	4.401	4.390	16.74	4.399
400.000	2.602	30	5	16.667	17	4.05	4.118	4.056	14.13	4.116
200.000	2.301	30	3	10.000	10	3.72	3.633	3.730	9.06	3.633
100.000	2.000	30	1	3.333	3	3.12	3.148	3.116	4.62	3.150

Y = -6.127167E-02 + 1.605525X

CHI-SQUARED IS .2587204 WITH 4 DEGREES OF FREEDOM

NO SIG HETEROGENEITY

LOG LD-50 IS 3.15241

LD-50 IS 1420.399

95% CONF LIMITS ARE 804.0775 TO 2509.127

Appendix Table-XXXXX: Dose-mortality effect of ethyl acetate extract of *Pleurotus ostreatus* against *A. salina* nauplii after 30h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1000.000	3.000	30	18	60.000	60	5.25	5.046	5.250	19.11	5.071
800.000	2.903	30	14	46.667	47	4.92	4.893	4.942	18.81	4.910
600.000	2.778	30	10	33.333	33	4.56	4.696	4.551	18.03	4.704
400.000	2.602	30	6	20.000	20	4.16	4.417	4.180	16.74	4.413
200.000	2.301	30	5	16.667	17	4.05	3.942	4.062	12.15	3.916
100.000	2.000	30	2	6.667	7	3.52	3.466	3.540	7.14	3.419

Y = .1144791 + 1.652055X

CHI-SQUARED IS 2.331625 WITH 4 DEGREES OF FREEDOM

NO SIG HETEROGENEITY

LOG LD-50 IS 2.957238

LD-50 IS 906.2294

95% CONF LIMITS ARE 619.9871 TO 1324.628

Appendix Table-XXXXXI: Dose-mortality effect of chloroform extract of *Pleurotus ostreatus* against *A. salina* nauplii after 12h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1000.000	3.000	30	5	16.667	17	4.05	3.971	4.062	12.15	3.994
800.000	2.903	30	4	13.333	13	3.87	3.849	3.873	11.10	3.867
600.000	2.778	30	2	6.667	7	3.52	3.693	3.529	9.06	3.704
400.000	2.602	30	2	6.667	7	3.52	3.472	3.540	7.14	3.473
200.000	2.301	30	1	3.333	3	3.12	3.095	3.135	3.93	3.079

Y = 6.378317E-02 + 1.310219X

CHI-SQUARED IS .3770208 WITH 3 DEGREES OF FREEDOM

NO SIG HETEROGENEITY

LOG LD-50 IS 3.767475

LD-50 IS 5854.292

95% CONF LIMITS ARE 483.129 TO 70939.13

Appendix Table-XXXXXII: Dose-mortality effect of chloroform extract of *Pleurotus ostreatus* against *A. salina* nauplii after 18h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1000.000	3.000	30	10	33.333	33	4.56	4.615	4.551	18.03	4.592
800.000	2.903	30	9	30.000	30	4.48	4.479	4.480	16.74	4.464
600.000	2.778	30	7	23.333	23	4.26	4.304	4.266	15.96	4.299
400.000	2.602	30	6	20.000	20	4.16	4.057	4.160	13.17	4.066
200.000	2.301	30	3	10.000	10	3.72	3.634	3.730	9.06	3.667
100.000	2.000	30	1	3.333	3	3.12	3.212	3.121	5.40	3.269

Y =.6220026+1.323507X

CHI-SQUARED IS .3230248 WITH 4 DEGREES OF FREEDOM

NO SIG HETEROGENEITY

LOG LD-50 IS 3.307875

LD-50 IS 2031.773

95% CONF LIMITS ARE 848.5294 TO 4865.007

Appendix Table-XXXXXIII: Dose-mortality effect of chloroform extract of *Pleurotus ostreatus* against *A. salina* nauplii after 24h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1000.000	3.000	30	15	50.000	50	5.00	4.933	4.990	19.02	4.930
800.000	2.903	30	13	43.333	43	4.82	4.797	4.818	18.48	4.794
600.000	2.778	30	10	33.333	33	4.56	4.622	4.551	18.03	4.619
400.000	2.602	30	7	23.333	23	4.26	4.375	4.266	15.96	4.371
200.000	2.301	30	5	16.667	17	4.05	3.953	4.062	12.15	3.949
100.000	2.000	30	2	6.667	7	3.52	3.531	3.519	8.07	3.527

