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Evaluation Of Antimicrobial And Antioxidant Activities Of Some Edible Mushrooms

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ABSTRACT

Fruit bodies, mycelial extracts and broth filtrate of five edible mushrooms namely *Pleurotus ostreatus*, *Pleurotus sajor caju*, *Agaricus campestris, Agaricus bisporous* and *Grifola frondosa* have been evaluated for their antimicrobial and antioxidant activities. Different solvents including water, ethanol, ethyl acetate, acetone, chloroform, and hexane were used to extract antimicrobial and antioxidant compounds of different mushrooms under investigation. The extracts were evaluated for their antimicrobial activities using the agar diffusion method against some pathogenic bacteria and fungi. The results revealed that the aqueous extract of *A. bisporous* fruit body exhibited the highest antimicrobial activity in comparison to all other solvent extracts. The antioxidant activities were analyzed using DPPH radical scavenging, reducing power and the total phenolic assay. Results revealed that also aqueous extract of *P. sajor caju* and *Grifola frondosa* fruit bodies exhibited the highest antioxidant activity. Fractionation of the aqueous crude extracts of *A. bisporous, P. sajor caju* and *G. frondosa* fruit bodies producing three purified bioactive proteineous compounds which have antimicrobial activities for the first mushroom and antioxidant activities for latter tested mushrooms.

INTRODUCTION

Mushroom is defined as a macro fungus with a distinctive fruiting body which can be hypogeous or epigeous, large enough to be seen with the naked eye and to be picked by hand (Chang and Miles, 2004).

The first mushrooms have received worldwide popularity in recent decades with realization to the fact that they are good source of delicious food with high nutritional values. Infectious diseases remain one of the major threats to human health. Although numerous antibiotics have been used against pathogens, antimicrobial resistance within a wide range of infectious agents is a growing public health threat of broad concern to countries and multiple sectors. Increasingly, governments around the world are beginning to pay attention to a problem so serious that it threatens the achievements of modern medicine. To combat the multidrug resistant organisms, production of new bioactive compound from new source is essential (WHO, 2014).

Certain species of edible, inedible and poisonous mushrooms are known to have important medicinal properties, and their extracts are also used for possible treatments of some diseases in the world (Emanuel, 2013). Mushrooms need antibacterial and antifungal compounds to survive in their natural environments. Therefore, antimicrobial compounds could be isolated from many mushroom species and could be of beneficial for humans (Lindequist *et al*., 2014).

Antioxidants are man-made or natural substances that may prevent or delay some types of cell damage such as vitamins C, E, selenium, carotenoids (beta-carotene), lycopene, lutein and zeaxanthin (Nakayama *et al.,* 2011). Oxidative stress is thought to play a role in a variety of diseases including cancer, cardiovascular diseases, diabetes, Alzheimer's disease, Parkinson's disease and eye diseases such as cataracts and age-related macular degeneration (Costa *et al.,* 2014).

Like plants, mushrooms accumulate a variety of secondary metabolites, including phenolic compounds, polyketides, terpenes, and steroids. Some mushrooms have been found to possess antioxidant activity that is well correlated with their total phenolic content. Mushrooms are considered to be a good source of antioxidants such as variegatic acid and diboviquinone, which have been found in mushrooms (Turkoglu *et al.,* 2007).

The aim of the present work is to evaluate the antimicrobial and antioxidant potential of fruit bodies, mycelia and culture filtrate of five tested mushroom species extract.

MATERIALS AND METHODS

Mushroom species:

Five Chinese mushroom species two of them belonging to genus *Pleurotus ; (Pleurotus ostreatus*, *Pleurotus sajor caju) and other two belonging to genus* Agaricus; (*Agaricus bisporous* and *Agaricus campestris*) were obtained from the Fujian Edible Mushroom Association, Fuzhon, China and the Fujinan Jun-Cao Develop Project Association of China. While *Grifola frondosa* was obtained from Amazon internet shipping.

Mushroom Cultivation

Spawn production:

The preparation of grain spawn was carried out according to the method stated by Zhanxi and Zhanhua, (2001).

Fruit bodies Production:

The Chinese compost medium in sterilized plastic bags was used for production of tested mushroom fruit bodies according to (Zhanxi and Zhanhua, 1999 and Baysal *et al.,* 2007)**.**

Mycelial production:

The potato dextrose liquid medium was used for cultivation of mushroom for mycelial production. The medium was dispensed in 100ml volume by 500ml flasks then sterilized for 20 min. at 121°C under 1.5 atmospheric pressures. After sterilization, the flasks containing the media were inoculated aseptically with mushroom spores then incubated for 10 days. After the end of incubation period, the mycelial growth was harvested and separated by filtration through Whatman no.1 filter paper and the resulting filtrates (Broth) were also collected separately (Samuel *et al*., 1986).

Preparation of crude extracts:

Mycelial growth was filtered and washed with deionized water and lyophilized. The fresh fruiting bodies were also washed and lyophilized. Six different solvents were used for extraction, from the lyophilized materials added at 1 g/10 ml (w/v) to water, ethanol, acetone, ethyl acetate, chloroform, and hexane. They were soaked for 24 hours, and then homogenized with a blender (Osteizer, Denzil Green, USA), and sonicated for an hour. The extraction process was repeated for three times, followed by filtration through a Whatman No. 4 filter paper to remove the debris. The filtrates from aqueous extract were concentrated by lyophilization and those from all other solvents were concentrated by air drying.

Also, the mushroom culture filtrates (broth) were concentrated, centrifuged at 10.000 rpm for 20 minutes. All dried extracts and broth samples were stored at -20°C until use.

