Screening of potent edible mushrooms and their mass production

Dissertation II Report

Submitted for dissertation

(In the partial fulfillment of degree of Master of Science in Botany)

In the faculty of Bioengineering and Biosciences

Lovely Professional University, Phagwara

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(Reg. No.11509887)

DECLARATION

I hereby declare that the project work entitled as 'Screening of potent edible mushrooms and their mass production' submitted to Lovely Professional University, is a record of an authentic work done by me, under the guidance of Dr. Devendra Kumar Pandey, Lovely Professional University, in order to fulfill the requirement of project. This work has not been copied from any source and whatever decoration and connection made in the circuit is a total dedicated work of mine.

Date-

Khozolu

Reg no. 11509887

CERTIFICATION

This to certify that Khozolu is undergoing her project title 'Screening of potent edible mushrooms and their mass production' under my guidance and supervision. To the best of my knowledge, the present work is the result of his original investigation and study. No part of the project has ever been submitted for any other degree at any University.

The project is for the as part of the requirement necessary for the awards for the degree M.Sc Botany (H).

Date-

Signature of Advisor

Dr.Devendra Kumar Pandey

Assisitant Professor

School of Bioengineering and Bioscience

ACKNOWLEDGEMENT

It is my honour to present my project on "*Screening of potent edible mushrooms and their mass production"*

I wish to express my sincere gratitude to my project supervisor Dr. Devendra kumar pandey, Assistant Professor, who gave me the motivation to take the project and inspired me each point of time. I deeply thank him for his dedication and patience provided for my project work.

Also, I sincerely thank all the faculty members who helped me at various stages of our project. My thanks also go out to Mr. Chirag Chopra, Head of Laboratory of Biotechnology for encouraging and supporting me during my project.

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Khozolu (11509887)

Introduction

Mushroom, a fleshy, spore-bearing fruiting body of a fungus, produced on soil above the ground and comes in a wide range of varieties. Mushrooms can be found anytime in the year, but mostly found during March-May and between September-October. Mushrooms can be found growing in open area, rubber, oil palm growing areas, primary and secondary forests, lowlands and highlands. Though the mushrooms are classified as vegetables in the food world, are not technically plants; but they provide several important nutrients which are highly poisonous and hallucinogenic, some are edible also. They play a vital role in forest ecosystem as saprobes, pathogens and symbionts. Mushrooms have high nutritional value with low content calorie with best quality vitamins and proteins. They are considered as an alternate source of food for the increasing population in the present era. It has bioactive properties which were screened, this bioactive and medicinal properties has been used since old ages by the people. Pharmaceutical company sees mushroom as they are potent sources of many valuable compounds which can help to fight against many diseases. They are the only vegetarian source of vitamin D (Pratima Bodh et al. 2016). It can be utilized as the alternative sources of medicine for their curative properties. They are the potent source of neutraceuticals and various bioactive molecules which bear anticancer and immunomodulatory properties. It also acts as antioxidant having a good effect on cardio vascular activity, acting against hypercholesterolemia and bearing antimicrobial, heptaprotective and anticancer activity.

In these modern century mushrooms are well known to all, it is much common in Asian countries as a major bio source of secondary metabolites which possess an extensive range of biological activities with chemically diverse, which are explored in traditional and in modern system of medicine also. The cultivation of mushroom was first started during the 17^{th} century in France. In India the cultivation was started in 1961 with the launch of a scheme of ICAR at Solan, Himachal Pradesh. The production and culture of new species of mushrooms is increasing and has significantly improved. Though many types of mushrooms are being commercially produced, the button mushroom (*Agaricus bisporus*), Oyster (*Pleurotus* sp), Paddy straw mushroom (*Volvariella volvacea*), milky mushroom (*Calocybe indica*) are the most popular with the growers and consumers in India. *Pleurotus ostreatus* mushroom cultivation is very popular and next to *Agaricus bisporus* mushroom in India in popularity and consumption. World production of mushroom is growing and now exceeds three million tonnes and *Agaricus bisporus* accounts for most of this production. Mushroom production in

India has been estimated at 48000 tonnes per annum. At present, China accounts for the highest production of mushrooms in the world followed by Italy and USA. Mushroom is a useful recycler of waste materials as various agricultural and wood wastes are being used for the cultivation of mushrooms. Growing mushrooms on waste materials is a traditional practice which is followed even in the present with certain modifications and improvements. The in vitro cultivation of mushrooms is done by using the mushroom strains culture in various media such as PDA, Oddoux, and MEA etc. The in vitro cultivation has much faster growing rate which may have industrial benefits and offer the possibility of cloning or genetic manipulation mycelium. The growth and development of current technologies, such as automated control, automatic mushroom harvesting, preparation and production of mushrooms in a compost and non-composted substrate, and novel method of spawn preparation, will improve and increase the yield of mushroom culture. However, before starting any method, adequate knowledge about the geographical position, climatic conditions, major crops and plantations are essential in order to find a suitable agro-wastes and suitable species for cultivation. All these aspects are essential for the production of mushrooms with improved taste, appearance, quality, nutritional values, and medicinal properties at low cost.

Scope of the study

This study is based on the edible mushrooms which are known to be rich in high quality protein, high proportion of unsaturated fatty acids and have vitamins and minerals content. Various compounds of medicinal value present in mushroom are used as neutraceutical and dietary supplements.

In this study the edible mushroom *Agaricus bisporus* and *Pleurotus ostreatus* was cultivated in agricultural waste using certain chemicals and optimum conditions for mass production. The invitro culture was also done in Potato Dextrose Agar and Potato Dextrose Brooth. The fruiting body of mushroom was collected from the locally available area and extraction and analysis of various compounds was done which will provide various health benefits and food security.

Mushroom cultivation gives economic help to the cultivators and its consumption is good for health too. Mushroom cultivation is a very easy process and they are eco friendly and healthy in nature as well. They contribute to nature as a natural decomposer which reduces the amount of agricultural waste by decomposing it and releasing the nutrients that are entrapped inside them. Cultivation of mushroom will provide a good source of income for unemployed youth. It will be an alternate for existing farming system and a source of financial help. Amount of money required to set up a mushroom production unit is less but profit is quite good.

Objectives of the study

This study is based on the following objectives which are mentioned below

1. Collection and identification of edible mushrooms.

2. Standardization of culturing aspects of edible mushrooms.

3. Analysis and screening of potent mushrooms on the basis of neutraceuticals .

Review of literature

Edible mushrooms

Berkeley (1856; 1867) gave an account of some mushrooms from India. Mushrooms have long been valued as highly nutritious tasty food items by many societies throughout the world (Oei 2003). Mushrooms are considered to be good sources of proteins which content vary from 10-40%. It contains all essential amino acids but limiting in cystine and methionine (Chang 1991, Breene 1990). (Bilgrami, Jamaluddin and Rizwi 1979 and 1991) gave an account of some edible mushrooms from India. (H a w k s w o r t h et al. 1995) listed many edible mushrooms in *Dictionary of Fungi*. Lakhanpal (1995) gave an account of mushrooms from North West Himalayas.

Nutritional and nutraceutical values

A large number of mushrooms possess tonic and medicinal properties in addition to edible and nutritious qualities. (Bahl 1983) reported that mushroom can cure diabetes, dysentery, gall bladder disease, liver disease, diarrhea, anesthesia, heart ailments, skin diseases, epilepsy and relieves cold, insomnia, stress and asthma. It also contains compounds with wide range of antifungal, antibacterial, antiviral, anti-inflammatory and antitumor (Wasser and Weis 1999). (Chang and Buswell 1996) described mushroom nutraceutical as a class of new compounds extractable from fruiting body and mycelium. The nutraceutical values of edible mushrooms such as fibre, fatty acids, proteins, peptides, amino acids, minerals, antioxidants, vitamins etc are described. (Gunde-Cimerman 1999, Ooi and Liu 1999, Ooi 2000, Wasser and Weis 1999 and 1999) isolated and identified compounds, originate from mushrooms which show quite significant and considerable medicinal properties, such as immunomodulatory, cardiovascular, liver protective, anti-fibrotic, anti-inflammatory, anti-diabetic, anti-viral and antimicrobial activities. Phenolic compounds present in mushrooms plays a very important role as it has properties of antioxidant, intitumour agents. Phenolic compounds include large subclass such as flavonoids, phenolic acids, tannins, lignans etc (Puttaraju et al. 2006). Turkoglu *et al*., 2007) gave an account of mushrooms which accumulate a range of secondary metabolites, including phenolic compounds, terpenes, polyketides, and steroids. (S. K. Sharma and N. Gautam 2015) evaluated the chemical, bioactive, and antioxidant activity of various wild edible culinary mushroom species consumed by the people of northern Himalayan regions. All the culinary species contained

important and useful nutraceuticals such as unsaturated fattyacids, phenolics, carotenoids, ascorbic acid, tocopherols. Besides these, some important amino acids were detected in these mushrooms which could be used for the purpose of useful ingredients. (Fedia Souilem 2016) conduct a study on bioactive properties and compounds of wild mushrooms grown *in vitro* under different conditions. (Pratima Bodh et al., 2016) described the deveolpment of a product rich in Vitamin D prepared from mushroom powder which are the only vegetarian source. (Agahar and Subbulakshmi 2005) described the nutritional and nutraceutical values of edible wild mushrooms which are collected from the Khasi hills of Meghalaya. Many of them are rich source of various bioactive molecules having anticancer and immunomodulatory potential. (Uppuluri V. Mallavadhani et al., 2006) gave the chemical and analytical screening of various vitamins present in some edible mushrooms. (Narayanan and Natarajan 2004) gave an account of some interesting agarics from South India. (Poonam Dehariya et al., 2013) gave a comparative study of nutraceuticals on different substrates on two edible mushroom species *Pleurotus sajor-caju* and *Agaricus bisporus*.