Y = .7205691 + 1.403094X

CHI-SQUARED IS .4953194 WITH 4 DEGREES OF FREEDOM

NO SIG HETEROGENEITY

LOG LD-50 IS 3.049997

LD-50 IS 1122.01

95% CONF LIMITS ARE 666.5686 TO 1888.638

Appendix Table-XXXXXIV: Dose-mortality effect of chloroform extract of *Pleurotus ostreatus* against *A. salina* nauplii after 30h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1000.000	3.000	30	20	66.667	67	5.44	5.372	5.422	18.48	5.365
800.000	2.903	30	17	56.667	57	5.18	5.218	5.202	18.81	5.213
600.000	2.778	30	14	46.667	47	4.92	5.020	4.925	19.11	5.018
400.000	2.602	30	12	40.000	40	4.75	4.741	4.740	18.48	4.742
200.000	2.301	30	8	26.667	27	4.39	4.263	4.388	15.09	4.271
100.000	2.000	30	3	10.000	10	3.72	3.786	3.720	10.08	3.800

Y = .6703076 + 1.564836X

CHI-SQUARED IS .4980374 WITH 4 DEGREES OF FREEDOM

NO SIG HETEROGENEITY

LOG LD-50 IS 2.766866

LD-50 IS 584.6101

95% CONF LIMITS ARE 431.108 TO 792.7683

Appendix Table-XXXXXV: Dose-mortality effect of pet-ether extract of *Pleurotus ostreatus* against *A. salina* nauplii after 12h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1000.000	3.000	30	14	46.667	47	4.92	4.753	4.922	18.48	4.754
800.000	2.903	30	10	33.333	33	4.56	4.636	4.551	18.03	4.636
600.000	2.778	30	8	26.667	27	4.39	4.485	4.390	16.74	4.484
400.000	2.602	30	6	20.000	20	4.16	4.272	4.150	15.09	4.269
200.000	2.301	30	5	16.667	17	4.05	3.909	4.062	12.15	3.903
100.000	2.000	30	2	6.667	7	3.52	3.546	3.519	8.07	3.536

Y = 1.100771 + 1.21773X

CHI-SQUARED IS 1.324588 WITH 4 DEGREES OF FREEDOM

NO SIG HETEROGENEITY

LOG LD-50 IS 3.202047

LD-50 IS 1592.379

95% CONF LIMITS ARE 739.299 TO 3429.83

Appendix Table-XXXXXVI: Dose-mortality effect of pet-ether extract of *Pleurotus ostreatus* against *A. salina* nauplii after 18h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1000.000	3.000	30	18	60.000	60	5.25	5.160	5.240	19.02	5.154
800.000	2.903	30	15	50.000	50	5.00	5.023	5.000	19.11	5.019
600.000	2.778	30	12	40.000	40	4.75	4.846	4.760	18.81	4.843
400.000	2.602	30	10	33.333	33	4.56	4.597	4.544	17.43	4.597
200.000	2.301	30	7	23.333	23	4.26	4.171	4.284	14.13	4.175
100.000	2.000	30	3	10.000	10	3.72	3.744	3.720	10.08	3.753

Y =.9499078 + 1.401496X

CHI-SQUARED IS .5048218 WITH 4 DEGREES OF FREEDOM

NO SIG HETEROGENEITY

LOG LD-50 IS 2.889835

LD-50 IS 775.9521

95% CONF LIMITS ARE 521.5273 TO 1154.497

Appendix Table-XXXXXVII: Dose-mortality effect of pet-ether extract of *Pleurotus ostreatus* against *A. salina* nauplii after 24h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1000.000	3.000	30	14	46.667	47	4.92	4.753	4.922	18.48	4.754
800.000	2.903	30	10	33.333	33	4.56	4.636	4.551	18.03	4.636
600.000	2.778	30	8	26.667	27	4.39	4.485	4.390	16.74	4.484
400.000	2.602	30	6	20.000	20	4.16	4.272	4.150	15.09	4.269
200.000	2.301	30	5	16.667	17	4.05	3.909	4.062	12.15	3.903
100.000	2.000	30	2	6.667	7	3.52	3.546	3.519	8.07	3.536