Screening of antimicrobial activity

The mushroom extracts from the six different solvents were tested for antimicrobial potentiality against some pathogenic microorganisms obtained from Antimicrobial Unit at Regional Center for Mycology and Biotechnology (RCMB), namely; *Pseudomonas aeruginosa* , *Staphylococcus aureus*, *Shigella sp*., *Escherichia coli* as pathogenic bacteria and *Candida albicans*, *Creptococcus neoformans*, *Aspergillus flavus* ,*Fusarium oxysporum*, *Penicillium expansum* as pathogenic fungi. Antimicrobial activity was determined using agar well diffusion method as described by Holder and Boyce (1994). Ampicillin and Nystatin were used as standard antibacterial and antifungal drugs, respectively (Cleidson *et al.,* 2012). 1 cm diameter wells cut by sterile cork borer in malt extract agar (for fungi) and nutrient agar (for bacteria) sterile plates (9cm), which has previously been seeded with fungal/bacterial test organism. The hole was filled with 100µl for each mushroom extracts, while control hole was filled with solvent used in the extraction. Plates were left in cooled incubator at 4 ±2°C for one hour to allow sample diffusion and then incubated at 28 ±2°C for fungi and at 37 ±2°C for bacteria and yeast strains.

Zones of inhibition produced by different extracts against different microorganisms were measured after 24-48 hours of incubation and subtracted from solvent inhibition zone to exclude any possible solvent effect.

Antioxidant assay:

Determination of free DPPH radical scavenging:

The 2, 2 diphenyl-1 picrylhydrazyl (DPPH) method is based on quantitative determination of the free DPPH radical scavenging activity that was used for assaying the antioxidant activity at concentration of 25 mg/100 ml methanol. Whereas 50 µl of the samples dissolved in methanol in a concentration of 50 µg/ ml were added in each well of 96 well plates then 150 µl were applied in each well followed by incubation for one hour in dark room at room temperature. The absorbance was measured at 517 nm in 300 sec intervals for 5 times with Microplate Reader (TECAN Inc, Männedorf/Switzerland). The samples were assayed in triplicate and compared with ascorbic acid as reference standard.

The percentage inhibition (PI) of the DPPH radical scavenging was calculated according to the formula: PI = [{(AC- AT)/ AC} x 100], Where *AC* = Absorbance of the control at t = 0 min and *A*T = absorbance of the sample + DPPH at t = 5 min.

The IC50 value, i.e., the concentration of the tested compound leading to 50% inhibition of the DPPH radical was estimated from graphical plots of DPPH radical scavenging vs. compound concentrations (Lee *et al.,* 2007).

Reducing power assay:

The reducing power of mushroom extracts was determined by the method of Oyaizu (1986) and (Arulpriya *et al.,* 2010) which depending on substances, which have reduction potential, react with potassium ferricyanide (Fe^{+3}) to form potassium ferrocyanide (Fe^{+2}), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm.

Estimation of total phenolic content:

The total phenolic content was determined by the spectrophotometric method according to Saeed *et al*., (2012).

Fractionation and isolation of bioactive compounds:

Gel filtration technique was used to purify antimicrobial and antioxidant compounds which might be protein, carbohydrate, glycoprotein or lipoprotein (polar active fractions) from the crude extract of the investigated mushrooms.

The Pharmacia column (40×2 cm) was packed by Sephadex G l50 after its soaking

with phosphate buffer (pH 7.2) overnight and the samples were mixed with blue dextran and bromophenol blue dyes, loaded on the top of the column and fractions were collected (5 ml of each fraction), fractionation was performed using HPLC system (pump model MINI Puls3, detector UV⁄VIS-151 FC 203B, Gilson)

Determination and estimation of total water soluble proteins:

The total protein of the active fractions was determined by the method of Lowry *et al.,* (1951) using serum albumin as a standard protein. The absorbance was measured at 750 nm using UV/VIS spectrophotometer (Milton Roy spectronic C 1201). After plotting the standard curve, the protein concentration of the samples was calculated.

Characterization of active compounds:

Characterization of proteinaceous active compounds (using LCMS and Amino acid sequencing):

The LC-MS system consisted of a Thermo Electron LTQ-Orbitrap hybrid mass spectrometer system with a nanospray ion source operated at 3.5 kV, interfaced to a Waters trap column and a Waters NanoAcquity C18 reversed-phase capillary column. An aliquot of the final solution was injected onto the trap column and washed, and then the peptides were eluted onto the analytical column. The peptides were eluted from the column by an acetonitrile/0.1% formic acid gradient at a flow rate of 0.4 µL/min over 60 minutes. The digests were analyzed using full scan mass spectra to determine peptide molecular weights and product ion spectra to determine amino acid sequence in sequential scans.

The data were analyzed by database searching using the Mascot search algorithm against NCBI's non-redundant database

Cytotoxicity assay on normal hepatocytes:

The isolated normal hepatocytes were suspended in medium at concentration $5x10^4$ cell/well and 200 µl/well dispensed into Corning 96-well tissue culture plates (Fisher Scientific Inc.). The cells were treated with the purified compounds then incubated for 24 hr at 37°C in humidified incubator (Forma Scientific water jacked incubator series II, Thermo Scientific Inc., Waltham, MA. USA) under 5% CO_2. Cells were viewed with an inverted microscope (Olympus, CKX41; Shinjuku, Tokyo, Japan) to confirm adherence and viability. The viability of the cells was measured using MTT assay (Mosmann, 1983 and Hay *et al*., 2000).

Statistical analysis:

The data were expressed as mean ±S.D. STATA statistical analysis package was used for the dose response curve drawing in order to IC_{50} calculations.

RESULTS AND DISCUSSION

Evaluation of the antimicrobial activity of different mushrooms species:

In the current study, five fungal species; *Pleurotus ostreatus, Pleurotus sajor caju*, *Agaricus bisporous*, *Agaricus campestris* and *Grifola frondosa* fruit bodies and mycelial extracts (some polar and non-polar solvents were used for the extraction) beside broth filtrate were evaluated *in vitro* for their antimicrobial activity against nine pathogenic microorganisms; four bacterial species and five fungal species.

