Screening of Mushrooms collected from Azad Jammu and Kashmir to highlight their immense therapeutic potential.

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“IN THE NAME OF ALLAH THE MOST

BENEFICENT, THE MOST MERCIFUL”

DEDICATION

I would like to dedicate my thesis to those who have been a source of support for me throughout this journey. My deepest appreciation goes to my supervisor, Dr. Samiullah Khan, and co-supervisor, Mehmoona Sharif for their continuous support, guidance, and motivation. My special thanks go to my parents who have been there for me like a beacon of light, always praying for me and facilitating me to the best of their capacity.

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DECLARATION

I, ***Umme Habiba Saeeda,*** student of MPhil from the Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan hereby declare that the work embodied in this thesis entitled **"Screening of Mushrooms collected from Azad Jammu and Kashmir to highlight their immense therapeutic potential"** has developed by me based on my efforts under the guidance of my supervisor ***Dr. Samiullah Khan***. It is further declared that it does not contain any text, graphics, or tables copied from any source unless specifically acknowledged and the source is detailed in this thesis in the reference section. It is the result of original research and has not been submitted for any degree in any other Institution.

Umme Habiba Saeeda

CERTIFICATE

This thesis submitted by ***Umme Habiba Saeeda*** is accepted in its present form by the Department of Microbiology, Quaid-i-Azam University, and Islamabad, Pakistan; as satisfying the thesis requirements for the degree of Master of Philosophy in Microbiology.

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Umme Habiba Saeeda

TABLE OF CONTENTS

List of Tables

List of Figures

List of Acronym/abbreviations

˚C	Degree Celsius
MRSA	Methicillin-resistant *Staphylococcus aureus*
DMSO	Dimethyl Sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
PBS	Phosphate buffer saline
MDR	Multi Drug-Resistant
AMR	Antimicrobial Resistance
mg	Milligram
mL	Milliliters
hr	Hours
E. coli	*Escherichia coli*
P. aeruginosa	*Pseudomonas aeruginosa*
B. subtilis	*Bacillus subtilis*
S. aureus	*Staphylococcus aureus*
K. pneumoniae	*Klebsiella pneumoniae*
C. albicans	*Candida albicans*
A. niger	*Aspergillus niger*
MHA	Muller Hinton agar
SDA	Sabouraud Dextrose agar
TLC	Thin Layer Chromatography
HPLC-MS	High Performance Liquid chromatography-mass spectrometry
Rf	Retention Factor

ABSTRACT

The alarming rise in antimicrobial resistance has raised serious health concerns across the world. Exploration of novel drug candidates has become the need of the day. Macrofungi are renowned for therapeutic potential which makes them an attractive candidate for drug discovery. This study was a deep dive into the screening of mushrooms collected from the AJK for their therapeutic potential. Bioactivity assays that have demonstrated concrete evidence of the desirable effects of mushrooms on human health were the most critical contribution of this study. *Trametes versicolor* has proven to be the most potent mushroom as a very substantial amount of activity has been reported by all its extracts against all tested MDR strains with a zone of inhibition in the range of 11mm to 22mm. The antifungal activity of macrofungal crude extracts was very negligible with the highest zone of inhibition of 12 ± 1.00mm recorded.

The antioxidant potential suggests how consuming mushrooms as a whole or supplements can help prevent oxidative stress-related diseases. The best antioxidant activity of 78 ± 1.00%, 72 ± 1.00%, 75 ± 2.08%, and 75 ± 1.00% was recorded by ethyl acetate, acetonitrile, ethanol, and methanol extracts of *Phellinus glivus*. Cytotoxic activity analysis paves the way for further investigation of mushrooms for the exploration of novel chemotherapeutic agents. The strongest cytotoxic activity was reported by the *Amanita phalloides* with maximum activity demonstrated by the ethyl acetate, acetone, acetonitrile, and ethanol extracts with 80% mortality at the concentration of 1mg/ml. The total flavonoid content in all mushroom species was fairly high with *Phellinus gilvus* showing the highest concentration in the range of 110 to 174mg QE/g of all seven fractions. The highest total phenolic content was reported in *Amanita phalloides* with values of 107, 94, and 85 mg GAE/g, recorded in methanol, hexane, and acetone extracts. Active fractions were subjected to elution column chromatography and further analyzed for antibacterial activity. Active fractions of purified compounds were further purified via thin-layer chromatography and subjected to antibacterial analysis. The findings of this research have laid the groundwork for further studies like characterization and artificial synthesis of these compounds.

1. Introduction:

The drastic changes in the lifestyle of man, from active to sedentary, and shifts in dietary habits, from consuming fresh and organic fruits and vegetables from farm to eating junk foods have made humans weak physically as well as immunologically thus making them vulnerable to diseases and infections that had no existence a few centuries back. Humans have been crudely using natural resources such as plants, weeds, etc. for the treatment of a plethora of diseases. With the advancement of scientific knowledge, they became more refined in strategizing and started approaching diseases in a more precise and targeted manner. Instead of just crushing some weeds and gulping down the throat with a mixture of useful and useless compounds, they started to identify the exact therapeutic molecules and packed them in the form of tablets and capsules. The discovery of Penicillin by Alexander Fleming in 1928 unlocked new avenues in the field of medicine (Bennett & Chung, 2001). The realization that some invisible microscopic creatures can produce molecules that are capable of healing triggered a revolution in the field of medicine. The time period between 1940-1962 is marked as the golden age of antibiotics as most of the antibiotics we use today such as salvarsan, prontosil, penicillin, and streptomycin were discovered in those days (Prescott, 2014). Unfortunately, due to the unattended use of antibiotics, pathogenic microbes gradually began to develop resistance against them. Certain modifications were made to those antibiotics so as to cater to this challenge resulting in the origin of semisynthetic antibiotics like clarithromycin and azithromycin with enhanced potency and a broader spectrum of activity. Nonetheless in this war of survival between humans and pathogens, none is willing to surrender (Kondo & Hotta, 1999). Despite the advancement of humans in knowledge and technology, we are incapable of keeping up with the pace at which bacteria are evolving and developing resistance against every class of antibiotic introduced. This calls for a more rigorous and robust approach to drug discovery. This can be actualized by exploring untapped sources of nature such as plants, prokaryotes, marines, animals, and mushrooms etc. (Béni et al., 2018).

Since immemorial times mushrooms have been of great value to humans and were utilized for their nutritional and therapeutic potential. Romans regarded mushrooms as the food of Gods. The extraordinary potential of metabolites from mushrooms has stirred scientists to carry out more aggressive research to highlight the potential of these sources and the possibilities to materialize them from raw form to refined products of great health as well as economic value. Mushrooms are deemed functional foods because they can be utilized as a normal diet to promote health (Reis et al., 2017). They are teeming with carbohydrates, proteins, minerals, vitamins, phenolic compounds, unsaturated fatty acids, carotenoids, tocopherols, and ascorbic acid which makes them a wholesome food supplement. Moreover, a wide range of activities of pharmacological importance have also been reported including but not limited to, antibacterial, antifungal, antioxidant, anti-tumor, anti-malarial, anti-HIV, anti-inflammatory, anti-fibrotic, anti-diabetic, immunomodulatory, liver protectant, blood-sugar reducing, and cholesterol-lowering etc. To date, these drugs have been utilized in a way that is not up to par with modern-day quality specifications and standards. European Union (EU) has predicted the rise in death toll to 10 million deaths per year by 2050 due to antimicrobial resistance. This has inspired researchers to discover novel drugs and alternative treatment strategies.(M. Kumar et al., 2023).