Extraction and estimation of neutraceuticals

Ruthes et al. (2015) gave a detailed account of extraction, purification and chemical characterization of neutraceuticals from the edible mushrooms. (N. Joy Dubost 2007) analyzed the quantification of polyphenols and ergiothioneine in cultivated mushrooms. (Ioannis Giavasis2014) describe the bioactive fungal polysaccharides as potential efficient ingredients in food and nutraceuticals. (Mara Kuka and IlzeCakste 2011) investigate the content of bioactive compounds of two widely used wild edible mushrooms. The cultivation, fruiting, watering and storage conditions affects the biologically active compounds because it affet the nutrient content in mushrooms (Mattila et al. 2002). (Chirinang et al., 2009) demonstrated antitumor, antifungal, antibacterial activities of mushrooms. (Vidovic et al., 2010) gave an account on compounds such as selenium, ergothioneine, phenolsfrom mushrooms which have powerful antioxidant properties. (Gan et al. 2013) analyse the antioxidant properties of different types of edible mushrooms.(K.Aarthy and K.Deivanal 2014) conducted a study was to test the presence of phytochemical constituents and antibacterial activity of five edible mushroom species. (Sharma et al. 2012) evaluated the composition of amino acid of wild edible mushrooms. Mushrooms contain all the essential amino acids but are limited in cysteine and methionine (Breene 1990, Chang 1991).

Cultivation

Worldwide commercial production of mushroom has improved progressively during the last decade, but only a few genera of basidiomycetes i.e., *Agaricus, Lentinula, Pleurotus, Auricularia, Volvariella, Flammulina, Tremella* and some others are cultivated industrially (Stamets, 1993). Mushroom cultivation is a million dollar industry in various countries including China, India and South East Asia where raw material and preparation of selective compost constitutes major cost input (Van Roestel 1988). (Straatsma et al., 2000) describe the process of production of *A. bisporus* from wheat straw, chicken manure, straw-bedded horse manure, and gypsum. (Sanchez 2004) describes the methods of modern aspects of mushroom culture technology. (Dhar et al., 2011) gave a detailed description on the cultivated edible mushrooms in India and EU countries. (Petre and Teodorescu 2010) gave an account of cultivation of edible and medicinal mushrooms through vineyard and winery wastes. (Colavolpe and Alberto 2014) determine the optimum condition needed to evaluate its biological efficiency and to determine the biodegradation of substrate and cultivation of mushroom. (Malayil et al., 2016) attempt a new use for lignin and N-rich biogas digester liquid (BDL) as a nutrient supplement for mushroom cultivation. (Fazaeli and Talebian Masoodi 2006) describe the cultivation of *Agaricus bisporus* on spent compost wheat straw, which constitutes a potential pollutant and is cost effective for disposal. (Peker et al., 2007) gave an account on Cultivation of *Agaricus bisporus* on agricultural based compost formulas to find out the pin head formation time and yield values of *Agaricus bisporus.* (Leiva et al 2015) analyse the life cycle assessment and various environmental related issues which affects the cultivation process of mushrooms. The environmental factor such as global warming, ozone layer depletion, human toxicity, acidification, eutrophication has a great impact on the growing process. Preparation of growing chambers, covering soils, ventilation and climate also affects the production process. The temperature must be kept below 28°c during incubation, however during the fruiting period 16-18°c are recommended and 21-25°c during the growth phase (Foulonge-Oriol et al., 2014, Largeteau et al., 2011). (Sharma et al. 2014 and 2015) contributed towards the study of mushrooms.

Equipments, materials and chemicals

List of equipments

Sl no	Equipments
1	Spectrophotometer
2	Water bath
3	Grinder
4	TLC plates
5	TLC chamber
6	Soxlet extractor
7	Rotary evaporator
8	Incubator
9	Centrifuge
10	Heating mantle
11	Weighing balance
12	Laminar air flow
13	Autoclave

Materials

Sl.no	**materials**
1	Wheat straw
2	Test tubes
3	Beaker
4	Conical flask
5	Cuvette
6	Test tube stand
7	Micropipette
8	Micropipette tips
9	Capillary tube
10	Petriplates
11	Glass rod
12	spatula

13	Inoculation loop

Chemicals

S. no	Chemical	company
1	Formaldehyde	Loba chemie
2	Wheat bran	Loba chemie
3	Sulphate of potash	Loba chemie
4	Urea	Loba chemie
5	Super phosphate	Loba chemie
6	Calcium ammonium nitrate	Loba chemie
7	Bovine Serum Albumin (BSA)	Loba chemie
8	Sodium carbonate	Loba chemie
9	Sodium hydroxide	Loba chemie
10	Anthrone	Loba chemie
11	Hydrochloric acid	Loba chemie
12	Sulphuric acid	Loba chemie
13	Glucose	Loba chemie
14	Butanol	Loba chemie
15	Methanol	Loba chemie
16	Chloroform	Loba chemie
17	Formic acid	Loba chemie
18	Metaphosphoric acid	Loba chemie
19	2, 6-dichlorophenol indophenols dye	Loba chemie
20	DPPH	Loba chemie
21	Gallic acid	Loba chemie
22	Quercetin	Loba chemie
23	Ethanol	Loba chemie
24	Aluminium chloride	Loba chemie
25	Potassium acetate	Loba chemie
26	Ascorbic acid	Loba chemie
27	Potato Dextrose Agar	Hi media
28	Potato Dextrose Brooth	Hi media

Miscellaneous

Sl.no	Miscellaneous
1	Cotton
2	Brown paper
3	Parafilm
4	Muslin cloth
5	Alluminium foil
6	Forceps
7	Thread

Materials and methods

Mass production of mushroom

Agaricus biosporus

Long method of composting formula, first successfully advocated by Mantel et al. (1972) in India, was used in the present study, with the use of synthetic compost for *Agaricus bisporus*. A steel barrel is washed with 2% formalin. 20 kg of wheat straw was soaked in water and kept it for 48hrs. Wheat bran (2kg), sulphate of potash (200g), urea (250g), super phosphate (600g), calcium ammonium nitrate (600g) was mixed properly with water. The chemicals are mixed with the wheat straw after one day. After the compost heap is complete, it is kept for 5 days. The temperature of the compost increases up to 70°c after 2-3 days.

Day 6 (First turning) - The compost heap is broken and mixed thoroughly. It is then sprayed with water after which it is stacked again.

Day 10 (Second turning) - The compost stack is broken and mixed thoroughly by sprinkling with water and stacked again.

Day 13 (Third turning) - The compost stack is broken and the composting substrates are mixed with gypsum. They are turned again and stacked.

Day 16 (Fourth turning) -The compost pile is break open and turned as followed in the preceding step. Water is required to add if the outer portions are dried up.

Day 19 (Fifth turning) - The compost pile is break open and turned as followed.

Day 22 (Sixth turning) - The compost pile is break open and turned as followed.

Day 25 (Seventh turning) - The compost pile is break open and turned as followed.

Day 28 (Eighth turning) – the pile is break open and turned with a fork. They are kept open for the next two days to remove excess ammonia formed during composting. After two days the compost is checked by smelling. If sweet smell appears then the compost is ready for spawning. The good quality compost is dark brown in colour and smells sweet. The spawn which is collected from PAU is used for spawning.

Pleurotus ostreatus

The cultivation of *Pleurotus ostreatus* (oyster) was done on wheat straw by the conventional method (Lelley 1991). A steel barrel is washed with 2% formalin. 20 kg of wheat straw was soaked in water and kept it for 48hrs. The next day the wheat straw is autoclaved at 15 psi pressure for 15 minutes. After the straw is cooled down, the straw was put in plastic bag in 4-

5 layers stack. Spawning is done in between the layers after which it was transferred to the cullture room.

Invitro culture of mushrooms

The strains of *Agaricus bisporus*, *Lentinula edodes* and *Pleurotus ostreatus* were collected from MTCC. The strains were cultured on Potato Dextrose Agar (PDA) with a temperature of 27°c which was then transferred to Potato Dextrose Brooth.

Extraction

The edible mushroom *Agaricus bisporus* is collected from the locally available market during the month of September 2016. The mushrooms were cut into small pieces and then shade dried and powdered using a grinder. The powdered mushroom was extracted in soxhlet extractor using 75% methanol. (Bouchet et al., 1998). The extract was concentrated by using rotary evaporator.