Evaluation of the antimicrobial activities of fruiting bodies extracts:

It can be deduced from Table (1) that extracts prepared by polar solvents (water, ethanol, acetone) were more potent in their antimicrobial activity than non-polar extracts (ethyl acetate, chloroform and hexane). The efficiency of polar solvent supported by many researches (Vamanu *et al.,* 2011; Nehra *et al.*, 2012). This may be due to the presence of more than one biochemical class of compounds of different polarities having antimicrobial effects as demonstrated by Pauliuc and Botău (2013).

Table 1. The inhibition zones produced by mushrooms fruit bodies extracts (using different solvent) against certain microbial species.

Mushrooms	Test organism	Bacteria				Fungi				
	solvent	*P. Aeruginosa*	*S. aureus*	*Shigella sp.*	*E. coli*	*C. albicans*	*C. neoformans*	*A. flavus*	*F. oxysporum*	*P. Expansum*
P.ostreatus	Water	14±1	12±1	12±1	15±1	14±1	13±1	12±1	12±2	11±2
	Ethanol	11±1	9±1	9±1	13±1	13±1	10±1	8±1	11±1	10±2
	Acetone	8±1	7±1	7±1	10±1	8±1	3±1	4±1	5±2	4±1
	Ethyl acetate	4±1	3±2	3±2	4±1	6±1	NIZ	NIZ	NIZ	NIZ
	Chloroform	NIZ	3±2	3±2	3±1	5±1	NIZ	NIZ	NIZ	NIZ
	Hexane	2±1	3±1	3±1	2±1	4±1	NIZ	NIZ	NIZ	NIZ
P. sajor caju	Water	15±1	18±1	16±1	19±1	17±1	16±1	15±1	15±2	15±2
	Ethanol	13±2	15±1	13±1	14±1	15±1	15±1	13±1	14±1	13±2
	Acetone	12±2	13±1	12±1	13±1	14±1	14±1	12±1	12±2	11±1
	Ethyl acetate	11±1	10±1	10±2	12±1	13±1	11±1	10±1	10±1	10±1
	Chloroform	8±1	9±2	8±2	9±2	10±1	7±1	8±1	7±2	9±2
	Hexane	5±1	5±1	6±1	6±2	6±1	7±2	5±1	5±1	6±1
A. bisporous	Water	25±1	30±1	18±1	21±1	21±1	19±1	21±1	20±2	19±2
	Ethanol	18±2	22±1	16±1	19±1	20±1	19±1	19±1	17±1	17±2
	Acetone	16±2	17±1	15±1	17±1	16±1	17±1	17±1	15±2	15±1
	Ethyl acetate	14±1	14±1	12±2	13±1	15±1	12±1	14±1	13±1	12±1
	Chloroform	13±1	12±2	10±2	11±2	13±1	11±1	12±1	11±2	11±2
	Hexane	9±1	10±1	8±1	10±2	9±1	8±2	10±1	8±1	9±1
A.campestris	Water	16±1	17±1	16±1	16±1	15±1	11±1	14±1	12±2	13±2
	Ethanol	13±2	14±1	12±1	11±1	12±1	8±1	11±1	9±1	8±2
	Acetone	8±2	9±1	9±1	8±1	10±1	7±1	9±1	8±2	7±1
	Ethyl acetate	7±1	8±1	6±2	7±1	7±1	5±1	7±1	6±1	5±1
	Chloroform	6±1	7±2	5±2	6±2	6±1	3±1	5±1	5±2	4±2
	Hexane	4±1	5±1	4±1	5±2	5±1	2±1	3±1	4±1	3±1
G. frondosa	Water	20±1	23±1	21±1	21±1	22±1	21±1	18±1	19±2	21±2
	Ethanol	18±2	22±1	17±1	18±1	20±1	19±1	17±1	14±1	16±2
	Acetone	16±2	17±1	15±1	16±1	14±1	12±1	12±1	12±2	13±1
	Ethyl acetate	14±1	13±1	12±2	13±1	11±1	10±1	10±1	9±1	11±1
	Chloroform	11±1	10±2	10±2	11±2	9±1	8±1	8±1	7±2	10±2
	Hexane	6±1	7±1	8±1	7±2	6±1	6±2	5±1	5±1	8±1
Standard drug		21±1	20±1	22±1	21±1	24±1	23±1	24±1	24±1	23±1

1- The standard drug used was ampicillin against the bacterial test microbes and nystatin against the fungal test microbes.

Aqueous extracts of all mushroom species fruiting bodies revealed superior antimicrobial activity than other polar extracts, this result agreement with results reported by Sivaprakasam *et al.* (2011) who demonstrated that aqueous extract of fruiting bodies from *Grifola lucidum* revealed strong antibacterial and antifungal activities against bacterial and fungal strains. Aqueous extract of *A. bisporous* fruit bodies achieved superior antimicrobial activity with inhibition zones similar to that of the standard drug, such in case of *E. coli* with inhibition zone 21 mm, or even exceeded the standard such in case of *P. aeruginosa and S. aureus* with inhibition zones 25 and 30 respectively. Aqueous extract of *A. bisporous* showed the highest antifungal effect comparing with the other extract against *C. albicans, C. neoformans, A. flavus, F oxyporum* and *P. expansum* with inhibition zones ranged from 19-21 mm. This result was in accordance with that of Jadadish *et al.* (2009) who demonstrated that *A. bisporous* polar extracts exhibited significant antibacterial activity against both Gram positive and Gram negative bacteria, as well as antifungal activity against *Candida albicans*, therefore, *A. bisporous* could be considered as a functional food with antimicrobial activity.