To date, approximately 10,000 species of mushrooms have been identified among which almost 60000 can produce visible fruiting bodies(Hawksworth, 2001). The versatile varieties of macrofungi scattered around the globe are a product of divergent evolution and belong primarily to two phyla; Ascomycota, and Basidiomycota though a few are found in Zygomycota as well. Mushrooms have a distinct fruiting body that can be epigeous or hypogeous. The fleshy fruiting bodies function as the sexual reproductive structure whereas sclerotia acts as the asexual reproductive structure. They exhibit diverse and remarkably distinct ecology (Afrin Joty et al., 2020). Mushrooms have dynamic metabolism and play a significant role in maintaining a balance of ecosystems. They are vital decomposers that play a significant role in the degradation of pectin and lignocellulosic components in the environment. Some of these exist in symbiotic

relationships with terrestrial plants and are identified as ectomycorrhizal fungi (Ismail et al., 2018).

It has been hypothesized that fungi share a closer genetic relationship with animals as compared to plants. The common infective agents of fungi and animals such as *Pseudomonas aeurignosa*, *Escherichia coli,* and *Staphylococcus aureus* make them strong candidates with the capacity to produce metabolites that are of high therapeutic value for humans (Barr et al., 2005). A myriad of important antibiotics such as penicillin, cephalosporin, griseofulvin, and amoxycillin have been derived from macrofungi (Kwon-Chung, 1994). The use of polysaccharides and glycoproteins such as lentinan, schizophyllan and krestin for their immunostimulating properties as adjunct in cancer treatment is very widespread in Asian countries. However, only a few compounds isolated from macrofungi have reached clinical trials such as an illudin S derivative that has reached phase 2 of human clinical trials (Jaspers et al., 2002).

A maximum proportion of ailments encountered in today's world such as gastroenteritis (AGE), bloodstream infections, colitis, meningitis, strep throat, urinary tract infections (UTIs), vaginosis, gonorrhea, syphilis, chlamydia, respiratory tract infections, bacterial food poisoning are caused by the common pathogens (Vouga & Greub, 2016). The giant strides made by medical science through antimicrobial discoveries have been saving lives. On the downside, the unattended use of commercial antibiotics has given rise to multi-drug resistant bacteria which is exacerbated by the ability of bacteria to transfer resistance genes among each other, rendering the newest generations of antibiotics totally obsolete. Other than antibiotic abuse certain other factors that have contributed prominently to antimicrobial resistance (AMR) are self-medication, consumption of expired antibiotics, and antibiotics with lower active ingredients (K. W. K. Tang et al., 2023). Additionally, biofilm production is one of the most significant processes that is utilized by bacteria to acquire resistance. Biofilm is a complicated and dynamic structure composed of colonies of bacteria of single or multiple species adhered to a solid surface in the form of a group. These cells are encapsulated by a matrix commonly known as extracellular polymeric substance (EPS) (K. W. K. Tang et al., 2023). It typically comprises proteins,

polysaccharides, and environmental DNA and is remarkably resistant to antibiotics. However, due to the dangerous rise in the level of AMR bacterial metabolic pathways are now being analyzed as a potential drug target site. Numerous natural sources such as prokaryotes, fungi, animals, plants, and marines are promising candidates that need to be investigated (Jorge et al., 2019).

Just like plants, mushrooms possess antioxidants thus, thus playing an important role in protecting organisms against oxidative stress which can be used as a chemo preventive measure for protection against diseases caused by reactive oxygen species (ROS). Research has highlighted the immense antioxidant potential of mushrooms attributed to the presence of vitamins A and C, beta-carotene, and secondary metabolites such as steroids, phenolic compounds, and terpenes (Tsao & Deng, 2004). Several mushrooms have been reported with prominent radical scavenging activities such as *Agaricus bisporus, Inontous obliqus, Ganoderma lucidum, Pleurots eryngii, Pleurotus ostreatus, Lentinus edodes, Agaricus blazei, Flammulina velutipes etc.* A vast number of diseases such as nephropathic, hepatopathic, Alzheimer's disease, retinopathic damage, cancer, diabetes, respiratory syndrome is caused by oxidative damage that can be prevented by consuming antioxdants. This is actualized by scavenging free radicals, obstructing lipid peroxidation chain reactions, and chelation of catalytic metals (Kostić et al., 2020).Mushrooms from Basidiomycota are considered a highly valuable source of molecules with therapeutic potential. They manifest vast structural diversity such as cyclic peptides, steroids, polysaccharides, and sesquiterpenes. (Money, 2016).

The endeavor commences with the harvest of mushrooms and initial identification on the basis of morphological features such as fruiting body color, size, presence or absence of cap, gills, stipe, rings, scales, reticulum, zonation, striation, and worts (M. K. Yadav et al., 2017). This is preceded by the drying, crushing, extraction, purification, activity analysis, and characterization of compounds. The cheap cost, natural origin, easy access, and enormous healing potential of mushrooms make them a very attractive target for the discovery of novel therapeutic molecules. The majority of biologically active molecules are the products of secondary metabolism and are non-nutrients in essence. They are

fundamentally produced to protect mushrooms from microbial infections, and insect attacks. Additionally, nutritional components like carbohydrates, proteins, lipids, etc. have also demonstrated promising pharmacological activities.

First of all, secondary metabolites such as flavonoids, alcohols, glycosides, terpenes, steroids, and saponins are extracted from the powdered biomass of mushrooms. This can be done via a myriad of extraction procedures such as maceration, percolation, infusion, Soxhlet extraction, decoction, microwave-assisted extraction, etc. The choice of appropriate extraction procedure is dictated by the nature of mushroom biomass, availability of instruments, type, and pH of solvents, and end use of the product (Palai & Shekhawat, 2022). Primary screening of mushroom extracts is carried out by checking their antimicrobial, antioxidant, antiviral, antitumor, and antidiabetic activities, etc. The potent extracts can be further purified via paper chromatography (PC), thin-layer chromatography (TLC), gas chromatography (GC), and high-performance liquid chromatography (HPLC) (Ruthes et al., 2015). Further characterization can be carried out via FTIR, LC-MS, GC-MS and NMR etc.

AIM AND OBJECTIVES:

Aim:

The central aim of this study was to assess the therapeutic potential and distribution of bioactive metabolites in mushrooms harvested from AJK, Pakistan.

Objectives:

The main objectives of this study were:

- Collection and Morphological identification of Mushrooms collected from Azad Jammu and Kashmir, Pakistan
- Extraction of Bioactive metabolites from the mushrooms
- Evaluation of Antibacterial potential of crude extracts of mushrooms
- Evaluation of Antifungal potential of crude extracts of mushrooms
- Determination of Antioxidant potential of crude extracts of mushrooms
- Determination of Hemolytic potential of crude extracts of mushrooms
- Assessment of mycochemical constituents of crude extracts of mushrooms
- Purification of bioactive extracts using Elution Chromatography and TLC.

2. Review of Literature:

2.1. Kingdom Fungi:

Fungi are eukaryotic, heterotrophic organisms with absorptive mode of digestion. They either have digestive enzymes bound to their cell wall or release them into the environment which convert food into simple, digestible molecules which are then absorbed by mycelia that are highly branched in order to provide high surface-area-to-volume-ratio to facilitate efficient absorption. They are often found growing in places enriched with organic matter with a cool and humid atmosphere (Richards et al., 2017). They can be conveniently spotted growing on forest floors and play a pivotal role in maintaining the equilibrium of the ecosystem by decomposing dead bodies of plants and animals. Based on the mode of nutrient acquisition and nature of interaction with other organisms' fungi are classified into numerous categories. Biotrophs fulfill their nutritional requirement from living hosts (plants and animals). Those who obtain their nutrition from dead organisms are termed saprotrophs. Necrotrophs obtain their nutrients from living organisms by killing their host cells. Fungi are one of the most versatile kinds of living organisms that are scattered all over the planet and can be found growing in a variety of environments from water and soil and in association with plants and animals that can be symbiotic or parasitic (Perotto et al., 2013).