Y = 1.100771 + 1.21773X

CHI-SQUARED IS 1.324588 WITH 4 DEGREES OF FREEDOM

NO SIG HETEROGENEITY

LOG LD-50 IS 3.202047

LD-50 IS 1592.379

95% CONF LIMITS ARE 739.299 TO 3429.83

Appendix Table-XXXXXVIII: Dose-mortality effect of pet-ether extract of *Pleurotus ostreatus* against *A. salina* nauplii after 30h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1000.000	3.000	30	18	60.000	60	5.25	5.160	5.240	19.02	5.154
800.000	2.903	30	15	50.000	50	5.00	5.023	5.000	19.11	5.019
600.000	2.778	30	12	40.000	40	4.75	4.846	4.760	18.81	4.843
400.000	2.602	30	10	33.333	33	4.56	4.597	4.544	17.43	4.597
200.000	2.301	30	7	23.333	23	4.26	4.171	4.284	14.13	4.175
100.000	2.000	30	3	10.000	10	3.72	3.744	3.720	10.08	3.753

Y = .9499078 + 1.401496X

CHI-SQUARED IS .5048218 WITH 4 DEGREES OF FREEDOM

NO SIG HETEROGENEITY

LOG LD-50 IS 2.889835

LD-50 IS 775.9521

95% CONF LIMITS ARE 521.5273 TO 1154.497

Appendix Table-XXXXXIX: Dose-mortality effect of hot water extract of *Pleurotus ostreatus* against *A. salina* nauplii after 12h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1500.000	3.176	30	5	16.667	17	4.05	4.018	4.037	13.17	4.031
1200.000	3.079	30	4	13.333	13	3.87	3.845	3.873	11.10	3.853
1000.000	3.000	30	3	10.000	10	3.72	3.704	3.720	10.08	3.708
800.000	2.903	30	2	6.667	7	3.52	3.531	3.519	8.07	3.531
600.000	2.778	30	1	3.333	3	3.12	3.308	3.148	6.24	3.302
400.000	2.602	30	1	3.333	3	3.12	2.994	3.172	3.30	2.979

Y = -1.788182 + 1.832167X

CHI-SQUARED IS .2777853 WITH 4 DEGREES OF FREEDOM

NO SIG HETEROGENEITY

LOG LD-50 IS 3.705003

LD-50 IS 5069.943

95% CONF LIMITS ARE 1100.691 TO 23352.88

Appendix Table-XXXXXX: Dose-mortality effect of hot water extract of *Pleurotus ostreatus* against *A. salina* nauplii after 18h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1500.000	3.176	30	7	23.333	23	4.26	4.328	4.266	15.96	4.327
1200.000	3.079	30	6	20.000	20	4.16	4.128	4.170	14.13	4.130
1000.000	3.000	30	5	16.667	17	4.05	3.964	4.062	12.15	3.969
800.000	2.903	30	3	10.000	10	3.72	3.764	3.720	10.08	3.772
600.000	2.778	30	2	6.667	7	3.52	3.505	3.519	8.07	3.518
400.000	2.602	30	1	3.333	3	3.12	3.141	3.116	4.62	3.159

Y = -2.135141 + 2.034756X

CHI-SQUARED IS .2232285 WITH 4DEGREES OF FREEDOM

NO SIG HETEROGENEITY

LOG LD-50 IS 3.506632

LD-50 IS 3210.936

95% CONF LIMITS ARE 1308.101 TO 7881.742

Appendix Table-XXXXXXI: Dose-mortality effect of hot water extract of *Pleurotus ostreatus* against *A. salina* nauplii after 24h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1500.000	3.176	30	10	33.333	33	4.56	4.539	4.544	17.43	4.531
1200.000	3.079	30	8	26.667	27	4.39	4.368	4.394	15.96	4.360
1000.000	3.000	30	6	20.000	20	4.16	4.228	4.150	15.09	4.221
800.000	2.903	30	5	16.667	17	4.05	4.056	4.037	13.17	4.050
600.000	2.778	30	4	13.333	13	3.87	3.835	3.873	11.10	3.830
400.000	2.602	30	2	6.667	7	3.52	3.524	3.519	8.07	3.520

Y = -1.058994 + 1.75991X

CHI-SQUARED IS .1198487 WITH 4 DEGREES OF FREEDOM

NO SIG HETEROGENEITY

LOG LD-50 IS 3.442786

LD-50 IS 2771.954

95% CONF LIMITS ARE 1219.288 TO 6301.815

Appendix Table-XXXXXXII: Dose-mortality effect of hot water extract of *Pleurotus ostreatus* against *A. salina* nauplii after 30h of exposure.