Determination of protein content in Mushroom

The protein content of mushroom in methanol and ethyl acetate extract was determined by Lowry's method using BSA (bovine serum albumin) as standard (Lowry et al., 1651). The method was based on the principle that different proteins contain different amount of aromatic residues which react with Folin Coicalteu's Reagent (Phenol reagent) and values are expressed as mg/ml of culture filtrate.

Preparation of standard BSA

The stock solution of standard was prepared by weighing 100gm of BSA and dissolving it in 100ml of distilled water.

Preparation of mushroom extract

1. Plant extract was prepared by Soxhlet extraction method.
2. 5g dried mushroom powder of the plant was extracted in 300ml of 75% methanol for 18hrs
3. The extract was evaporated in rotary evaporator at 65˚C followed by preparation of 50mg/ml test sample in methanol.

Preparation of reagents

Reagent A - 2% Na_2CO_3 dissolve in 0.1N NaOH.

Reagent B - 2% sodium potassium tartarate and 1% CuSO4.

Reagent C - 50ml of reagent A and 50ml of reagent B.

Reagent D - Mixing of Folin Coicalteu with distilled water in 1:1 ratio.

Procedure

1. The standard protein ranging from 200µl to 1000µl was taken in separate test tubes and the volume made upto 1 ml with distilled water.
2. A test tube with 1 ml distilled water served as blank.
3. 4.5 ml reagent C was added to each tube including the blank and after proper mixing, the solutions were allowed to stand in water bath at 35^0c for 30 min.
4. 0.5 ml reagent D was added and mixed properly.
5. The tubes were kept in a water bath at 35^0c for 60 min. Blue colour was developed in the solution.
6. The absorbance was taken at 660 nm in UV- visible spectrophotometer.
7. Estimation of protein from the sample was done by pipetting out 200 µl of test sample of mushroom in test tubes and the volume was made upto 1 ml with distilled water.
8. Rest of the procedure was same as followed for the standard protein. With the help of the standard curve the amount of protein in mushroom was estimated.

Determination of carbohydrates

The carbohydrate content of mushroom was determined by using Anthrone's test using glucose as standard.

Preparation of mushroom test sample

100mg of shade dried mushroom was taken in a test tube and add 1ml of concentrated Hcl and 4ml of distilled water. The test tube was kept in hot water bath at 100^0c for 1 hour.

Preparation of standard

The stock solution of glucose was prepared with 100mg of glucose dissolve in 100ml of distilled water.

Preparation of reagent

Anthrone solution was prepared by weighing 200mg of anthrone which was dissolved in 95ml of concentrated H_2SO_4 and 5ml of distilled water.

Procedure

1. The standard solution ranging from 20µl to 100µl was taken in separate test tube and the volume was made up to 1ml with distilled water.
2. 1ml of distilled water was served as blank.
3. 4ml of anthrone solution was added to each test tube including the blank after which it was kept in water bath for 10 minutes.
4. Deep blue green colour was developed in the solution and absorbance was measured at 630nm.
5. 20µl of mushroom test sample is taken in a test tube and the volume was made up to 1ml with distilled water.
6. 4ml anthrone solution was added by which deep blue green colour appears and the absorbance was measured at 630nm. The standard graph is plotted in which the total carbohydrates are estimated in the sample.

Determination of Ascorbic acid (vitamin C)

The ascorbic acid content was determined by using the direct calorimetric determination. The chemical method of estimation are based on the reduction of 2, 6 – dichlorophenol indophenol by ascorbic acid.

Preparation of reagents

1. 2% metaphosphoric acid was prepared in glass distilled water.
2. Dye solution was prepared by dissolving 50mg of 2, 6- dichlorophenol indophenol dye and 42 mg of sodium bicarbonate in hot distilled water, cool and make up to 100 ml. Filter and dilute 25ml to 500ml with distilled water.
3. The standard ascorbic acid solution was prepared by weighing 20mg in 20ml of 2% HPO_3. 4ml of this solution is dilute to 100ml of 2% HPO_3. (1ml=40µg of ascorbic acid)
4. Test sample is prepared by taking 10g powdered sample, blend with 2% HPO_3 and make up to 100ml with 2% HPO_3 and centrifuge.

Procedure

1. To dry test tubes, pipette the requisite volume of standard ascorbic acid solution 1, 2, 3, 4,5ml and make up to 5ml with requisite amount of 2% HPO_3.

2. Add 10ml of dye with a rapid delivery pipette shake and take readings within 15-20 seconds.
3. A test tube consisting of 5ml of 2% HPO_3 and 10ml of distilled water is served as blank.
4. Measure the red colour at a wavelength of 518nm.
5. Plot the absorbance against concentration.
6. Place 5ml of the sample extract in a dry test tube and add 10 ml of dye and measure as in standard. Note the concentration of ascorbic acid from the standard curve and calculate the ascorbic acid content in the sample.

Determination of antioxidant activity

The antioxidant activity of mushroom was estimated using DPPH antioxidant assay after following the method of Padmanadhan and Jangle (2012) with slight modifications.

Preparation of DPPH

1. 0.1mM DPPH solution was prepared by taking 3.94mg of DPPH in 100ml absolute methanol.
2. The solution was kept in dark for 30 minutes at room temperature.
3. Optical Density was measured using spectrophotometer at 517nm.

DPPH Antioxidant Assay

1. Different concentrations of sample were taken, ranging from 0.25-1.25 mg/ml. All the concentrations were prepared in triplicates.
2. 2 ml of DPPH methanol solution were added in each test tube.
3. The mixture was kept at room temperature covered with black paper or kept in dark for 5-10 minutes.
4. Absorbance was recorded at 517nm.
5. All the tests were done in triplicates.
6. DPPH radical scavenging activity was calculated using the following formula

$$\text{Scavenging effect (\%)} = \left(1 - \frac{As}{Ac}\right) \text{x } 100$$

Where, A_S = OD of sample with DPPH solution

A_C = OD of DPPH methanol solution.

Estimation of Total Phenolic Content

Folin-Ciocalteu Colorimetric method was used (Ainsworth and Gillespie, 2007) for total phenolic content estimation.

Preparation of stock solutions

4. 5mg/ml Gallic acid solution was prepared by taking 100mg Gallic acid in 20ml distilled water.
5. 20% sodium carbonate was prepared by dissolving 20g in 100ml distilled water.
6. Plant extract was prepared by taking 1g dry mushroom powder in 60ml methanol and kept in water bath for 60 minutes at 40˚C followed by filtration.

Preparation of working solution

Table 1. Different concentrations of Gallic acid were prepared in beakers as follows:

Gallic acid (ml)	Distilled water (ml)	Concentration of gallic acid (mg/100ml)
2	98	10
4	96	20
6	94	30
8	92	40
10	90	50

1. 20µl of gallic acid was taken from each beaker to test tubes.
2. For test sample, 20µl extract was taken.
3. 1.58ml of distilled water was added in each test tube followed by 100µl Folin-Ciocalteu and kept it for 5 minutes.
4. 300µl of sodium carbonate and kept in water bath for 30 minutes at 40˚C.
5. OD was recorded at 765nm.
6. Distilled water was used as reference.
7. All the tests were done in triplicates.

Estimation of Total Flavonoid Content

Aluminum Chloride Colorimetric method (Bag et al., 2015) was used for Total Flavonoid Content estimation.

Preparation of stock solutions

1. 0.1mg/ml Quercetin solution was prepared in methanol.
2. 10% aluminum chloride solution was prepared in distilled water.
3. 1M Potassium Acetate solution was prepared by taking 9.82g Potassium acetate in 100ml distilled water.
7. Plant extract was prepared by Soxhlet extraction method.

8. 5g dried mushroom powder of the plant was extracted in 300ml of 75% methanol for 18hrs
9. The extract was evaporated in rotary evaporator at 65˚C followed by preparation of 20mg/ml test sample in methanol.

Preparation of working solution

Table 2: Different concentrations of Quercetin was prepared in beakers as follows:

Quercetin (ml)	Methanol (ml)	Concentration of quercetin (µg/ml)
2	8	20
4	6	40
6	4	60
8	2	80
10	0	100

1. 500µl of quercetin was taken from each beaker to test tubes.
2. For test sample, 500µl extract was taken.
3. 1.5ml of methanol was added in each test tube.
4. 100µl Aluminum chloride was added and kept it for 5 minutes.
5. 100µl of potassium acetate was added followed by addition of 2.8ml distilled water.
6. OD was recorded at 415nm.
7. Aluminum chloride was replaced by distilled water for reference.
8. All the tests were done in triplicates.

Thin layer chromatography analysis for amino acids

The technique involve two phase which are mobile phase and the stationary phase it works on the principle of equilibrium distribution between the two components in the phase it separate according to that and the Rf value is noted down. The experiment is conducted to determine different amino acids and vitamins present in the sample extract for which the analysis is done on two plates one is the silica plate and the other is the commercially available TLC plate. The mobile phase prepared is the mixture of Chloroform, methanol and formic acid in the proportion of 35:15:1, the silica gel is prepared in the ratio of 2:1 in distilled water and plates were made which were kept under air for drying and after that in the oven for activation. The methanol extract of the sample is allowed to run in the plates along with the standard prepared amino acid solution (glycine, tyrosise and trypton) and after that the reagent i.e. ninhydrin is sprayed and dried for getting different readings of amino acid in the sample (Bhawani. et al. 2012).