The results revealed that the aqueous extract of *G. frondosa* fruit bodies gave significant antimicrobial activity against all tested microorganisms. *S. aureus* and *C. albicans are* the most susceptible tested microorganisms to the aqueous extract of *G. frondosa* fruit bodies with 23 and 22 mm diameter inhibition zones, respectively, the antimicrobial efficiency of this extract agreement with Usman *et al.* (2012) who revealed that *Grifola frondosa* extracts had significant antibacterial effects against group of various Gram positive and Gram negative bacteria where antibacterial activity was higher against Gram positive than Gram negative

bacteria.

Evaluation of the antimicrbial activities of mycelial extracts*:*

As fruit bodies extracts the aqueous mycelial extracts exhibited the highest inhibition zones compared to other corresponding extracts (Table 2). *G. frondosa* mycelial aqueous extract demonstrated the highest antimicrobial activity which has been achieved by inhibition zone range from 17-23 mm, followed by *A. bisporous* aqueous extract (12-20 mm). The least antimicrobial activities are shown by mycelial aqueous extract of *P. ostreatus* (6-9 mm) against all tested microorganisms.

The non-polar extracts of *P. ostreatus* mycelia did not show any antifungal activities against all tested fungi except *C. albicans*, while the antimicrobial activities for all non-polar extracts of all tested mushrooms were more or less weak.

It was noticed that all mycelial extracts of different mushrooms had lower antimicrobial activity than their corresponding fruit bodies extracts which in agreement with Kalyonecu *et al.* (2010) who demonstrated the weakness of mycelia extract than corresponding fruit bodies of some mushrooms.

Evaluation of the antimicrobial activities of broth:

It can be deduced from Table (3) that the antimicrobial activity of broth(s) of different mushrooms under investigation was weak to moderate against all the tested microorganisms. The trend of antimicrobial efficacy of different mushrooms broth was as: *P. ostreatus* > *P. sajor caju* > *A. campestris* > *A. bisporous* > *G. frondosa* against all tested microorganisms.

Table 2: The inhibition zone produced by mushrooms mycelial extracts (using different solvent) against certain microbial species.

Mushrooms	Test organism	Bacteria				Fungi				
	Test solvent	*P. aeruginosa*	*S. aureus*	*Shigella sp.*	*E. coli*	*C. albicans*	*C. neoformans*	*A. flavus*	*F. Oxysporum*	*P. Expansum*
P. ostreatus	Water	8±1	9±1	7±1	9±1	8±1	7±1	7±1	6±2	7±2
	Ethanol	5±2	7±1	6±1	7±1	6±1	4±1	5±1	5±1	4±2
	Acetone	4±2	5±1	5±1	5±1	5±1	2±1	3±1	3±2	3±1
	Ethyl acetate	3±1	4±1	4±2	4±1	4±1	NIZ	NIZ	NIZ	NIZ
	Chloroform	NIZ	NIZ	3±2	3±2	4±1	NIZ	NIZ	NIZ	NIZ
	Hexane	2±1	2±1	3±1	2±2	3±1	NIZ	NIZ	NIZ	NIZ
P. sajor caju	Water	13±1	15±1	12±1	14±1	13±1	12±1	12±1	11.5±2	12±2
	Ethanol	10±2	11±1	10±1	12±1	11±1	10±1	9±1	10±1	11±2
	Acetone	8±2	9±1	8±1	10±1	9±1	8.5±1	8±1	8±2	9±1
	Ethyl acetate	7±1	8±1	7±2	8±1	8±1	8±1	6±1	7±1	7±1
	Chloroform	6±1	5±2	5±2	6±2	6±1	7±1	5±1	6±2	6±2
	Hexane	5±1	4±1	4±1	5±2	4±1	5±2	4±1	5±1	5±1
A. bisporous	Water	16±1	20±2	14±1	14±1	13±1	12±2	12±1	12±1	13±1
	Ethanol	12±2	19±1	12±2	12±1	11±1	10±1	9±1	11±1	12±2
	Acetone	11±2	15±1	10±1	10±1	9±1	9±1	8±1	11±2	11±1
	Ethyl acetate	9±1	10±1	7±2	8±1	8±1	8±1	6±1	9±1	9±1
	Chloroform	8±1	9±2	6±1	6±2	6±1	7±1	5±1	8±1	8±2
	Hexane	6±1	7±1	5±1	5±2	4±1	4±1	4±1	5±1	6±1
A.campestris	Water	12±1	14±1	14±1	14±1	13±1	12±1	11±1	12±2	13±2
	Ethanol	11±2	12±1	11±1	11±1	12±1	10±1	9±1	10±1	8±2
	Acetone	7±2	9±1	6±1	6±1	7±1	6±1	7±1	8±2	5±1
	Ethyl acetate	5±1	8±1	5±2	5±2	6±1	4±1	5±1	6±1	5±1
	Chloroform	4±1	7±2	5±1	5±1	5±1	3±1	4±1	5±2	4±2
	Hexane	3±1	3±1	4±1	4±1	4±1	2±2	3±1	4±1	3±1
G. frondosa	Water	18±1	23±1	18±1	21±1	19±1	18±1	18±1	17±2	18±2
	Ethanol	17±2	20±1	16±112±1	19±1	17±1	15±1	15±1	13±1	13±2
	Acetone	14±2	13±1	10±2	11±1	10±1	12±1	10±1	11±2	11±1
	Ethyl acetate	11±1	12±1	8±2	9±1	8±1	11±1	8±1	9±1	8±1
	Chloroform	7±1	6±2	6±1	7±2	6±1	8±1	6±1	7±2	6.5±2
	Hexane	4±1	3±1	18±1	5±2	5±1	6±2	4±1	5±1	3±1
Standard drug		21±1	20±1	22±1	21±1	24±1	23±1	24±1	24±1	23±1

1- The standard drug used was ampicillin against the bacterial test microbes and nystatin against the fungal test microbes.