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
1500.000	3.176	30	16	53.333	52	5.05	5.017	5.050	19.11	5.018
1200.000	3.079	30	13	43.333	41	4.77	4.758	4.766	18.48	4.757
1000.000	3.000	30	10	33.333	31	4.50	4.547	4.488	17.43	4.544
800.000	2.903	30	8	26.667	24	4.29	4.288	4.286	15.09	4.284
600.000	2.778	30	5	16.667	14	3.92	3.955	3.924	12.15	3.948
400.000	2.602	30	3	10.000	7	3.52	3.484	3.540	7.14	3.475

Y = -3.516904+2.687124X

CHI-SQUARED IS .1143532 WITH 4 DEGREES OF FREEDOM

NO SIG HETEROGENEITY

LOG LD-50 IS 3.169524

LD-50 IS 1477.487

95% CONF LIMITS ARE 1126.617 TO 1937.631

Appendix Table-XXXXXXIII: ANOVA with arcsin transformed data for the repellent activity of *Pleurotus ostreatus* methanol extracts on *T. castaneum* adults.

ANOVA: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Row 1	5	183.76	36.752	477.8239
Row 2	5	250.17	50.034	193.5242
Row 3	5	169.59	33.918	85.04712
Row 4	5	105.29	21.058	111.1161
Row 5	5	105.58	21.116	151.0343
Column 1	5	169.91	33.982	526.659
Column 2	5	185.86	37.172	264.7311
Column 3	5	93.9	18.78	146.4674
Column 4	5	177.98	35.596	281.5216
Column 5	5	186.74	37.348	227.536

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	2940.088	4	735.022	**4.129958**	0.017389	3.006917
Columns	1226.61	4	306.6526	1.723026	0.194108	3.006917
Error	2847.572	16	177.9733			
Total	7014.271	24				

Appendix Table-XXXXXXIV: ANOVA with arcsin transformed data for the repellent activity of *Pleurotus ostreatus* ethyl acetate extracts on *T. castaneum* adults.

ANOVA: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Row 1	5	238.57	47.714	48.00463
Row 2	5	111.31	22.262	23.05947
Row 3	5	65.81	13.162	210.8102
Row 4	5	66.31	13.262	63.50567
Row 5	5	105.83	21.166	151.081
Column 1	5	117.8	23.56	403.1722
Column 2	5	76.34	15.268	573.7051
Column 3	5	114.29	22.858	152.9907
Column 4	5	151.98	30.396	115.8724
Column 5	5	127.42	25.484	107.3659

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	4024.94	4	1006.235	**11.60356**	0.00013	3.006917
Columns	598.3589	4	149.5897	1.725017	0.193679	3.006917
Error	1387.485	16	86.71782			
Total	6010.784	24				

Appendix Table-XXXXXXV: ANOVA with arcsin transformed data for the repellent activity of *Pleurotus ostreatus* chloroform extracts on *T. castaneum* adults.

ANOVA: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Row 1	5	187.11	37.422	165.9879
Row 2	5	258.8	51.76	53.9958
Row 3	5	156.32	31.264	200.6534
Row 4	5	222.95	44.59	57.23605
Row 5	5	124.91	24.982	55.82187
Column 1	5	199.87	39.974	146.7052
Column 2	5	195.03	39.006	226.4851
Column 3	5	190.8	38.16	194.6645
Column 4	5	157.94	31.588	337.7856
Column 5	5	206.45	41.29	116.8917

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	2239.709	4	559.9272	**4.841515**	0.009443	3.006917
Columns	284.3605	4	71.09011	0.614694	0.658247	3.006917
Error	1850.42	16	115.6512			
Total	4374.489	24				

Appendix Table-XXXXXXVI: ANOVA with arcsin transformed data for the repellent activity of *Pleurotus ostreatus* pet-ether extracts on *T. castaneum* adults.

ANOVA: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Row 1	5	262.77	52.554	62.47623
Row 2	5	219.11	43.822	10.27592
Row 3	5	179.13	35.826	45.96108
Row 4	5	186.64	37.328	85.59282
Row 5	5	154.47	30.894	26.73288
Column 1	5	170.95	34.19	45.4212
Column 2	5	186.64	37.328	85.59282
Column 3	5	193.96	38.792	154.3971
Column 4	5	223.65	44.73	143.6426
Column 5	5	226.92	45.384	34.45568

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	1398.279	4	349.5697	**12.27209**	9.38E-05	3.006917
Columns	468.3967	4	117.0992	4.110916	0.017685	3.006917
Error	455.759	16	28.48494			
Total	2322.434	24				

Appendix Table-XXXXXXVII: ANOVA with arcsin transformed data for the repellent activity of *Pleurotus ostreatus* hot water extracts on *T. castaneum* adults.