Result and discussion

Cultivation

The cultivation of button mushroom in the long method of synthetic compost gives response to mycelium growth from 10-15 days after spawning. 25°c

Fig 1: Growth of mycelium of *Agaricus bisporus*

For oyster mushroom, the mycelium growth takes place after 10 days after which the plastic bag is cut open for the growth of fruiting body.

Fig 2: Growth of mycelium of *Pleurotus ostreatus*

Invitro culture was done in Potato dextrose agar media at 27°c. *Agaricus bisporus* and *Pleurotus ostreatus* gives positive response whereas *Lentinula edodes* did not give any response as it requires a very low temperature to grow which cannot be maintained. The cultures were then transferred to Potato Dextrose Brooth which yields a good response. The mycelium was separated from the brooth by filtering, cleaned it with distilled water and dried

at room temperature. The dry weight of *Agaricus bisporus* obtained was 3.46grams and *Pleurotus ostreatus* was 2.50grams.

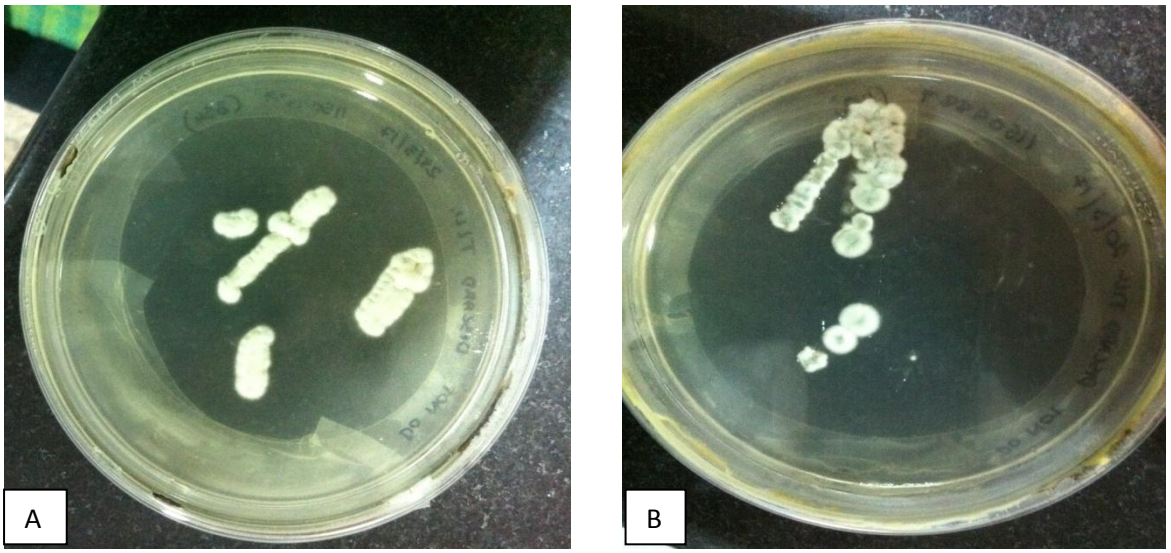

Fig 3: Growth of mycelium in PDA (A) *Agaricus bisporus* (B) *Pleurotus ostreatus*

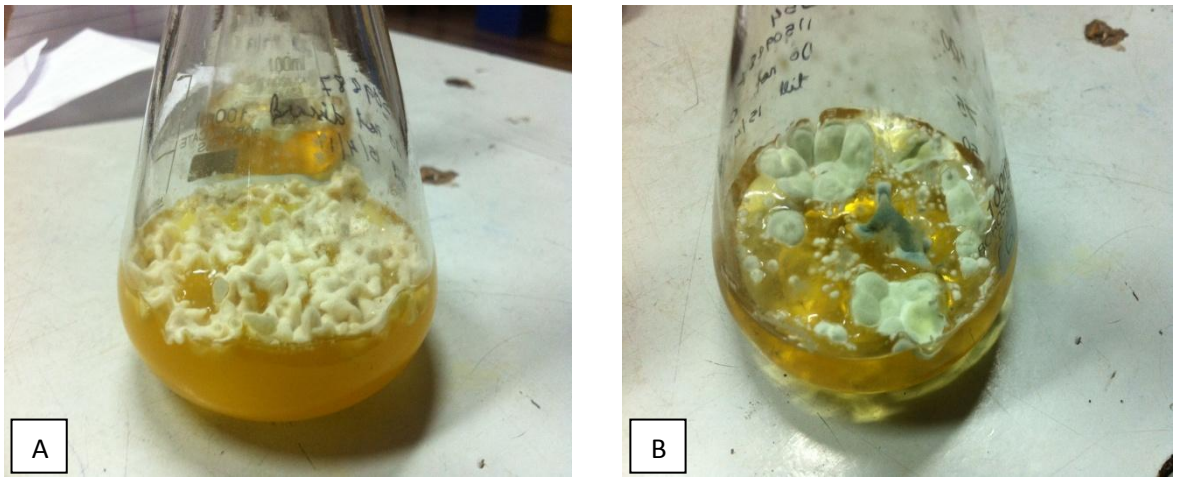

Fig 4: Growth of mycelium in Potato dextrose brooth (A) *Agaricus bisporus* (B) *Pleurotus ostreatus*

Microscopic studies

The mycelium from the culture was taken for microscopic studies. They were stained with cotton blue and the observations are showed below.

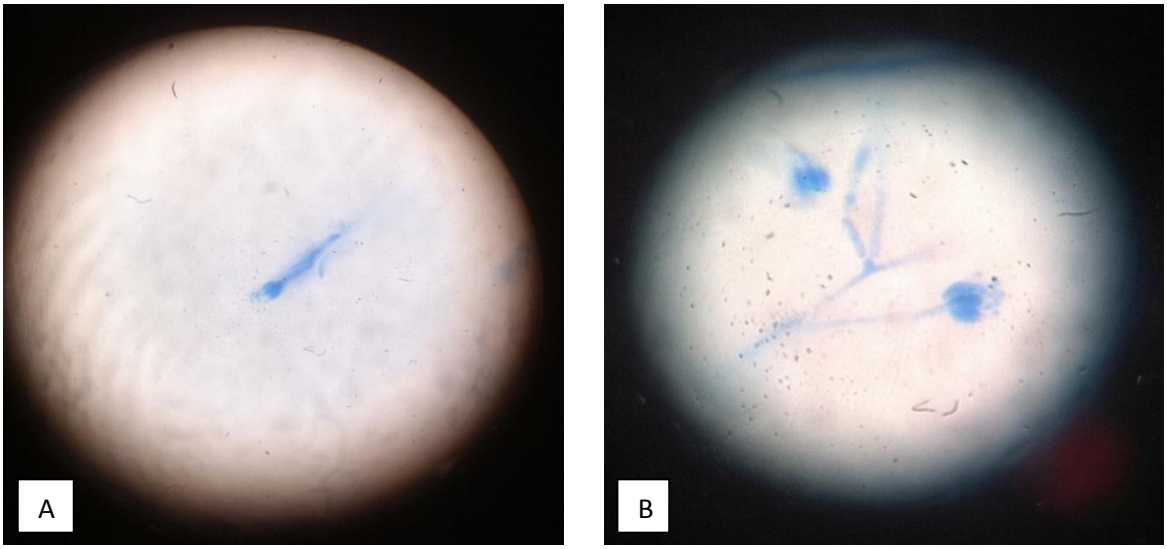

Fig 5: (A) *Pleurotus ostreatus* (B) *Agaricus bisporus*

Protein estimation

The determination of proteins in methanol and ethyl acetate extract of mushrooms is done by Lowry's method using BSA as standard. The observations and graph are given below-

Table 3: Absorbance of standard BSA

Sl. no	Vol. of BSA(ml)	Conc. of BSA(µg)	Vol. of d.w(ml)	Vol. of reagent C(ml)		Vol. of reagent D		O.D at 660nm
1	Blank	0	1ml	4.5ml	Water bath at 35^0c for 30 minutes.	0.5ml	Water bath at 35^0c for 1 hour.	0
2	0.2ml	200µg	0.8	4.5ml		0.5ml		0.212
3	0.4ml	400 µg	0.6	4.5ml		0.5ml		0.41
4	0.6ml	600 µg	0.4	4.5ml		0.5ml		0.545
5	0.8ml	800 µg	0.2	4.5ml		0.5ml		0.698
6	1ml	1000 µg	0	4.5ml		0.5ml		0.846