2- Data are expressed as means of inhibition zone diameters (mm) ± SD.

3- NIZ: No inhibition zone detected under screening conditions.

The most susceptible microorganisms to *P. ostreatus* (broth) were *S. aureus*, *E. coli*, and *C. albicans* with 13 mm inhibition zones against all of them. By comparing broth of each mushroom with their fruit bodies and mycelial aqueous extracts, it was found that broth has the least antimicrobial activity for all mushroom species except for *P .ostreatus* since the broth had higher antimicrobial activity than mycelial aqueous extract but lower than that of fruit bodies' aqueous extract.

Table 3: The inhibition zones produced by mushroom broth against certain microbial species

Mushroom broth / Test microorganisms	*P. ostreatus*	*P. sajor caju*	*A. campestris*	*A. bisporous*	*G. frondosa*	Reference standard
Bacteria	Diameter of inhibition zone (mm)					Ampicillin
P. aeruginosa	10±1	9±2	6±2	5±1	4±1	21±1
S. aureus	13±1	8±1	5±1	4±1	3±2	20±1
Shigella sp	11±1	9±1	6±1	5±1	3±2	22±1
E. coli	13±1	11±1	5±1	7±1	5±1	21±1
Fungi	Diameter of inhibition zone (mm)					Nystatin
C. albicans	13±1	11±1	7±1	5±1	4±1	24±1
C. neoformans	9±1	8±1	6±1	4±1	3±1	23±1
A. flavus	9±1	7±1	5±1	4±1	3±1	24±1
F. oxysporum	10±2	8±1	6±2	3±1	2±1	24±1
P. expansum	9±2	8±2	5±1	3±1	2±1	23±1

1-Data are expressed as means of the inhibition zone diameters (mm) ± SD.

Antioxidant activity:

In this study, the level of antioxidants activity of fruit bodies, mycelial extract and broth of the tested mushrooms were evaluated using three different methods; percentage of DPPH scavenging, total phenolic content and reducing power.

Antioxidant activities of mushrooms fruit bodies extracted by different solvents:

All results indicated that aqueous extracts is the most potent solvent for all tested mushrooms fruit bodies followed by ethanol, ethyl acetate, acetone chloroform and finally hexane (Table 4). Dong and Yao (2008) had demonstrated that water extract from natural and cultured mycelium of *C. sinensis* had a better superoxide scavenging effect than methanolic extract. Also, Puttaraju *et al*. (2006) have reported that antioxidant activity of water extract from *Termitomyces* species was better than alcoholic extract. The aqueous extracts gave the highest % DPPH scavenging activities of 83.5, 76.1, 71.8, 71.5 and 57.87 % for *G. frondosa, P. sajor caju, A. campestris*, *P. ostreatus,* and *A. bisporous,* respectively. This result implies that although both mushroom species can inhibit free radical formation and scavenging activity, significant variation exists between the two species evaluated. Similar results were previously reported in literature (Unekwo *et al.,* 2014).

The highest DPPH scavenging activities were detected under these screening conditions for *Pleurotus sajor caju* and *G. frondosa* fruit bodies aqueous extracts. On the other hand the aqueous extract expressed the highest phenolic contents of tested mushrooms fruit bodies. The results of reducing power revealed that aqueous extracts of *G. frondosa* have the highest reducing power followed by *P. sajor caju, A. campestris, P. osteatus* and finally *A. bisporous*.

The results showed that the aqueous extract of *Grifola frondosa* fruit bodies showed the highest antioxidant activities among the investigated mushrooms these results were in accordance with Jan-Ying *et al.* (2011) who reported that antioxidant properties and antioxidants compounds of various extracts (ethanolic, cold water and

hot water) from the edible basidiomycetes *Grifola frondosa* showed high reducing power and chelating abilities on ferrous ions than ethanolic and hot water extracts. Ethanolic extract was revealed more scavenging DPPH than hot and cold water respectively, it was found that *Grifola frondosa* display a potent antioxidant properties due to the high content of total phenolic compounds

.

Table 4: Percentages of DPPH radical scavenging activities, total phenolic content, and reducing power of different fruiting bodies and mycelial extract of tested mushrooms.