ANOVA: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Row 1	5	169.55	33.91	93.31915
Row 2	5	57.92	11.584	119.1471
Row 3	5	59.56	11.912	44.34242
Row 4	5	125.64	25.128	131.1812
Row 5	5	125.64	25.128	131.1812
Column 1	5	87.72	17.544	244.8698
Column 2	5	117.52	23.504	467.9907
Column 3	5	81.2	16.24	9.1125
Column 4	5	119.73	23.946	57.07543
Column 5	5	132.14	26.428	106.3577

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	1852.922	4	463.2305	**4.388984**	0.013855	3.006917
Columns	387.982	4	96.9955	0.919006	0.476942	3.006917
Error	1688.703	16	105.5439			
Total	3929.607	24				

Appendix Table-XXXXXXVIII: Repellency of *T. castaneum* by methanol tract of with percent repulsion and arcsine transformation data.

Dose (mg/ml)	Insects used	Replication	Hourly observation					Average of hourly observation (Nc)					Percent Repulsion (PR) PR = (Nc – 5) ×20%					Arcsin trasformation data				
			1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h
10	10	R1	9	6	0	10	8	8.33	6.66	5	8	7.33	66.66	33.32	0	60	46.66	54.70	35.24	0	50.77	43.05
		R2	8	8	7	9	7															
		R3	8	6	8	5	7															
5	10	R1	8	9	0	7	7	8	9	6	8.33	8.33	60	80	20	66.66	66.66	50.77	63.43	26.57	54.70	54.70
		R2	7	8	8	9	9															
		R3	9	10	10	9	9															
2.5	10	R1	7	8	6	8	8	7.33	5.66	6.33	6.33	7.33	46.66	13.32	26.66	26.66	46.66	43.05	21.39	31.05	31.05	43.05
		R2	7	9	7	5	8															
		R3	8	0	6	6	6															
1.25	10	R1	6	6	7	7	6	5.66	5	4.33	5.33	5.33	13.32	0	-6.66	6.66	6.66	21.39	39.23	14.89	14.89	14.89
		R2	5	3	2	4	4															
		R3	6	6	4	5	6															
0.625	10	R1	4	5	6	5	5	5	4	5.66	6	6.33	0	-20	13.32	20	26.66	0	26.57	21.39	26.57	31.05
		R2	5	3	7	7	7															
		R3	6	4	4	6	7															

Appendix Table-XXXXXXIX: Repellency of *T. castaneum* by E. Acetate tract of with percent repulsion and arcsine transformation data.

Dose (mg/ml)	Insects used	Replication	Hourly observation					Average of hourly observation (Nc)					Percent Repulsion (PR) PR = (Nc – 5) ×20%					Arcsin trasformation data				
			1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h
10	10	R1	9	10	9	9	6	8.33	8.33	8	7.66	7	66.66	66.66	60	53.32	40	54.70	54.70	43.05	46.89	39.23
		R2	8	8	7	7	7															
		R3	8	7	8	7	8															
5	10	R1	6	8	6	5	7	6	5.66	5.33	5.66	6	20	13.66	6.66	13.66	20	26.57	21.64	14.89	21.64	26.57
		R2	5	5	6	6	5															
		R3	7	4	4	6	6															
2.5	10	R1	6	6	5	7	8	5	5	5.33	6.66	7.33	0	0	6.66	33.32	46.66	0	0	14.89	21.64	15.68
		R2	5	4	7	6	8															
		R3	4	5	4	7	6															
1.25	10	R1	6	5	6	6	6	5.33	5	5.33	5.66	5.33	6.66	0	6.66	13.66	6.66	14.89	0	14.89	21.44	14.89
		R2	8	6	6	7	5															
		R3	2	4	4	4	5															
0.625	10	R1	6	5	6	7	7	5.66	5	6	66	6.33	13.66	0	20	20	26.66	21.64	0	26.57	26.57	31.05
		R2	6	4	6	5	5															
		R3	5	6	6	6	7															

Appendix Table-XXXXXXX: Repellency of *T. castaneum* by Chloroform extract with percent repulsion and arcsine transformation data.