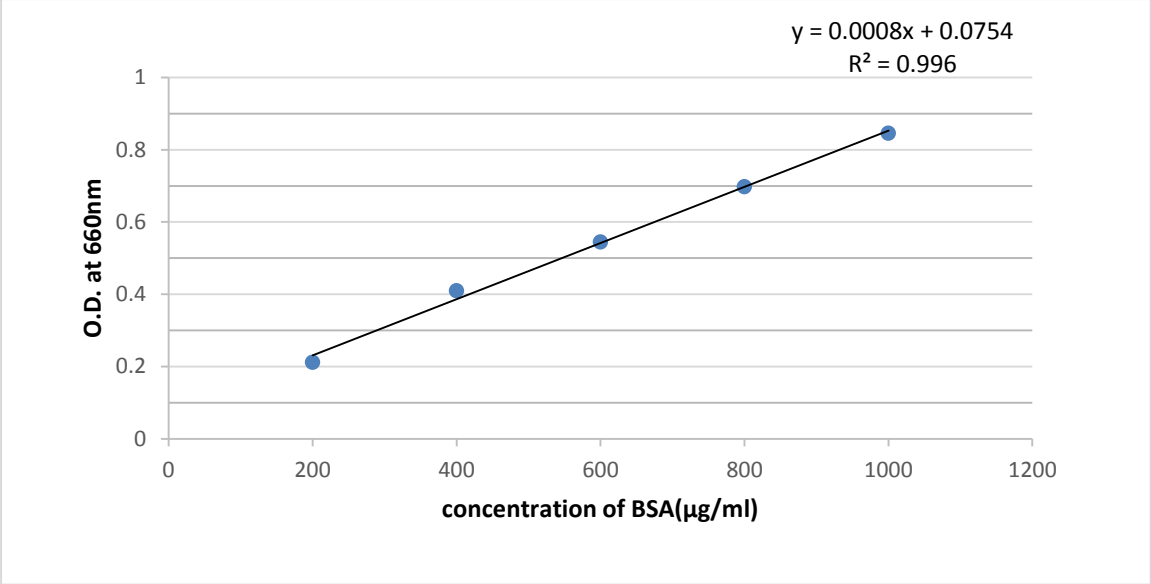

Fig 6: Standard graph of protein (BSA)

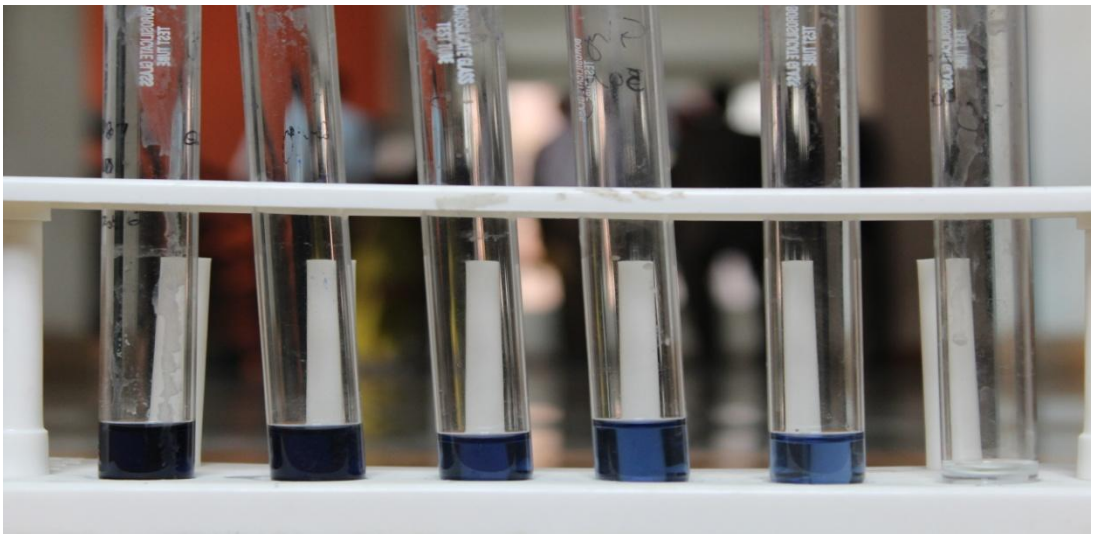

Fig 7: Test tubes containing blank solution and different concentration of standard BSA with reagents.

Table 4: Absorbance of methanol extract of mushroom

Sl. no	Vol. of methanol extract(ml)	Conc. of protein(µg)	Vol. of d.w(ml)	Vol. of reagent C(ml)		Vol. of reagent D		O.D at 660nm
1	0.2ml		0.8ml	4.5ml	Water bath at 35^0c for 30 minutes.	0.5ml	Water bath at 35^0c for 1 hour.	0.473

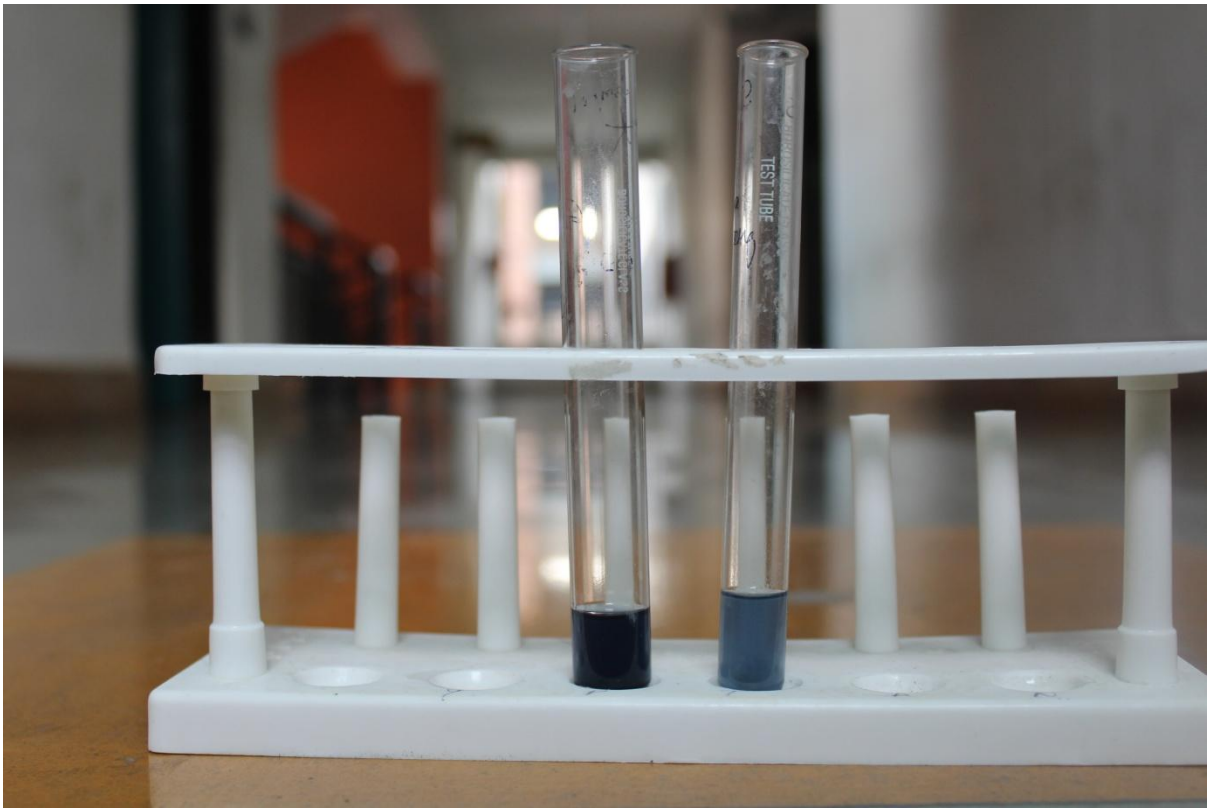

Fig 8: Test tubes containing methanol and extract of mushroom with reagents.

Concentration of protein in the sample is 497µg BSA equivalent per gram dry weight of sample. The percentage of protein in the mushroom sample is 9.94%.

Carbohydrate estimation

The concentration of carbohydrates in mushroom is determined by Anthrone test using glucose as a standard. Given below are the observation and graph-

Table 5: Absorbance of standard glucose

Sl. no	Vol. of glucose (µl)	Conc. of glucose (µg)	Vol. of d.w(µl)	Vol. of Anthrones olution(ml)		O.D at 630nm
1	Blank	0	1000 µl	4ml	Water bath for 10 minutes.	0
2	20µl	20µg	980 µl	4ml		0.105
3	40µl	40 µg	960 µl	4ml		0.226
4	60µl	60 µg	940 µl	4ml		0.349
5	80µl	80 µg	920 µl	4ml		0.450
6	100 µl	100 µg	900 µl	4ml		0.540