2.2. Morphology:

Until the end of the 20th century fungi was part of the kingdom Plantae. However, later they were recognized as absolutely distinct entities which led to the emergence of a separate kingdom of their own. The fundamental feature that separates them from plants and animals is their unique mode of nutrition. Plants synthesize food for themselves on their own, animals ingest food acquired from other living organisms whereas fungi have an absorptive mode of nutrition. There is a sharp contrast in chemical composition as well. For instance, fungal cell membrane contains ergosterol whereas animal cell membranes are composed of cholesterol. Likewise, the cell wall of plants is composed of cellulose whereas

fungi contain chitin, chitosan, mannans, glucan, and glycoproteins in their cell walls whereas animals have no cell wall at all. Fungi may be unicellular or multicellular. Their cells are filamentous structures composed of long, thread-like filaments that are connected by the ends and are called hyphae. Bundles of hyphae connected with one another are called mycelium. In the sexual phase of the fungal life cycle, reproductive hyphae are produced which organize to form a fruiting body, also called sporocarp that bears spore-producing structures like asci and basidia. It is solely produced for the production and release of spores. Production of the fruiting body occurs in the sexual phase, and the remaining life cycle entails vegetative mycelial growth (Gow et al., 2017).

These filamentous cells may be coenocytic; cells that are not individually distinct and have multiple nuclei as in zygomycetes and chytrids. Whereas basidiomycetes and ascomycetes are septate, that is, their cells are separated by cross-walls known as septa. There are considerable variations in the structure of these septa which have great taxonomic significance (Free, 2013). Majority of fungi completely lack flagella at all stages of their life cycles with chytrids being the only exception that produces flagellated gametes. This feature has a huge impact on the dynamics of the fungal mode of reproduction and makes direct physical contact between two organisms mandatory in the case of sexual reproduction. Fungi that exist in unicellular form reproduce via budding. The newly produced bud may remain attached or detach from the parent cell and gives rise to another bud in a similar fashion and the process goes on.

Fungi may reproduce sexually as well as asexually. Mitotic division of the cells gives rise to identical structures called conidia or spores. Whereas sexual reproduction is characterized by the meiotic division of the cells that involves the fusion of nuclei from two distinct cells. However, some species of fungi undergo self-fertilization, thus giving rise to sexual structures within the same thallus, such fungi are called homothallic. However, the majority of them are heterothallic which requires an intimate connection between two compatible fungal strains thus causing the fusion of haploid nuclei followed by meiosis. In some fungi production of these haploid spores occurs in microscopic

structures while in mushrooms they are produced in macroscopic structures called fruiting bodies (Chamberlain & Ingram, 1997).

2.3. Classes of Fungi:

Based on mode of reproduction and molecular data, fungi are classified into five phyla. According to a crude estimation, there are approximately 20 million species of fungi out of which only 100,000 are known which is increasing perpetually with the discovery of round about 1000 novel species annually. Chytridiomycota (Chytrids), Zygomycota (Conjugated Fungi), Ascomycota (Sac Fungi), Basidiomycota (Club Fungi), and Glomeromycota are regarded as true phyla whereas Deuteromycota is specified for a group of unrelated species that share some mutual characteristics with fungi possessing asexual mode of reproduction(Brandt & Warnock, 2011).

2.4. Macrofungi:

The term macrofungi alludes to all fruiting body-producing fungi. They have versatile ecological roles and are food sources for a wide variety of animals and are key decomposers of the ecosystem. The majority of macrofungi exhibit a fleshy fruiting body that acts as sexual reproductive structures. However, the asexual reproductive stage is represented by another visible structure known as sclerotia. Their fruiting body may be located below the ground (hypogenous) or above the ground (epigeous). These fruiting bodies are composed of reproductive hyphae, whose filamentous structures are tightly intertwined to form mycelium which is then packed in the form of fruiting bodies whose primary function is the formation and release of spores. The number of macrofungi in the world is huge. Spores are small unicellular, desiccated and dormant structures with a diameter in the range of 1-100µm, capable of surviving harsh environmental conditions and responsible for propagating fungal species across the planet. Out of 1000,000 species of fungi that have been documented, almost 6000 are macrofungi (Tuo et al., 2022). They exhibit diverse lifestyles ranging from saprophytic and symbiotic to parasitic and predatory. Most of them are not self-sufficient, especially the symbiotic species like ectomycorrhiza need the help of their host partners to reproduce and disperse.

Macrofungi can be spotted growing on a myriad of terrestrial habitats from grassland to forests. They are often found sprouting on the fallen tree trunks, old stumps, and roots of trees. They typically grow in clusters of three to six, however, at times a tuft of about thirty mushrooms can be found growing together. In a natural environment, macrofungi have a shorter life span and decay as swiftly as they grow and are conveniently tattered by rain. Generally, macrofungi tend to grow well in dark places with moderate temperature and high moisture. High temperatures and low relative humidity inhibit spore germination and fungal growth (Lu et al., 2020).

These fungi have a very divergent evolutionary history. They primarily belong to two main phyla, Ascomycota, and Basidiomycota. The largest group of macrofungi is Ascomycota comprising almost 6400 species with a characteristic cup-shaped fruiting body known as ascoma that bears spore-producing structures known as ascus (Balmford et al., 2000). Almost 3100 species are present in the phylum Basidiomycota with a characteristic umbrella-shaped fruiting body known as basidioma dangling on a cylindrical structure called stalk or stipe.

It has been estimated that roughly 1.5 million species of fungi are present on the surface of earth out of which presumably 140,000 are macrofungi. Out of these almost 10% have been identified of which 16% are culturable (Zhang et al., 2012). Macrofungi have multifaceted commercial applications. Some like truffles, shiitake, and king oyster mushrooms are very heartily savored delicacies by food lovers. Others like shiitake, maitake, and *Ganoderma lucidum* are very important pharmacologically because of their numerous bioactive properties like antibacterial, antifungal, antioxidant, anticancer, and so on. Others like turkey tail, lion's mane, and reishi are used as dietary supplements. However, some like *Amanita phalloides* can produce deadly toxins like amatoxin henceforth, they must be dealt with great caution (Meng et al., 2016).

Numerous agrochemical fungicides and drugs including antibiotics, immunosuppressive drugs, and cholesterol-lowering agents are the product of fungal secondary metabolism. Certain mycotoxins obtained from macrofungi like ergot alkaloids are used for treating migraine and other neurological diseases. Macrofungi have been traditionally manipulated

to treat diseases for ages. As the second most diverse organism with only a few species explored for their therapeutic potential, an extensive study needs to be carried out to tap into the potential of this diverse and enriched reservoir of bioactive metabolites (Meng et al., 2016).

2.5. Metabolites:

The multitude of chemical reactions taking place in an organism to convert food into energy is known as metabolism. There is a complex network of distinct pathways allocated for the production of compounds required for the growth, development, and protection of organisms. These pathways are intertwined in a very complicated yet elegant manner such that the product of one reaction serves as the reactant of the next. The entire network of metabolic pathways and the interaction between them is known as metabolome. The intermediates and end products of these biochemical pathways are known as metabolites. Some of the metabolites are produced within the cell however some of them like vitamins, essential amino acids are acquired from external sources like diet (De Silva et al., 2013).

2.5.1. Primary metabolites:

Metabolites like protein, peptides, amino acids, polysaccharides, fatty acids, alcohols, lactic acid, ethylene etc., typically produced during the growth phase of cell cycle are necessary for the growth, development and reproduction of an organism are known as the primary metabolites (Zaynab et al., 2019).

2.5.2. Secondary metabolites:

Initially, it was observed that there are certain metabolites produced by bacteria, fungi, and plants that are not imperative for the growth and development of organisms hence, named secondary metabolites (SM). However, SM is anything but secondary, and now the term: specialized metabolites" is being used to describe them. It has been established that SMs play a vital role in the survival of organisms as they play a key role in dictating the dynamics of the interaction of an organism with its environment. Oftentimes products of primary metabolism act as precursors for secondary metabolism. For instance, tryptophan,

a primary metabolite, is required for the synthesis of actinomycin, a secondary metabolite. They are generated during the secondary phase of the cell cycle. Alkaloids, pigments, terpenes, and polyketides are some of the examples of secondary metabolites. They are especially required by organisms in competitive environments. SMs have great commercial value and are being used in the food, pharmaceutical, and cosmeceutical industry (S. Agostini-Costa et al., 2012).