Mushroom	Solvent	Fruit bodies extract			Mycelial extract		
		DPPH radical scavenging (%)	Total phenolic. (mg/gm)	Reducing power (absorbance)	DPPH radical scavenging (%)	Total phenolic. (mg/gm)	Reducing power (absorbance)
P. ostreatus	Water	57.7 ± 0.75	9.63±0.86	2.81± 0.19	25.25±1.98	7.3±0.45	2.3±0.12
	Ethanol	50.8 ± 2.23	5.3±0.98	2.40±1.5	19.42±1.52	5.4±0.26	2.04±0.96
	Ethyl acetate	38.8 ±1.91	4.48±1.3	2.11±0.62	10.16±0.98	6.1±1.2	1.81±0.16
	Acetone	38.8 ±1.91	2.18±0.24	2.06±0.19	6.42±1.23	4.3±1.05	1.61±0.98
	Chloroform	12.7 ± 1.87	0.77±0.14	1.74±0.97	3.7±1.5	0.87±0.21	1.2±0.98
	Hexane	3.7 ± 0.85	0.81±0.85	1.53±0.98	2.5±1.02	1.25±0.18	0.98±0.17
P. sajor caj	Water	76.1 ± 1.92	12.25±0.74	2.99±1.3	42±1.58	10.82±0.96	2.5±0.89
	Ethanol	70.8 ± 2.20	7.5±0.12	2.51±0.89	35±0.97	8.4±1.12	2.21±0.51
	Ethyl acetate	18.5 ± 1.48	5.19±0.47	2.25±0.87	29±0.87	6.7±1.8	2.011±0.75
	Acetone	37.7 ± 1.72	4.00±0.61	1.92±0.51	9.5±0.96	5.9±0.96	1.8±0.61
	Chloroform	16.2 ± 1.71	1.92±0.87	1.3±0.73	0.67±0.25	0.98±0.08	1.2±0.95
	Hexane	1.7 ± 0.85	1.21±0.48	0.79±0.09	0.98±0.12	0.87±0.1	1.09±0.97
A.bisporous	Water	71.5 ± 1.42	10.24±0.31	2.31±0.74	39.1±0.98	8.1±0.12	2±0.45
	Ethanol	69.1 ± 2.10	5.54±1.5	2.16±0.93	23±0.61	7.6±0.97	1.81±0.74
	Ethyl acetate	20.4 ± 0.71	3.6±1.4	1.81±1.2	13.4±1.7	5.8±0.84	1.5±0.15
	Acetone	58.4 ± 1.21	1.74±0.18	1.54±1.3	6.5±1.8	6.4±1.34	1.3±0.85
	Chloroform	16.6 ± 0.65	0.12±0.04	1.23±0.97	2.3±0.98	0.81±0.11	1.1±0.98
	Hexane	0.8 ± 0.21	0.99±0.14	1.11±0.97	2.1±0.36	0.99±0.03	0.95±0.11
A.campestris	Water	71.8 ± 1.01	9.4±1.2	2.87±0.86	32±0.31	9.3±0.54	2.2±0.95
	Ethanol	68.4 ± 2.00	4.2±0.74	2.40±0.45	19±1.2	7.3±0.34	2.01±0.72
	Ethyl acetate	58.1 ± 1.90	6.5±0.14	1.8±0.76	12.13±1.9	6.8±0.75	1.95±0.31
	Acetone	56.3 ± 1.41	2.4±0.74	1.5±0.26	6.15±0.91	3.45±0.38	1.5±0.61
	Chloroform	21.5 ± 1.68	0.79±0.07	1.31±0.74	3.2±0.19	0.93±0.15	1.2±0.19
	Hexane	3.4 ± 1.16	0.75±0.05	1.11±0.56	1.5±0.51	0.98±0.03	1.02±0.72
G. frondosa	Water	83.5± 0.97	13.89±1.5	3±1.09	50±1.64	11.5±0.42	2.6±0.95
	Ethanol	72.6± 2.48	7.3±0.65	2.61±0.18	39±1.97	10.26±0.94	2.32±0.12
	Ethyl acetate	55.12± 2.31	5.1±0.14	2.21±0.51	16.94±2.1	8.61±0.64	1.95±0.10
	Acetone	31.4±1.45	3.65±0.64	1.92±0.98	12.19±2.00	6.45±0.28	1.76±0.45
	Chloroform	22.1±0.97	1.4±0.25	1.61±0.56	3.4±2.00	1.15±0.06	1.2±0.13
	Hexane	5.1± 0.94	1.12±0.54	1.3±0.47	1.65±0.31	1.06±0.11	1.11±0.18

1-The data represent the means ± standard deviation from three replicates tested of 50 ug/ml.
2-Total phenolic content was measured as mg of gallic acid equivalent per mg of dried mushroom extract.

Antioxidant activities of mushrooms mycelial extracted by different solvents:

The results indicated that the polar extracts gave the highest percentage of DPPH radical scavenging activities than non-polar extracts for all tested mycelial mushrooms extract. *G. frondosa* and *P. sajor caju* aquatic mycelial extracts gave the highest percent of DPPH scavenging activity among the tested mushrooms .The highest total phenolic content found in aqueous mycelial extract of *G. frondosa* followed by *P. sajor caju*. The descending trend of aqueous extracts reducing power was *G. frondosa* > *P. sajor caju* > *P. ostreatus* > *A. campestris* > *A. bisporous*. Also the ethanolic extracts had the same arrangement but with lower O.D. This study was supported by Jagadish *et al*. (2011); Gan *et al*. (2013) and Ruksiriwanich *et al*. (2014) in which their findings had showed that water contributed to the highest yield of total phenolic content in mushrooms under the same extraction condition.

Antioxidant activities of mushrooms broth:

The results of percentage of DPPH radicals scavenging, total phenolic content and reducing power of tested mushrooms broth were illustrated in (Table 5). The broth of *G. frondosa* is the most potent antioxidant activity where expressed the highest

percentage DPPH radical scavenging, total phenolic contents and reducing power (41.1%, 12.41 mg GAE/g DW and 2.26 O.D.) respectively. On the other hand, the lowest percentage of the DPPH radical scavenging percentage and total phenolic content were demonstrated for *P. ostreatus* broth was with (15.12 % and 6.14 mg GAE/g DW) respectively, while the lowest reducing power was 1.75 O.D. for *A. bisporous* broth.

Table 5: The percentage % of DPPH radical scavenging activities, total phenolic content, and reducing power of the broth of different mushroom species.

P. ostreatus	15.12±1.3	6.14±0.61	1.95±0.96
P. sajorcaju	39.25±1.2	9.45±0.74	2.05±0.34
A. bisporous	22.47±2.3	6.24±0.45	1.75±0.75
A. campestris	17.36±1.8	8.6±0.37	1.95±0.18
G. frondosa	41.1±1.4	12.41±0.95	2.26±0.67

1-The numbers represent the means ± standard deviation from five samples

Fractionation of selected active extracts:

The previous experimental results cleared that the most active antimicrobial extracts was the aqueous extract of *A. bisporous* fruit bodies and the aqueous extracts of *G. frondosa and P. sajor caju* fruit bodies were the most antioxidant active extracts. So, the concentrated aqueous extracts of these mushrooms were fractionated separately through column chromatography packed with Sephadex G150 and the resulted fractions were evaluated for their antimicrobial activity against *S. aureus* and *C. albicans* in case of *A. bisporous* extract and for antioxidant activity in case of *G. frondosa* and *P. sajor caju.*

Bioguided fractionation of *A. bisporous* antimicrobial active extract:

The inhibitory effect of *A. bisporous* fractions against *S. aureus* indicated that 14 fractions were active with inhibition zone ranged from 3-25 mm. The highest activity was observed for fraction no. 10 with inhibition zones of 25mm.