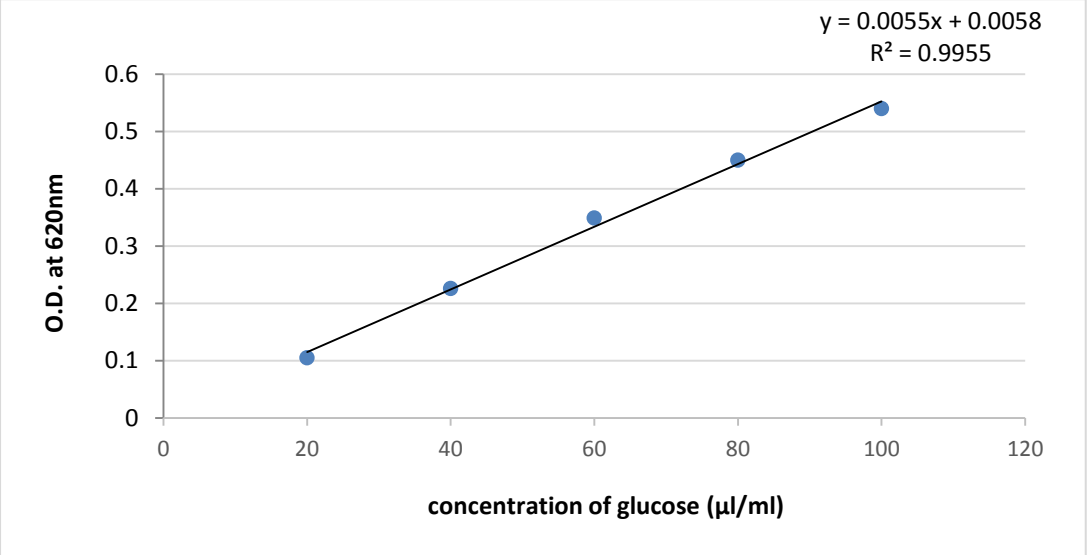

Fig 9: Standard graph of glucose

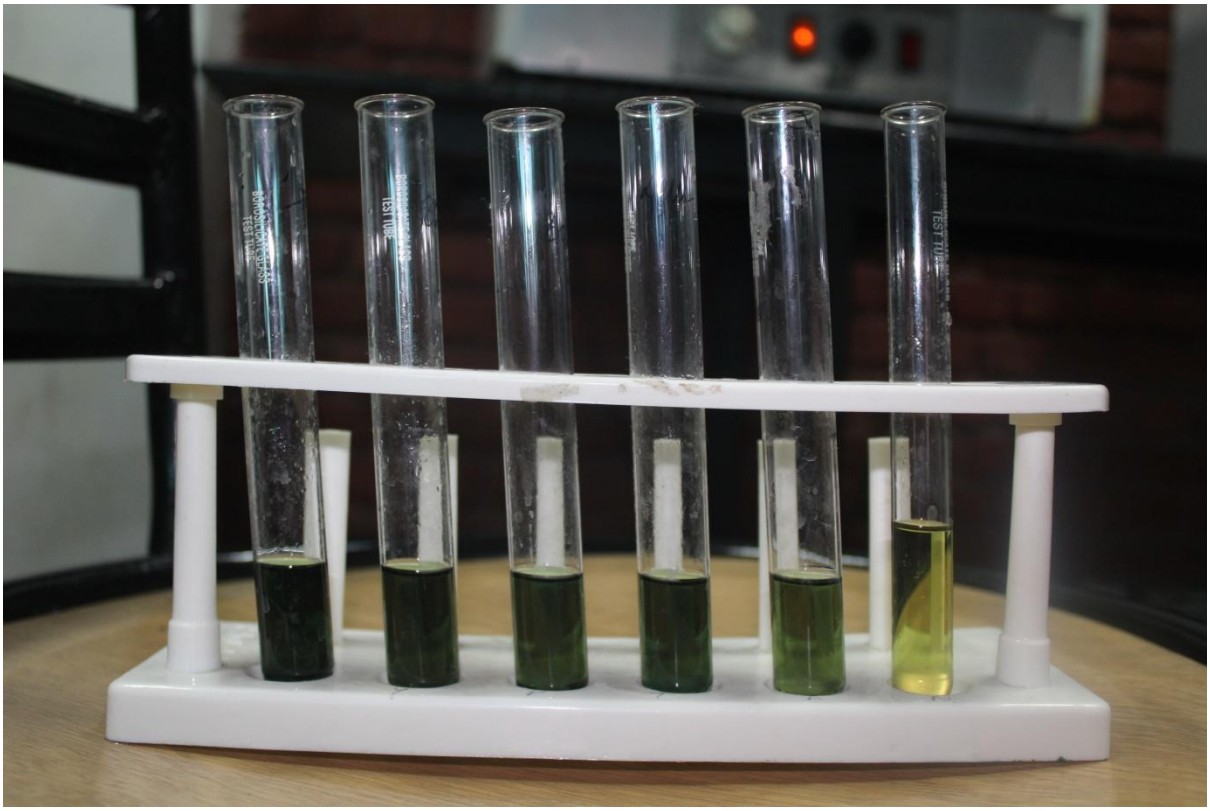

Fig 10: Test tubes containing blank solution and standard glucose of different concentrations with Anthrone solution.

Table 6: Absorbance of mushroom at 630nm

Sl. no	Vol. of mushroom (µl)	Conc. of carbohydrates(µg)	Vol. of d.w(µl)	Vol. of Anthrone solution(ml)		O.D at 630nm
1	20 µl		960 µl	4ml	Water bath for 10 minutes.	0.320

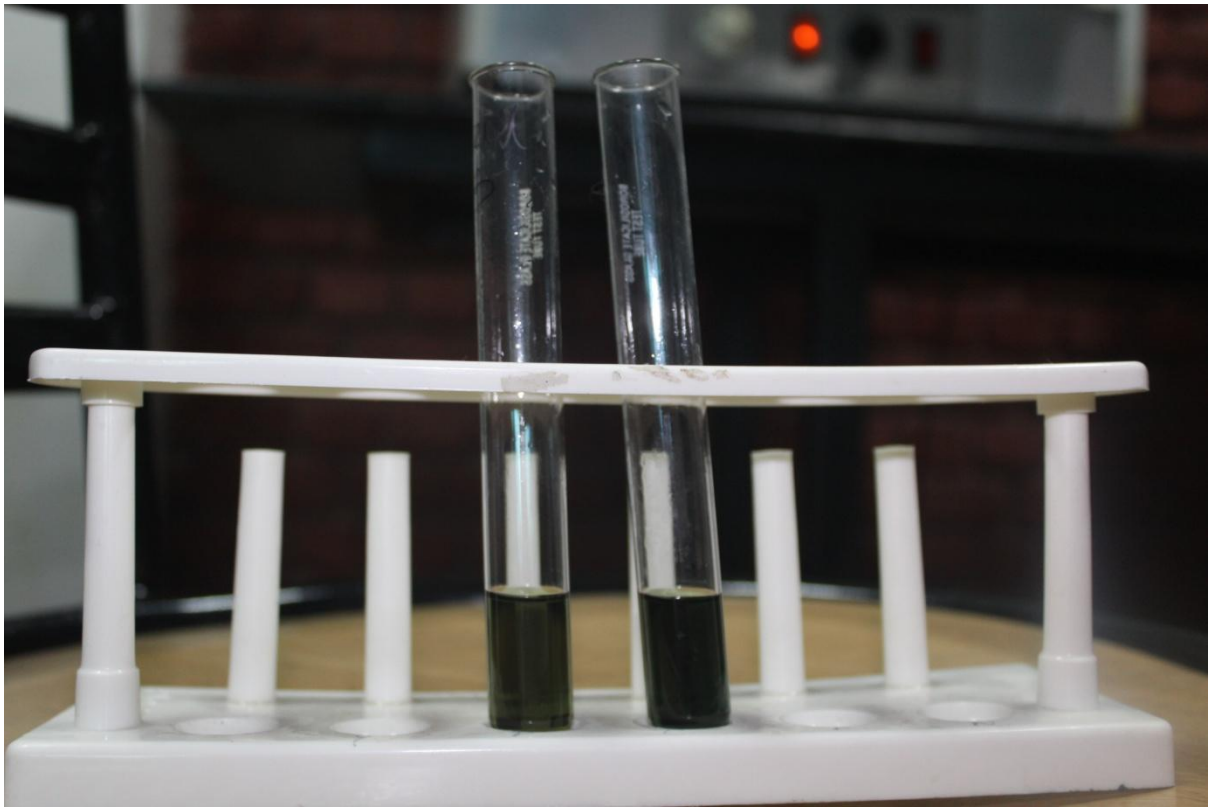

Fig 11: Test tube containing replicates of mushroom extract with Anthrone solution.

Concentration of carbohydrates is 63 µg glucose equivalents per gram dry weight of sample. The percentage of carbohydrate in the present study sample is 0.315%

Determination of Ascorbic acid

The determination of ascorbic acid is done by direct calorimetric method. Ascorbic acid is used as a standard for the preparation of standard curve.