2.6. Nutritional composition of mushrooms and their bioactive properties:

Mushrooms have been described as an incredible source of nourishment. Carbohydrates constitute the principal nutritional component of dry matter of mushrooms which can be digestible (glucose, glycogen, trehalose, mannitol, glycogen) and non-digestible (β-glucan, chitin, mannnas). The proportion of polyunsaturated fatty acids (PUFA) is prominently higher than the saturated fatty acids constituting almost 75% of total fatty acids most of which are oleic and linoleic acids. Their protein contains all the essential amino acids and is the only vegetative source of vitamin D as well as vitamin B required by the human body in order to function optimally. Furthermore, they are a good source of minerals such as potassium, phosphorous, zinc, iron, copper, and calcium. Alongside these mushrooms also contain numerous bioactive components such as peptides, proteins, polysaccharides, proteoglycans, terpenes, lectins, phenolic compounds, etc. that are responsible for healing properties of mushrooms like antioxidant, anti-tumor, anti-cancer, anti-inflammatory, hepatoprotective, anti-diabetic, detoxicating, analgesic etc. They can either be consumed directly as whole mushrooms or in individuals as fractions numerous bioactive components are now available in the market. The increasing trend of consuming healthy and functional food has led to a global increase in demand for mushroom cultivation. The most cultivated species across the globe are *Agaricus bisporus, Lentinus edodes, Flaminula velutipe,* and *Pleurotus* species. As compared to commercial varieties wild varieties of mushrooms possess higher quantities of phenols, proteins, PUFA and α-tocopherolas compared to the commercial mushroom varieties. Growth, yield, quality and nutritional composition of mushrooms are greatly influenced by the composition and nutritional status (C; N ratio,

macro and microelements, phytohormones, vitamins) of mushrooms. An elaborate description of certain components of mushrooms is presented in the following section (S. Agostini-Costa et al., 2012).

2.6.1. Carbohydrates:

Carbohydrates comprise almost 50–65 g 100 g−1 of dry weight of mushrooms. The most found monosaccharides in mushrooms are glucose, galactose, fructose, fucose, xylulose, mannose, rhamnose, trehalose, arabinose, and mannitol. Almost 80% of the total free sugar is mannitol, therefore, is also called mushroom sugar. These compounds confer numerous biological functions upon mushrooms such as anticancer, antidiabetic, anti-hypoxia, anti-inflammatory, antilipidemic, hypoglycemic, immunomodulatory, antilipidemic, antimicrobial, and antioxidant. The indigestible carbohydrates, chitin, and β-glucan are the principal components of mushroom cell walls and confer several physiological functions (Béni et al., 2018). A major proportion of polysaccharides is dietary fibers that have a very positive influence on the distribution of gut microbiota. These polysaccharides are degraded into metabolites such as short-chain fatty acids (SCFA) by the gut microbiota and help maintain intestinal homeostasis. Consumption of β-glucan supplemented diet strengthens the immune system thus helping combat upper respiratory infections, seasonal allergies, obesity-related disorders, and osteoarthritis and minimizes the side effects of chemotherapy in cancer patients (H. Kumar et al., 2021).

2.6.2. Proteins:

Proteins comprise approximately 19–35 g 100 g− 1 dry weight of the mushroom. The amount of protein may vary according to the specie, time of harvest, pileus size, and substrate composition. Proteins are fundamental bioactive components and are of great value because of their pharmacological properties such as immunomodulatory, anti-inflammatory, antimicrobial, etc. All essential 9 amino acids required by humans such as lysine, isoleucine, valine, histidine, threonine, phenylalanine, and methionine are present in mushrooms in sufficient quantity (Wong et al., 2010). Henceforth they can be used for the fortification of several foods as well as a substitute for animal protein. Alongside, ornithine, γ-aminobutyric acid (GABA), has also been extracted from mushrooms which

have important applications in the detoxification of toxins and liver function enhancement. Another novel class of proteins known as Ribosome inactivating proteins (RIPs) with remarkable healing potential has also been discovered. RIPs have the capability to inactivate ribosomes via the removal of adenosine residues from rRNA. Moreover, mushroom proteins have displayed a good effect on the treatment of gut-related disorders. Mushroom proteins have a remarkable influence on the composition of the microbiota and their metabolite production. Additionally, bioactive peptides in mushrooms have demonstrated great pharmacological effects such as antioxidant, antibacterial, and antihypertensive properties (X. Xu et al., 2011).

2.6.3. Lipids:

A very sparse amount of lipids is present in mushrooms comprising about 2–6 g 100 g− 1 of dry matter. The content of unsaturated fatty acids is higher in contrast to saturated fatty acids. To be precise, linoleic acid (C18:2) and oleic acid (C18:1) are the most widespread. Linoleic acid acts as the precursor for the synthesis of 1-octen-3-ol, an aromatic compound that is responsible for imparting specific flavor in mushrooms (HANUŠ et al., 2008). Ergosterol and tocopherol, which have strong antioxidant potential are also present in its lipid fractions (Atila et al., 2017).

2.6.4. Vitamins:

Edible mushrooms with their rich arsenal of vitamins are a promising candidate for nutraceutical formulations. The wide variety of vitamins present in mushrooms are vitamin D2, ascorbic acid, niacin, folate, thiamine, tocopherol, riboflavin, and thiamine. The amount of vitamin B12 in mushrooms is comparable to the amount present in fish, liver, and beef with impressive bioavailability which is of special value for people who are vegetarian (Mattila et al., 2001). Low-level of vitamin D is reported in cultivated mushrooms. Nevertheless, they do have a fair quantity of provitamin like ergocalciferol. Production of vitamin D2 is carried out via photo-conversion of ergosterol into vitamin D2. Significant quantities of vitamin D2 can be acquired from UVB-irradiated cultivated mushrooms like *Pleurotus and Agaricus* (Cardwell et al., 2018).

2.6.5. Minerals:

Significantly higher proportions of minerals like magnesium, potassium and phosphorous are present in mushrooms. However, calcium and sodium are present in relatively moderate and low quantities respectively which makes them a safe choice for hypertensive people. For instance, the low quantity of sodium in *L.edodes* makes it a suitable choice for people with diabetes. Potassium is the primary constituent of mushrooms that is dispersed heterogeneously in various regions of fruiting body with the highest stock in cap, preceded by comparatively lower concentrations in stipe, spore forming region and least in spores. Mushrooms ae rich in dietary selenium with the highest concentration present in *Bloteus edulis* (Mallikarjuna et al., 2013).

2.7. Mycochemical diversity in mushrooms:

2.7.1. Phenolic compounds:

Flavonoids, hydroxycinnamic acids, lignans, tannins, oxidized polyphenols, tannins, phenolic acids, stilbenes, lignans, and hydroxybenzoic acids are all categorized as phenolic compounds. Phenolic compounds in mushrooms perform a spectrum of functions such as free radical inhibitors, oxygen-scavengers, and metal-inactivators. Additionally, they have demonstrated anti-microbial, anti-cancer, and anti-inflammatory properties with concomitant protection from several degenerative disorders such as cardiovascular diseases, aging, and brain dysfunction. Certain phenolics isolated from mushrooms such as syringic and chlorogenic acids manifest antiangiogenic effects (Palacios et al., 2011). They can improve blood vessel synthesis by preventing vascular endothelial growth factor (VEGF). The highest quantity of syringic and chlorogenic acids (0.31 and 0.45 mg g− 1 DW, respectively) was acquired from *Lentinus edodes* with a promise in the enhancement of osteoporosis treatment. Ethanolic extracts of *Ganoderma lucidum* were rich in flavonoids such as rutin, naringenin, morin, myricetin, and hesperetin and are amenable for their anti-proliferative properties(Abdelshafy et al., 2022).