Antimicrobial activity of *A. bisporous* fractions against *C. albicans* revealed that only 11 fractions out of total 23 fractions were active with inhibition zone ranged from 5 - 17mm. Fraction no.10 exhibited the highest antimicrobial activity.

The spectroscopic technique of the active fraction no. 10 revealed that it is a pure proteinic in nature compound. The estimation of the total soluble protein of the fraction no. 10 yielded 130.2µg/ml. Thus, this active pure compound was designated as compound I.

Bioguided fractionation of *P. sajor caju* and *G. frondosa* antioxidant active extract:

Antioxidant activity of *P.sajor caju* extract gave antioxidant activities with 20 fractions of total 24 fractions and these values ranged from 1.3 to 62.02 %.

The highest DPPH scavenging activity was observed for fraction no. 12 (62.02 %). The estimation of the total soluble protein in this fraction yielded 84.27µg/ml. The spectroscopic technique (LC-MS) revealed that this fraction contained one compound that was protein in nature and the pure compound designated as compound II.

The water extract of *G. frondosa* fruit bodies that exhibited antioxidants activity was subjected to sephadex G150 column fractionation. Biological evaluation of the antioxidant activities obtained from *G. frondosa* extract showed that 21 fractions of total 24 fractions gave antioxidant activity, their values ranged from 1.98 to 69.14 % which reflects that fraction no. 13 was the highest antioxidant activity (69.14 %). The estimation of the total soluble protein in this fraction yielded 97.06 µg/ml.

The spectroscopic technique (LC-MS) revealed that this fraction contained one compound that was protein in nature and the pure compound designated as compound III.

Efficiency of the purified compounds:

Antimicrobial activity of the purified compound I:

The antimicrobial activity of the compound I against *P. aeruginosa, S. aureus, Shigella sp,* and *E. coli* resulted in inhibition zones of 23, 25, 16 and 17mm respectively. When compare the results of antimicrobial activity with the ampicillin standard it is clear that the protein compound have higher activity than the standard against the *S. aureus* and *P. aeruginosa* (Table 6).

Regarding the results of the purified compound against the fungal tested organisms, the inhibition zones diameter decreased slightly for the purified compound than the nystatin standard for all tested fungal species.

Table 6: Inhibition zones produced by different doses of compound I:

compound / Test organisms	Compound I	Reference standard Concen.
Bacteria		Ampicillin
P. aeruginosa	23±1	21±1
S. aureus	25±0	20±1
Shigella sp.	16±0	22±1
E. coli	17±1	21±1
Fungi		Nystatin
C. albicans	20±0	24±1
C. neoformans	21±0	23±1
A. flavus	22±1	24±1
F. oxysporum	18±0	24±1
P. expansum	20±1	23±1

1-The data expressed as the means of inhibition zones (mm) ± Standard deviation from three replicates.

In this study, purified peptides showed higher antibacterial activity than the standard antibiotic (ampicillin). These results revealed the promising antimicrobial activity of the purified peptide which are in agreement with Liu *et al*. (2000); Vizioli and Salzet, (2002) results which demonstrated that the native peptide molecules which known as antimicrobial Peptides (AMPs), were active against a large spectrum of microorganisms, including bacteria and filamentous fungi.

Percentages of DPPH radical scavenging activities of different concentrations of compounds II and III:

The antioxidant activities of compound II and III isolated from *P. sajor caju,* and *G. frondosa* were evaluated using DPPH assay. Results in Table (7) revealed that the percentage of DPPH radical scavenging followed a concentration dependent manner. It can be deduced that the percentage of DPPH radical scavenging percentages ranged from 95.13-43.76% for compound II and ranged from 98.47 to 36.47% for compound III.

Table 7: Percentages of DPPH radical scavenging activities of different concentrations of compounds II and III.

compound / Con.(µg /ml)	Compound II	Compound III	Standard (Ascorbic)
100	95.13±0.69	98.47±0.42	95.14±0.84
50	92.62±0.36	96.52±0.52	92.48±0.24
25	89.77±0.25	85.42±0.47	78.91±0.67
12.5	86.28±0.14	79.15±0.96	56.5±0.81
6.25	78.64±0.17	72.31±0.46	43.82±0.35
3.12	62.89±0.95	52.18±0.28	23.78±0.43
1.56	55.89±0.84	42.91±0.19	17.49±0.85
0.78	43.76±0.34	36.47±0.47	9.62±0.18
IC_{50}	2.4	1.2	9.3

On the other hand it is clear that there was a slight difference between the percentages of DPPH radical scavenging percentage for both compounds, thus the estimated IC_{50} values were 2.4 and 1.2µg/ml for compound II and compound III respectively ,which were potent than the tested ascorbic acid standards (9.3µg/ml).

Cytotoxic activity of the purified compounds against mouse hepatocyte:

The cytotoxic effect of the purified compounds was evaluated against normal mouse hepatocyte cells using MTT assay. No cytotoxic effects were observed for compounds I, II and III Even when tested at high concentrations 400 µg/ml (Table 8).

Table 8: Cytotoxic activity of the purified compounds obtained from different mushroom species on the normal mouse lymphocyte.