Table 7: Readings of standard ascorbic acid at 518nm

Sample (ml)	HPO_3(ml)	Dye (ml)	Conc. of sample (mg/ml)	1st	2nd	3rd	Mean±SD
1	4	10	0.04	0.461	0.453	0.443	0.452±0.009
2	3	10	0.08	0.349	0.312	0.329	0.33±0.01
3	2	10	0.12	0.231	0.247	0.213	0.23±0.01
4	1	10	0.16	0.149	0.167	0.134	0.15±0.01
5	0	10	0.20	0.016	0.029	0.017	0.02±0.00
(sample) 2ml	3	10		0.332	0.329	0.324	0.328±0.004

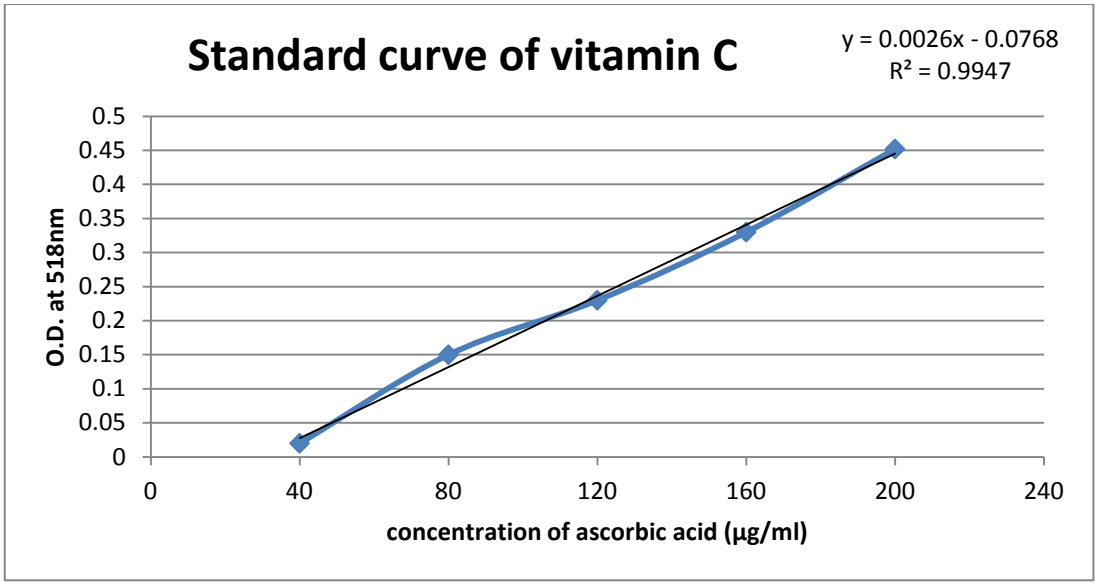

Fig 12: standard curve of ascorbic acid

Fig 13: Test tube containing working solutions of ascorbic acid with dye

The concentration of ascorbic acid calculated from the standard graph is 110.5µg/ml. The content of ascorbic acid in mushroom sample is 2.76mg per 100g dry weight.

DPPH Antioxidant assay

Concentration of stock solution= 5mg/ml

OD of DPPH at 517nm= 0.709

Table 8: Readings of absorbance at 517nm

Sample (µl)	Methanol (µl)	DPPH (ml)	Conc. of sample (mg/ml)	1st	2nd	3rd	Mean±SD	% Scavenging Activity
100	400	2	0.25	0.510	0.507	0.512	0.509±0.002	28.20
200	300	2	0.5	0.422	0.428	0.418	0.422±0.005	40.47
300	200	2	0.75	0.355	0.366	0.363	0.361±0.005	49.08
400	100	2	1.0	0.312	0.318	0.310	0.313±0.001	55.85
500	0	2	1.25	0.276	0.271	0.269	0.272±0.001	61.63

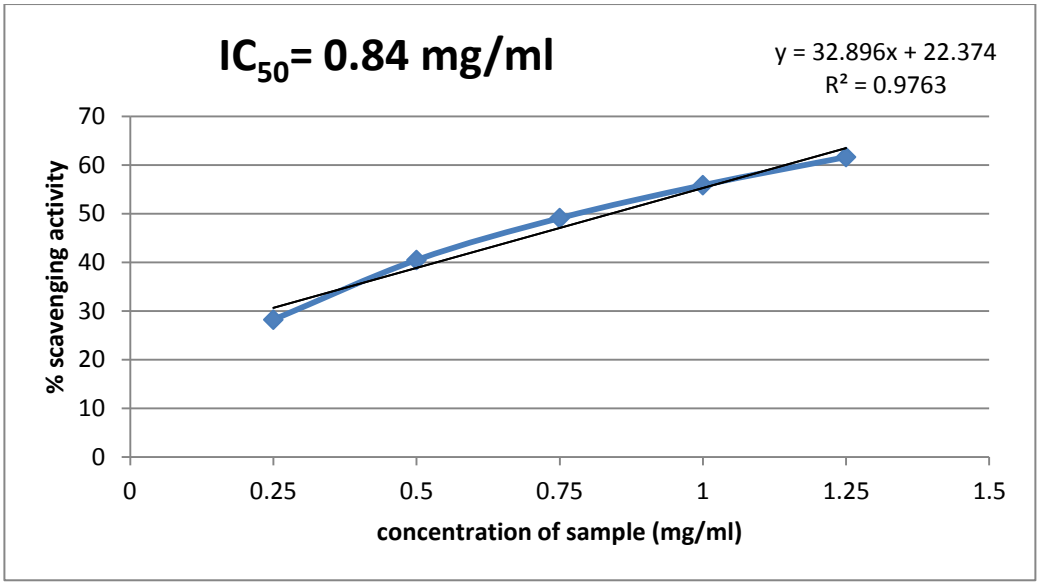

Fig 14: Graph of % scavenging activity

The percentage of scavenging activity was 61.63% when tested at highest concentration of 1.25mg/ml. The half maximal inhibitory concentration IC_{50} is 0.84mg/ml. This result shows a very significant antioxidant inhibitor.

Estimation of total phenolic content

Table 9: Readings of absorbance at 765nm

Standard (Gallic acid)	Concentration of gallic acid (mg/ml)	1st	2nd	3rd	Mean±SD
1	0.002	0.114	0.115	0.117	0. 115±0.001
2	0.004	0.232	0.229	0.217	0. 226±0.007
3	0.006	0.354	0.346	0.349	0. 349±0.004
4	0.008	0.416	0.420	0.422	0.419 ±0.003
5	0.010	0.542	0.537	0.533	0. 537±0.004
Test sample		0.144	0.132	0.132	0. 136±0.006

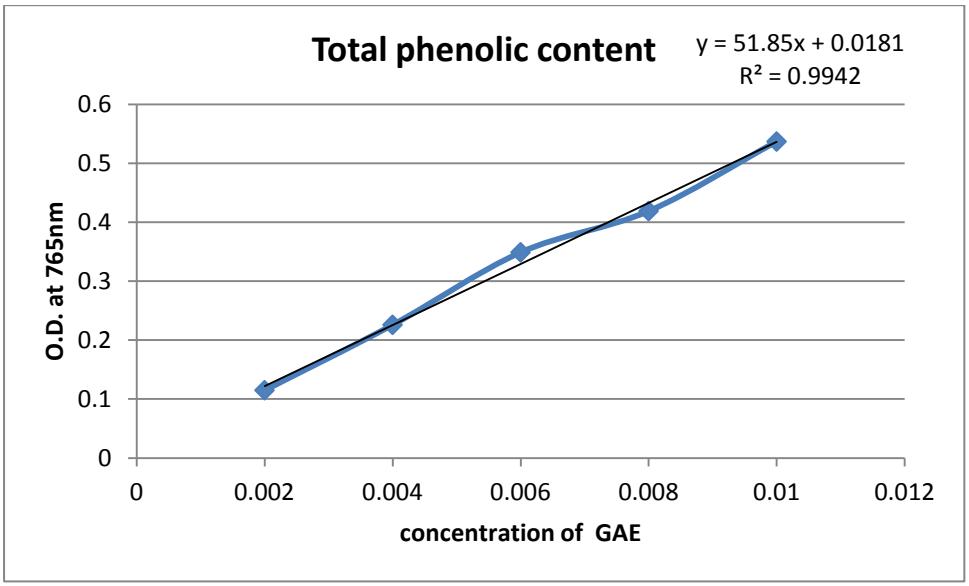

Fig 15: standard graph of phenolic

The concentration of Gallic acid in the mushroom sample is 10mg GAE per gram dry weight of sample.