2.7.1. Phenols:

Phenols are the product of the secondary metabolism of mushrooms and exhibit a wide array of therapeutic properties such as antimicrobial, antioxidant, antitumor, anti-

inflammatory, etc. They have a single aromatic ring with one or more hydroxyl groups. They exhibit a spectrum of structures ranging from simple molecules to complicated polymers. Numerous phenolic compounds like vanillic, salicylic, syringic, gallic, coumaric cinnamic, caffeic and chloro-genic acids have been isolated from several mushroom extracts (Tuladhar et al., 2021). Because of these therapeutic properties, there is an increasing interest in the incorporation of phenols in health-promoting products. Phenolics owe their antibacterial properties to their phenol hydroxyl group. Their mechanism of action includes disturbing the metabolism of microorganisms by interfering with oxidative phosphorylation via nonspecific interactions, inhibition of microbial enzymes, and withdrawal of substrate required for microbial growth. Several studies highlight that the antimicrobial activity exhibited by numerous mushroom extracts such as *Sarcodon imbricatus, Ganoderma lucidum,* and *Lactarius deliciosus* is because of the presence of flavonoids and total phenol content. A detailed investigation of *Ganoderma* species has revealed that their antimicrobial activity is because of the presence of compounds like ganomycin A and B and hydrohynon composition (Kuhar & Papinutti, 2014).

2.7.2. Tannins:

Tannins are polyphenolic biomolecules that cause precipitation of organic compounds like amino acids, and proteins alkaloids. Therefore, the term tannin is broadly used for polyphenolic compounds containing sufficient functional groups like hydroxyl and carboxyl to form complexes with numerous macromolecules. Tannins are extensively distributed in plants and mushrooms where they help regulate growth and provide protection against predation by insects. The molecular weight of tannins generally ranges from 500 to 3000 Daltons. The dry and puckery sensation caused in the mouth by chewing an unripened fruit is because of astringency from tannins. The modification and destruction that occurs in the structure of tannins with the passage of time play a key role in dictating harvest time (Yıldız et al., 2017).

Tannins are categorized into two main groups, the ones made by an assemblage of flavonoid monomers called condensed tannins and the hydrolyzed tannins that are esters of phenolic acids and sugars. The capability of tannins to bind and precipitate organic

molecules confers their antimicrobial properties as it binds and disables adhesions, transport proteins located in the cell membrane of microorganisms, and form complexes with metal ions and polysaccharides. The tannic acid from the extracts of *Lentinus edodes* has demonstrated antimicrobial activity against the fungus *Candida albicans* and bacteria, *B. Cereus* and *M. leteus* (Song et al., 2020).

2.7.3. Coumarins:

Coumarins are polyphenolic compounds that belong to a group of crystalline, colorless, and oxygenated heterocyclic compounds. They were first isolated from the plant *Dipteryx odorata*. Coumarin (1,2-benzopyrone or 2H-1-benzopyran-2-one) and coumarin derivatives are widely distributed in plants and mushrooms. Almost 800 natural coumarin derivative compounds have been isolated from 600 genera of 100 families. The vast majority of coumarins are isolated from vascular plants and fungi from basidiomycetes and the family Xylariaceae (Ascomycetes) however, others like coumermycin, aflatoxin, and novobiocin are of microbial origin (Küpeli Akkol et al., 2020). Steroid-like substances such as 5,8-epidioxy-5α,8α-ergosta-6,22-dien-3β-ol have been isolated from macrofungi *Ganoderma applanatum* demonstrating antibacterial activity against gram-positive and gram-negative bacteria. These compounds are gaining enormous attention due to their immense therapeutic properties like anti-HIV, anti-tumor, antibacterial, antioxidant, antifungal, anti-inflammatory, and anticoagulant properties. In addition, coumarins have wide applications in the agrochemical and cosmetic industries (Fotopoulos & Hadjipavlou-Litina, 2020).

2.7.4. Terpenes:

Terpenes comprise a class of unsaturated, lipophilic, bioactive secondary metabolites built up from a hydrocarbon made up of five carbon atoms bonded with eight hydrogen atoms (C_5H_8) called isoprene. By extension this term is also used for oxygenated derivatives of these hydrocarbons called terpenoids. Terpenoids are formed by the removal and transfer of the methyl group and the addition of oxygen in hydroxyl and carbonyl groups. Terpenes belong to the subclass prenyllipids and may have a monocyclic, bicyclic, and acyclic structure (Kostić et al., 2020).

They are hydrocarbons of great pharmacological importance owing to the properties they can impart such as anti-microbial, antimalarial, anti-inflammatory, anticholinesterase etc. The mechanism of action of terpenes in microorganisms is not clear yet. However, because of their lipophilic nature, it is proposed that they may act by interfering with the functionality of membranes or by enhancing the permeability of antimicrobial substances. They are classified on the basis of a number of carbon atoms such as hemiterpenes (C5), sesquiterpenes (15), diterpenes (C20), triterpenes (C30), and tetraterpenes (carotenoids) (C40). It has been found that mushrooms are cholesterol-free thus numerous terpenoids such as carotenoids are instrumental in the prevention of atherosclerotic abrasion. Monoterpenes, triterpenoids, and sesquiterpenoids from mushrooms have shown cytotoxic characteristics when analyzed against several cancer cell lines (Abdelshafy et al., 2022).

A triterpenoid, Ganoderic acid A isolated from Ganoderma mushroom helped sort out renal damage by lowering kidney fibrosis and levels of creatinine, urea, and uric acid in serum. Despite being a rich source of nutritional and bioactive compounds, mushrooms must be thoroughly analyzed for the presence of any toxic compounds that can cause a toll on human health. Moreover, mushrooms grown on improper media can soak in heavy metals that are prone to exhibit binding affinity with alkali-soluble polysaccharides thus reducing its bioavailability and efficacy. Although only a minuscule number of reports have demonstrated the adverse effects of mushroom consumption on human health. However, alongside a rise in demand for mushrooms as functional food there is a demand for toxicity and safety assessment. Effective processing can significantly reduce safety hazards by minimizing microbial contaminations and associated toxins (H. Kumar et al., 2021).

2.7.5. Sesquiterpenes:

Sesquiterpenes are composed of three isoprene units and have the molecular formula, $C_{15}H_{25.}$ They may exhibit a cyclic structure or contain rings just like monoterpenes. Sesquiterpenoids are produced by biochemical modifications like oxidation etc. They are widely distributed in higher plants, marine organisms, and fungi. In nature, they are found as hydrocarbons or in oxygenated forms such as alcohols, aldehydes, ketones, and lactones. Higher fungi employ several defense strategies and produce numerous secondary

metabolites such as toxins to shield their fruiting body against parasitic and microbial attack (Tao et al., 2016). Sesquiterpenes have been recognized as not only a vital chemical defense molecules but also a potential candidate for novel drug development. Basidiomycetes contain humulene, a sesquiterpene of protoilludane type formed by cyclization of farnesylpyrophosphate. Sesquiterpenoids found in mushrooms also have a role in protection against insect attacks and enhancing nerve growth factors. Additionally, they exhibit potent biological activities such as Collybial (α, β-unsaturated aldehyde) isolated *from Collybia confluens* has demonstrated high antibacterial, antiviral, cytotoxic, and low antifungal activity. Caryophyllene isolated from *Hypholoma fasciculare* has antibacterial activity. Certain sesquiterpene alcohols esterified with fatty acids are produced by certain species of genera *Lactarius and Russula*. Though they don't exhibit high antibacterial activity, upon damage to the fruiting body breakdown of ester bonds occurs releasing alcohols that are very lethal to microorganisms. Henceforth, these esters are considered precursors of biologically potent molecules that are transformed into active molecules through several metabolic processes (Wu et al., 2022).