Compounds / Conc. (µg/ml)	I	II	III
400	0	0	0
200	0	0	0
100	0	0	0
50	0	0	0
25	0	0	0
12.5	0	0	0

Characterization and Identification of the purified biologically active compounds:

Chemical characterization of compound I:

The antimicrobial active compound I obtained from *A. bisporous* fruit bodies was injected into LCMS system resulted in single pure compound that separated and characterized to determine the molecular weight and the amino acid sequence as shown in (Fig. 1). The molecular weight of the compound I is 16,242 Da which described as peptide with exclusive unique peptides and exclusive unique spectra, (28144 amino acids).

Beyond the antimicrobial activity of AMPs, these peptides are known to be multi-functional. In fact, it has been demonstrated their multiple roles as mediators of inflammation with effects on epithelial and inflammatory cells and the impact these roles have over such diverse processes as proliferation, immune induction, wound healing, cytokine release, chemotaxis, protease–antiprotease balance, and redox homeostasis (Ganz, 2002; Cole *et al*., 2003; Com *et al*., 2003; Liu *et al*., 2003).

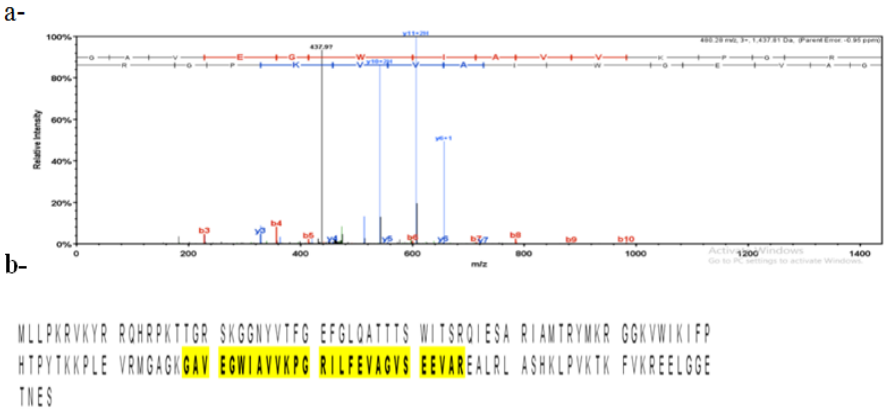

Fig. 1: Characterization of compound I (protein antimicrobial agent); (a) Mass spectrum; (b) amino acid sequence

Chemical characterization of compound II:

Characterization and separation of

compound II obtained from *P. sajor caju* fruit bodies using LCMS revealed the presence of on pure peptide as shown in (Fig. 2). The molecular weight of this protein is 43,411 Da with unique spectrum that contain 31412 amino acids.

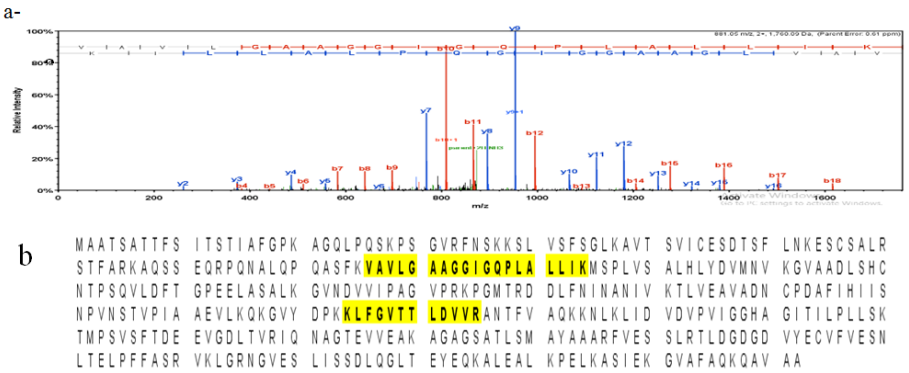

Fig. 2:Characterization of compound II, (a protein antioxidant agent) ; (a) Mass spectrum; (b) amino acid sequence.

Chemical characterization of Compound III:

In order to characterize the chemical constituents of compound III purified from *G. frondosa* fruit bodies aqueous extract, the mass spectrum was analyzed using LCMS followed by determination of the amino acid sequence. The molecular weight of this protein is 57,754 Da that has unique peptide with unique spectrum containing 2754 amino acids (Fig. 3).

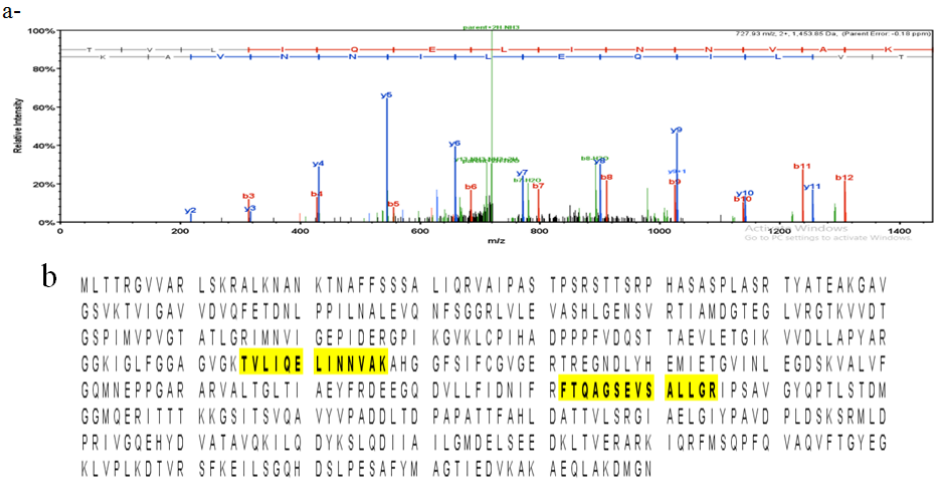

Fig. 3: Characterization of compound III, (a protein antioxidant agent); (a) Mass spectrum; (b) amino acid sequence.

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