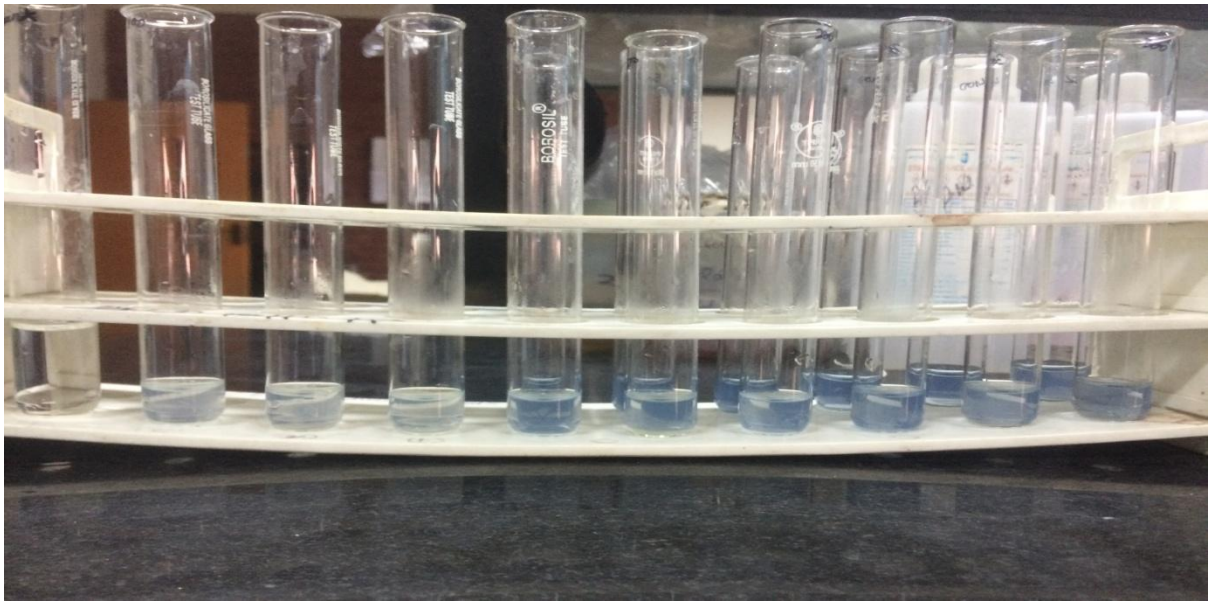

Fig 16: Test tube containing different concentrations of GA with reagents

Estimation of total flavonoid content

Table 10: Readings of absorbance at 415nm

Standard (quercetin)	Concentration of quercetin (mg/ml)	1st	2nd	3rd	Mean±SD
1	0.002	0.215	0.216	0.220	0. 217±0.002
2	0.004	0.444	0.475	0.482	0. 467±0.020
3	0.006	0.672	0.668	0.682	0. 674±0.007
4	0.008	0.845	0.896	0.895	0.879±0.029
5	0.010	1.120	1.130	1.125	1.125±0.005
Test sample		0.671	0.631	0.660	0. 654±0.022

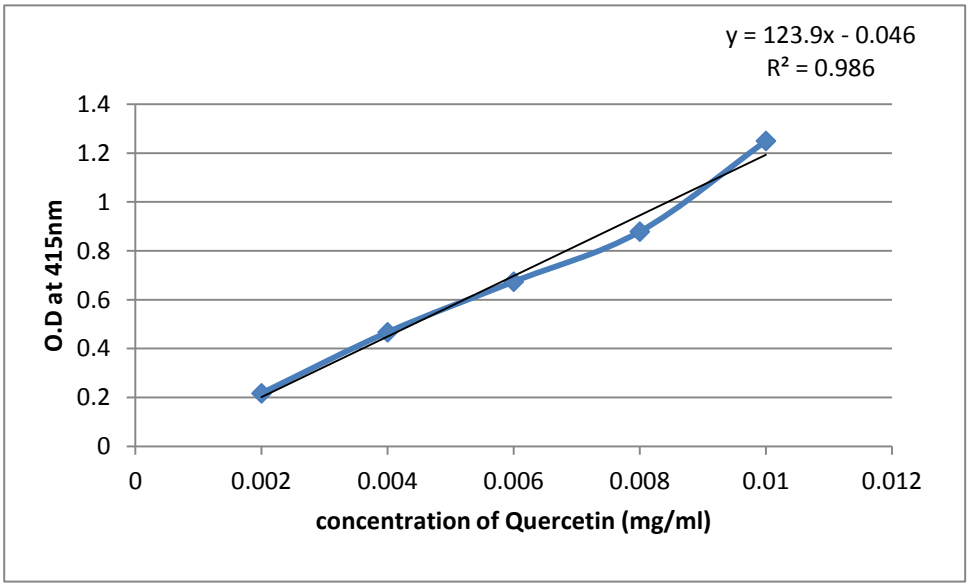

Fig 17: standard graph of flavonoid

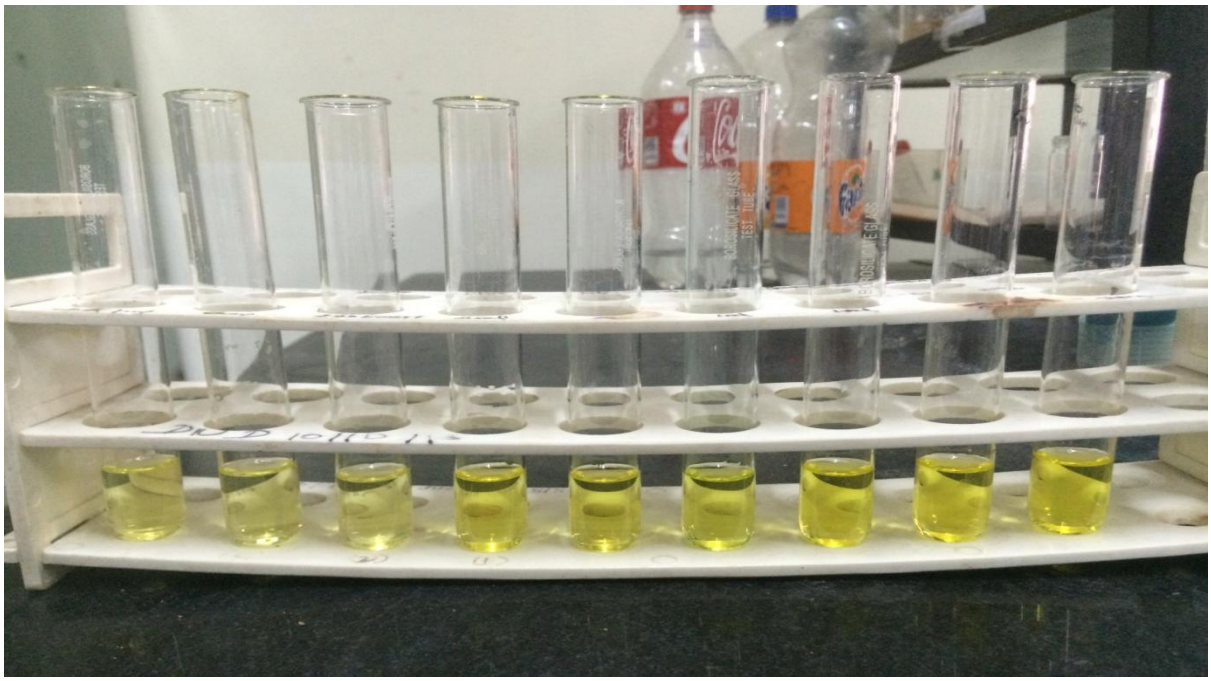

Fig 18: Test tube containing different concentrations of Quercetin with reagents.

The concentration of Quercetin in the mushroom sample is 2.5mg Quercetin equivalent per gram dry weight of sample.

TLC analysis

The analysis of the extracted sample on silica plate run in the solvent chloroform, methanol and formic acid in the ratio of 35:15:1 shows two amino acids. The result was analysed by measuring the retention factor of the amino acid travelled in the plate and by comparing it with standard that were simultaneously run in the plate.

Distance travelled by the solvent =6.3cm

Distance travelled by sample 1 = 1.6

2 = 2.7

The values were calculated using the formula of Retention factor which is distance travelled by solute divided by distance travelled by solvent. The value of sample 1 is 0.25 and sample

2 is 0.42 which are very much close to the value of glycine (0.26) and tyrosine (0.45) respectively.

Discussion

The content of ascorbic acid in mushroom sample is 2.76mg per 100g dry weight. This study is similar to that of Barros et al. 2008 (2.6-3mg/gdw). However the study reported by Mattila et al. 2001 is much higher (17mg/gdw). The percentage of scavenging activity was 61.63% when tested at highest concentration of 1.25mg/ml. The half maximal inhibitory concentration IC_{50} is 0.84mg/ml. This result shows a very significant antioxidant inhibitor. The results obtained in this study is much higher than methanolic extracts from Portugal (IC_{50}= 1.80) (Reis et al., 2012) and France (IC_{50}= 1.77) (Savoie et al. 2008). The concentration of Gallic acid in the mushroom sample is 10mg GAE per gram dry weight of sample. The present study is similar to that of N.J Dubost et al. 2007 which reported 8-10.7mg GAE/gdw. The value of the present study is much higher as compared to the value reported by Ramirez- Anguiano et al.2007 (4.5mg GAE/gdw) and (2.5-4.5mg/gdw) reported by Palacios et al 2011. The concentration of Quercetin in the mushroom sample is 2.5mg Quercetin equivalent per gram dry weight of sample. The present study is similar to the study reported by Barros et al. 2008 which is 0.67-3.40mg/gdw.

Conclusion

From this experiment we conclude that mushrooms contain a good nutritional value. It also contains various bioactive compounds which have been shown to act as antioxidant and often attribute to health benefits.

Sl.no	Nutritional composition	*Agaricus bisporus*	*Pleurotus ostreatus*
1	Protein	9.94%	10.26%
2	Carbohydrates	0.315%	1.34%
3	Ascorbic acid	2.75mg/100g	0
4	Phenolic	10mg GAE/gdw	1.01
5	Flavonoid	2.5mg GAE/gdw	0.14
6	Antoxidant	IC_{50}=0.84mg/ml	IC_{50}=1. 67mg/ml

Button mushroom has good nutritional properties. In addition to it, it has vitamin C which is absent in Oyester mushroom. Vitamin C contributes to the antioxidant properties and nutritional values.

The cultivation of mushroom is a very easy process which does not require much investment. It also helps reduce the amount of agricultural waste by decomposing it. It can also be a good source of income for unemployed youth or an alternative source of income.

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