2.7.6. Diterpenes:

Four isoprene units are clustered together to form diterpenes with the molecular formula $C_{20}H_{32}$. They are synthesized in plants, fungi, and animals via the HMG-CoA reductase pathway. They are divided into several classes based upon the structure of their skeleton such as linear, bicyclic, tricyclic, tetracyclic, or macrocyclic. Several biologically important compounds such as phytol, retinal, and retinol are formed on account of diterpenes. They exhibit antibacterial and anti-inflammatory properties. Tricholamalides and cyathane, extracted from mushrooms, have an important role in the treatment of neurological disorders such as Alzheimer's disease. Eight polyoxygenated cyathane known as neocyathins K-R (1–8) were identified in *C. africanus* (Akihisa et al., 2005).

2.7.7. Triterpenoids:

Triterpenoids are composed of six isoprene units with molecular formula C30H48. Based on chemical structure they are classified into linear and cyclic compounds. *Ganoderma lucidum* is a very rich source of triterpenoids which are called ganoderic acids.

Approximately 40 triterpenoids have been isolated from *G. lucidium* with potent activity against human breast carcinoma and hepatocellular carcinoma cell lines. Additionally, Ganoderic acid has demonstrated anti-tumor and anti-inflammatory, anti-HIV, anti-malarial, antimicrobial activities. GA are secondary metabolites that are produced in stress situations thus, their yield can be enhanced by exposing them to artificial elicitors or signaling molecules. Lanostane triterpenoids, astraeusins M–Q (1–5), 26-epiastrasiaone (7) and 26-epi-artabotryol C1 (6) extracted from *Astraeus asiaticus* have demonstrated antibacterial as well as antimalarial activity (Chen et al., 2009).

2.7.8. Tetraterpenes:

When eight isoprene units are interlinked together, they form tetraterpenes also called carotenoids with a molecular formula C40H64. These are colorful compounds with a spectrum of colors ranging from orange and light yellow to deep red. They are produced by plants, macrofungi as well as other microorganisms. Carotenoids are divided into two categories: carotenes such as α-carotene, β-carotene, γ-carotene, and lutein and xanthophylls such as canthaxanthin and cryptoxanthin. Carotenoids have a role in several key biological functions like light-capturing reaction centers, protection against free radicals, synthesis of plant hormones, and certain structural components of membranes. Because of their robust biological activities such as anticancer, anti-HIV, and antioxidant, they are high-value compounds for the pharmaceutical, nutraceutical, cosmeceutical, and food industries (Reis et al., 2017).

2.8. Lectins:

Lectins are non-immunoglobulin, proteinaceous structures that attach a wide array of sugar structures with a high level of selectivity and stereospecificity without conferring any changes on the covalent structure of glycosyl ligands. Lectins have vital implications in several biological processes such as cellular signaling, cell-cell interactions, malignancy, metastasis, inflammation, and the immune system. Lectins are often referred to as hemagglutinins because of their ability to agglutinate erythrocytes via the reversible binding of sugars present on the cell surface. However, not all hemagglutinins are lectins (Hassan et al., 2015). High levels of lectins have been detected in mushrooms that are

deemed to have a role in their defense system. Furthermore, an essential role of lectin in the symbiotic relations of fungi with other organisms like algae and plant roots is emerging. Also, they are key players in other vital functions like mating, flocculation, mycelial aggregation, etc. Lectins acquired from numerous mushroom species exhibit variations in their carbohydrate specificity, molecular mass, and subunit number. In addition, lectins have been isolated from several parts of mushrooms like caps, stalks, and mycelia. However, level of expression may vary with respect to environmental influences such as location, season and age of fruiting body etc. For instance, the expression of lectin is very high in adult mushrooms of *Laccaria laccata* however, others like *Amanita muscaria*, *Tricholomopsis rutilans* produce higher quantities in young mushrooms (Kostlánová et al., 2005).

Phallin was the first ever lectin isolated from *Amanita phalloides. Almost 105 lectins have been recognized from several mushroom species. The highest number of lectins have been described in Lactarius preceded by Agaricus, Amanita, Boletus and Pleurotus etc. Lectins with differing carbohydrate specificities such as those from Agaricus arvensis* and *Agaricus bitorquis* that only bond with polysaccharide inulin and lactase to lectins from *Coprinus atramentarius and Lactarius vellereus that bind with galactose and sialic acid respectively. Moreover, several properties of high medicinal value such as anti-proliferative, amitogenic/ antimitogenic, immunomodulatory and antiviral activities have been highlighted (Varrot et al., 2013).* Regardless of versatile bioactivity ascribed to lectins data regarding structure, functions and mechanisms of biological activity is significantly lacking. In order to reap maximum benefits from the extraordinary activities of lectins further research needs to be conducted to fill these gaps in knowledge. Now a days majority of lectins are isolated from medicinally important mushrooms found in nature which has major limitations like low yield, high cost, batch variations and time-consuming. Henceforth there is a dire need to develop systems to produce functional lectins via cloning. However, the tools and techniques for recombinant lectin production are still in infancy. For potential therapeutic uses of lectins extensive research and development is required to refine techniques for their post-translational modifications (Kobayashi et al., 2014).

2.9. Dietary fibers:

Edible portion of a plant or mushrooms that cannot be digested and absorbed in the small intestines of humans are known as dietary fibers. They are carbohydrate polymers composed of ten or more monomeric units. These non-digestible carbohydrates (NDC) are naturally found in food and have been extracted from it through numerous chemicals, physical and enzymatic means. It has been highlighted through extensive research that dietary fibers have important health implications and can help prevent several diseases like diabetes, rectal and colon cancer, cardiovascular diseases, etc. (Cheung, 2013). Depending on the origin DF can have varying structures and physico-chemical properties which in return impart different physiological and nutritional benefits. Edible mushrooms are an affluent source of unique DFs with compelling health benefits however, they are very underutilized in comparison to other traditional sources like cereals, legumes, vegetables and fruits. The components of mushroom cell wall such as polysaccharides like (1→3)-*β*-*D*-glucans, mannans and chitin (a straight-chain (1→4)-*β*-linked polymer of *N*-acetyl-glucosamine) are non-digestible carbohydrates henceforth, they serve as Dietary fiber. Carbohydrates are the most abundant ingredients of a mushroom, comprising almost 35% to 70% of its dry weight, the majority of which are non-digestible dietary fibers (NDF) like trehalose, oligosaccharides and cell wall polysaccharides mentioned earlier. Dietary fiber content in different mushroom species vary greatly. Water-insoluble dietary fibers (IDF) like chitin and *β*-glucans are the most abundant one water soluble one (SDF) comprise less than 10% of total. Daily consumption of edible mushrooms can fulfill up to 25% of the recommended intake of dietary fibers (Galisteo et al., 2008).

2.10. Fungal immunomodulatory proteins:

Fungi contain a myriad of bioactive proteins such as lectins, laccases, nuclease, glycoproteins, immunomodulatory proteins, ribosome-inactivating proteins, etc. A group of proteins found in fungi with very pronounced immunomodulatory activity are known as Fungal immunomodulatory proteins (FIPs). They are a collection of amino acid with high valine and asparginine content whereas other like histidine, methionine and cysteine are in relatively lesser amount. The first ever FIP, Ling-Zhi 8 (LZ-8) was discovered from

Ganoderma lucidum. Until now more than 38 types of FIP have been discovered. On the basis of protein identity and conserved structure FIPs have been categorized into five subtypes: Cerato-type FIPs (Pfam PF07249), PCP-like FIPs, Fve-type FIPs (Pfam PF09259), TFP-like FIPs, and unclassified FIPs. Fve-type FIPs are the most frequently studied for their anti-cancer, hemagglutinating and immunomodulating properties. Generally, they have a molecular weight of ~13 kDa comprising a string of 110-125 amino acids (Liu et al., 2020).