Dose (mg/ml)	Insects used	Replication	Hourly observation					Average of hourly observation (Nc)					Percent Repulsion (PR) PR = (Nc – 5) ×20%					Arcsin trasformation data				
			1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h
10	10	R1	7	6	8	7	9	7.33	7	7.66	5.33	7.33	46.66	40	53.33	6.66	46.66	43.05	39.23	46.89	14.89	43.05
		R2	8	8	9	8	10															
		R3	7	7	6	1	3															
5	10	R1	8	9	9	8	9	7.66	9	7.66	7.66	8.33	53.39	80	53.33	53.33	66.66	46.89	63.43	46.89	46.89	54.70
		R2	6	8	7	8	8															
		R3	9	10	7	7	8															
2.5	10	R1	8	6	6	8	8	6.33	6	6.66	5.33	6.	26.66	20	33.32	6.66	20	31.05	26.57	35.24	14.89	26.57
		R2	4	5	6	0	2															
		R3	7	7	9	8	8															
1.25	10	R1	7	8	8	9	7	6.66	7	7.66	8.33	7.66	33.32	40	53.33	66.66	53.33	35.24	39.23	46.89	54.70	46.89
		R2	6	7	7	8	8															
		R3	7	6	8	8	8															
0.625	10	R1	6	4	8	5	5	5.66	4	5.33	6	6.66	13.66	-20	6.66	20	33.32	21.64	26.57	14.89	26.57	35.24
		R2	6	3	5	5	7															
		R3	5	5	5	6	6															

Appendix Table-XXXXXXXI: Repellency of *T. castaneum* by Pet. Ether tract of with percent repulsion and arcsine transformation data.

Dose (mg/ml)	Insects used	Replication	Hourly observation					Average of hourly observation (Nc)					Percent Repulsion (PR) PR = (Nc – 5) ×20%					Arcsin trasformation data				
			1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h
10	10	R1	7	7	8	9	7	7.33	7.66	8.33	9	8.66	46.66	53.33	66.66	80	66.66	43.05	46.89	54.70	63.43	54.70
		R2	8	10	10	10	10															
		R3	7	6	7	8	9															
5	10	R1	7	8	8	8	7	7	7.66	7.66	7.33	7.33	40	53.33	53.33	46.66	46.66	39.23	46.89	46.89	43.05	43.05
		R2	6	9	8	9	9															
		R3	8	6	7	5	6															
2.5	10	R1	7	8	9	7	7	6.33	6	7	7	7.33	26.66	20	40	40	46.66	31.05	26.57	39.23	39.23	43.05
		R2	5	5	7	8	7															
		R3	7	5	5	6	8															
1.25	10	R1	8	7	6	7	7	6.33	6.66	6	7.66	57.66	26.66	33.32	20	53.33	53.33	31.05	35.24	26.57	46.89	46.89
		R2	6	7	5	8	7															
		R3	5	6	7	7	8															
0.625	10	R1	6	6	5	6	6	6	6.33	6	6.33	7	20	26.66	20	26.66	40	26.57	31.05	26.57	31.05	39.23
		R2	7	8	6	7	7															
		R3	5	5	7	6	8															

Appendix Table-XXXXXXXII: Repellency of *T. castaneum* by Hot Water tract of with percent repulsion and arcsine transformation data

Dose (mg/ml)	Insects used	Replication	Hourly observation					Average of hourly observation (Nc)					Percent Repulsion (PR) PR = (Nc – 5) ×20%					Arcsin trasformation data				
			1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h
10	10	R1	7	7	9	5	6	7.33	7.33	5.66	6	6.33	46.66	46.66	13.66	20	33.32	43.05	43.05	21.64	26,57	35.24
		R2	8	8	5	6	6															
		R3	7	7	6	7	7															
5	10	R1	4	5	6	6	0	5	5	5.33	5.66	4.33	0	0	6.66	13.66	-13.64	0	0	14.89	21.64	21.39
		R2	4	4	3	7	6															
		R3	7	6	7	4	7															
2.5	10	R1	5	5	4	5	4	4.66	5	5.33	5.33	4.66	-6.66	0	6.66	6.66	-6.66	14.89	0	14.89	14.89	14.89
		R2	3	3	5	5	5															
		R3	6	7	7	6	5															
1.25	10	R1	5	3	5	5	4	4.66	3	4.66	3.33	4.33	-6.66	-40	-6.66	-33.33	-33.33	14.89	39.23	14.89	35.24	21.39
		R2	4	6	4	5	5															
		R3	5	0	5	0	4															
0.625	10	R1	4	1	4	4	5	4.66	3.33	4.66	4.33	3	-6.66	-33.34	-6.66	-13.34	-13.34	14.89	35.24	14.89	21.39	39.23
		R2	5	5	6	5	0															
		R3	5	4	4	4	4															