Among the five subgroups, Fve-type FIPs are the most studied for their hemagglutinating, immunomodulating, and anti-cancer properties. In general, these small proteins consist of 110–125 amino acids, with a molecular weight of ~13 kDa. The other four subgroups are relatively less studied, but also show a noticeable influence on immune cells. Proteins from the other four FIP subgroups have manifested visible impact on immune cells. However, they haven't been studied extensively yet (Ullah et al., 2022).

Owing to their immunomodulating properties FIPs have numerous pharmacological applications. They have remarkable implications in chemopreventive therapy of cancer as they can help prevent tumor growth by diminishing their migration and causing apoptosis and autophagy of cancer cells. The precise mechanism that could elucidate the dynamics of FIP bioactive functions is yet to be known. Further studies need to be executed on effect of structure and modifications like glycosylation modifications on the bioactivity of FIPs that will potentially enable their effective use in therapy (X. Xu et al., 2011).

2.11. Medicinal potential of mushroom bioactive components

Mushrooms are a rich source of bioactive components that can act as a vital source of pharmaceutically important ingredients such as steroids, lectins, statins, phenols, polyphenols, alkaloids, triterpenes, lactones, and antibiotics. Several minerals found in mushrooms such as selenium and ergothioneine are antioxidants that can protect human cells from oxidative injury. Pantothenic acid (vitamin B5) is important for the functioning of the nervous system and β-glucans strengthen the immune system by provoking innate and adaptive immune responses. In addition to demonstrating antibacterial, antifungal,

antioxidant, anticancer, and antihemolytic activities they can be employed to mitigate health hazards such as hypertension, obesity, hyperlipidemia, etc. (Upadhyaya, 2018). Certain compounds from mushrooms such as proteases, protease inhibitors, lectins, and lignocellulose degrading enzymes are being employed for drug development. For instance, by manipulating *G. frondose, Hericium erinaceus*, and *A blazei* a product, AndosanTM has been manufactured that exhibits anti-inflammatory, anticancer, and antiallergenic properties because of high β-glucans and isoflavonoid content. The current literature points out the enormous medicinal potential of mushrooms however a detailed investigation of the underlying mechanism is necessary in order to bring it to practical use (D. Yadav & Negi, 2021).

2.11.1. Anti-microbial agents:

A host of antibacterial and antifungal compounds are produced by mushrooms for their natural defense. These antimicrobial agents offer a promising alternative to synthetic preservatives whose safety and health risks have not been settled still. Numerous mushrooms have shown remarkable antibacterial activity against multi-drug resistant human pathogens like Mycobacterium tuberculosis. These results allow treatment to be carried out with mushroom extracts with no harmful side effects thus protecting patients from, the collateral damage of commercially available antibacterial drugs (Fogarasi et al., 2020).

A very optimal MIC in the range of 0.1–0.2 mg ml− 1 has been detected from acetone extracts of *Craterellus cornucopioides* against Gram-positive (*Bacillus subtilis, Bacillus cereus, Staphylococcus aureus*) and Gram-negative (*Escherichia coli* and *Proteus mirabilis*). A large number of mushroom extracts have been isolated and characterized with potent antimicrobial activities and application as organic food preservatives.

Polysaccharides, proteins (peptide, lectin, nebrodeolysin, lentinan and ubiquitin-like proteins) carbohydrate-binding proteins (peptidomannan, polysaccharopeptide, acidic polysaccharides, sulfated polysaccharide), triterpenoids, triterpenes, polyphenols, and numerous other bioactive compounds have shown strong antiviral activity (Venturella et al., 2021). In a concentration range of 1.6nM to 1,3M, lectins have shown strong antiviral

potential against several viruses including hepatitis C, HIV, Influenza A/B, SARS virus, Japanese encephalitis virus, herpes simplex 1 and 2. It prevents viral cell invasion by specific attachment with viral envelope glycans. These studies point out the vast reservoirs of untapped potential in whole mushrooms as well as their isolated components with a host of applications in the pharmacological industry.

2.11.2. Antioxidants:

The metabolic reactions going on in our cells to maintain life produce reactive oxygen species (ROS) and reactive nitrogen species (RNS) as a by-product. These are highly unstable and reactive species that can degenerate the very fabric of cells thus resulting in many diseases associated with oxidative stress such as dementia, Alzheimer's disease, Parkinson, depression, and aging of skin, etc. Antioxidants are compounds that have the potential to cease the lethal chain reaction of free radicals by scavenging their unpaired valence electron. Numerous edible mushrooms have strong antioxidant potential owing to the presence of compounds such as vitamins, polysaccharides, tocopherols, minerals, carotenoids, and polyphenols. They also possess potent reduced coenzymes, antioxidant enzymes, and various phenolic compounds that can act as electron donors. They have an affluent supply of vitamins A, C, and E which possess formidable redox and antioxidant potential (Mwangi et al., 2022).

Lentinan acquired from *L.edodes* can protect against β-cell apoptosis in the pancreas by inhibiting ROS synthesis. Likewise, polysaccharides from *Morecella esculenta* have been found to accelerate the activity of hepatic antioxidant enzymes in mice models that help reduce lipid peroxidation. Flavonoids obtained from *Fomitopsis offinalis* have the potency to enhance the functionality of superoxide dismutase (SOD) and catalase (CAT) in lever glutathione peroxidase (GSH-Px) in tissues of the brain. This evidence testifies to the remarkable antioxidant potential of mushrooms that can be manipulated to boost human health by minimizing the damaging effects of oxidative stress (Ferreira et al., 2009).

2.11.3. Anticancer agents:

The recent trend of exploring mushrooms for novel drugs has resulted in the identification of numerous compounds such as polysaccharide-protein and polysaccharide-peptide complexes, shizophyllan, grifolan, lentinan with incredible anti-malignant properties. In fact crude extracts of several mushroom species such as ethanolic extracts of *Russula alatoreticula*, and methanolic extracts of *Lepistanuda* have demonstrated remarkable in-vitro anticancer activity (Hyder & Dutta, 2021).

In conventional Chinese medicine *C. militaris* has been used for its anticarcinogenic potency. Research has now highlighted that it is because of the presence of Cordycepin that has the capability to inhibit cell apoptosis and cancerous transformation and reduction in cell mitosis. Polysaccharides from mushrooms such as β-glucan stimulate an immune response in the host that can impart an anti-cancer effect (Sun et al., 2019). The consumption of polysaccharopeptide extracts of *T. versicolor* has been reported to minimize the side effects of chemotherapy and the survival of cancer patients. A myriad of cancers such as gastric, lung, cervical, lung, ovarian, nasopharyngeal, ovarian, non-lymphoma (NHL), Hodgkin's, and colorectal now employ Lentinan as a cancer adjuvant treatment in clinical trials. Patients subjected to a 12-week treatment of 4 mg d− 1 of Lentinan have manifested a dramatic rise in the proliferation of cytotoxic T cells, CD3+, CD56+, and NKT cells followed by a collateral rise in levels of IL-12, TNF-α, IFN-γ (Küpeli Akkol et al., 2020).

2.11.4. Anti-aging agents:

Aging is characterized by retardation in the physiological mechanisms of the body, consequently increasing vulnerability to age-related disorders, the most lethal one being neurodegenerative diseases. The primary factor responsible for aging is free radical accumulation and oxidative stress which can be counteracted by the application of antioxidants that are found in abundance in mushrooms. Mushrooms can activate several cellular pathways that have propensity to reverse the aging process. Furthermore, a spectrum of biological compounds often found in the fruiting body of mushrooms such as polysaccharides, polyphenols, selenium, vitamins, and terpenoids have antiaging

properties (Taofiq et al., 2016). A change in the expression of genes playing a vital role in the aging of nematodes was observed upon treatment with *G.lucidum* via S6K and Mtor signaling. It has been reported that a decrease in cellular energy that follows increasing age can be ameliorated by ethanolic extracts of *Ganoderma lucidum* and is actualized by increasing the number of respiratory chain complexes and mitochondrial enzymes in the heart of aged mice. Likewise, the replicative lifespan of yeast was profoundly enhanced by anti-aging ergosterols (Ganodermasidase A and B) acquired from the methanolic extracts of *G. Lucidum*. This is achieved by attuning the expression of UTH1 in genes responsible for oxidative stress management. Methanol and hot water extracts of *Phellinus vaninii* have reported remarkable antioxidant, anti-tyrosinase and anti-collagenase properties (Wang et al., 2017).

2.12. Mushroom in gut health:

The human gut is swarming with trillions of microorganisms that play a pivotal role in health maintenance as they facilitate nutrient absorption and compete with pathogens thus preventing infection and helping strengthen the immune system. Probiotics, prebiotics, and synbiotics play a significant role in maintaining the optimal amount and diversity of gut microbiota by stabilizing the overall intestinal environment thus preventing metabolic disorders. Involvement of gut microbiota has been spotted in numerous physiological processes such as lipid, glucose, energy metabolism, immunity, inflammation, cancer, etc. (Jayachandran et al., 2017) that are paramount for human health. Edible mushroom polysaccharides (EMPs) act as prebiotics for the gut microbiome as they are resistant to enzymatic degradation and are passed on intact in the caecum and colon where they are fermented by microorganisms that result in the production of beneficial metabolites such as SCFA such as propionic acid, butyric acid, and acetic acid. Henceforth, the outcome of several diseases can be influenced by intaking different kinds of dietary fibers which will ameliorate metabolite distribution and gut microbiota composition. The chemical nature and physical structure of dietary fiber play a crucial role in dictating the aforementioned functions. Significant rises in levels of propionic acid, butyric acid, and acetic acid have been detected after the consumption of soluble dietary fibers from *Lentinu edodes* (Ma et

al., 2022). The preservation of colonic epithelia morphology and functionality of distal intestines is greatly influenced by SCFA as they have a vital contribution to reducing osmotic pressure in intestines and maintenance of energy storage. Healing of a disrupted gastrointestinal tract can be affected via the prebiotic action of mushrooms. Polysaccharides isolated from the *G. lucidum* have been found to enhance the proliferation of gut microbiota and regulate immunological barrier function in rats. Intake of a mushroom-supplemented diet by C57BL/6J mice has resulted in an increase in the abundance of SCFA-releasing bacteria (*Bifidobacterium, Allobaculum, and Ruminococcus*). Henceforth, mushrooms can be regarded as a highly promising source of prebiotics that can enhance health by preventing infections, and gastrointestinal disorders, and maintenance of a healthy gut microbiota. The precise interaction between gut microbiota and mushroom components has not been elucidated yet however, certain mechanisms like oxidation, reduction, hydrolysis, isomerization, condensation, and rearrangement are assumed to be processes that are employed to transform bioactive phytochemicals from mushrooms into health-benefiting products (Li et al., 2021).

3. Materials and Methods

3.1. Study Area:

This research was performed in the Applied and Environmental Laboratory (AEG), Department of Microbiology, Quaid-i-Azam University, Islamabad.

3.2. Sample Site:

The mushrooms were harvested during the months of July to September 2022 from Azad Jammu and Kashmir (AJ&K) (Table 3.1).

Table 3.1. List of macrofungi collected from distinct locations of Azad Jammu and Kashmir, Pakistan during the year 2022.

S. No	Sample Code	Scientific Name	Common Name	Location	Edibility
1.	K5	*Amanita phalloides*	Death cap	Mehmood Gali	Toxic
2.	K17	*Trametes versicolor*	Turkey tail	Hajira, Poonch	Toxic
3.	K20	*Phellinus gilvus*	Mustard yellow polypore	Hajira, Poonch	Toxic

3.3. Collection and Morphological Identification of Mushrooms:

Freshly germinated mushrooms were collected from Azad Jammu and Kashmir (AJ&K) during the months of July to September (Fig 3.1). Initial identification was carried out on the basis of morphological features such as the color, shape, and size of the cap; stem; whether there are gills, pores, or teeth on the underside of the cap; the presence or absence of a veil, volva, reticulum, striation, zonation, rings, and scales, etc.

Fig 3.1. Collection of different mushrooms such as **A)** *Amanita phalloides*, **B)** *Trametes versicolor,* **C)** *Phellinus gilvus*

3.4. Processing of Mushroom Samples:

First of all, mushrooms were dried by placing them in a hot air oven at 40 to 45℃ or by exposing them to sunlight. This was done to remove moisture content to an optimal level so that microbial activity of its metabolites could be checked. This is followed up by crushing dried biomass into a fine powder via a blender or pestle and mortar (Fig 3.2).

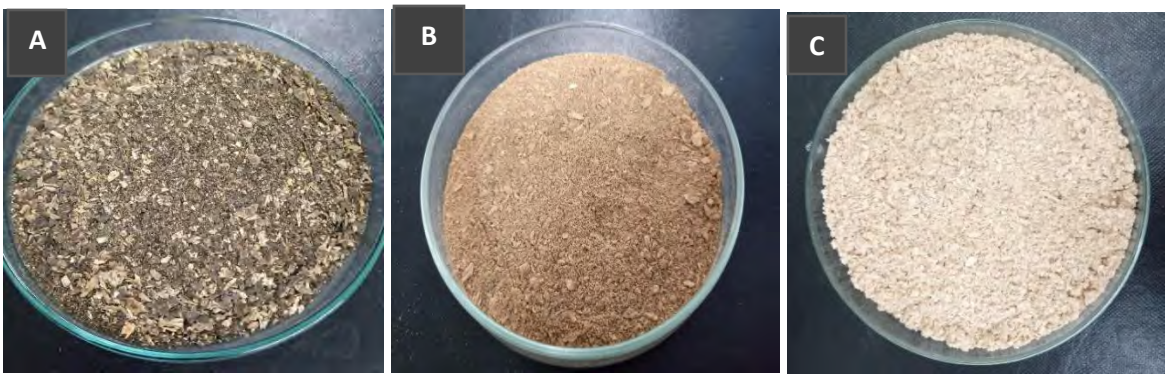

Fig 3.2. Powder of dried biomass of mushrooms labeled as **A)** *Amanita phalloides*, **B)** *Trametes versicolor,* **C)** *Phellinus gilvus.*

3.5. Extraction of Bioactive Metabolites from Mushrooms:

The powdered biomass of mushrooms was treated with a series of solvents from non-polar to polar such as n-hexane, chloroform, ethyl acetate, acetone, acetonitrile, ethanol, and methanol. A definite amount of biomass was mixed with a fixed quantity of solvent and kept at room temperature for 24 hours. This was followed up by filtration of extracts via Whatman filter paper no 4 which was then further dried by using a rotary evaporator at 45 to 50℃ (De Silva et al., 2013)..

3.6. Antibacterial potential of Crude Extracts of Mushrooms:

Antibacterial activities of crude extracts were determined against gram-positive and gram-negative bacteria on Mueller Hinton Agar (MHA) media using well plate diffusion.

3.6.1. Test Strains

- **Gram Negative**

  Escherichia coli

  Klebsiella pneumoniae

  Pseudomonas aeurignosa

- **Gram Positive:**

  Bacillus subtilis

  Staphylococcus aureus

3.6.2. Protocol

Bacterial strains were refreshed on selective media; EMB was used for *ii coli. P. aeruginosa,* and *K. pneumoniae*, MSA for *S. aureus* and LB medium for *B. subtilis.* The bacterial suspensions were prepared by mixing inoculum from freshly prepared plates in a 0.9% solution of normal saline. To make a lawn, tested bacteria were swabbed over respective MHA plates via sterilized cotton swabs. This was followed up by making wells on MHA using a 6mm sterile cork borer the base of which was sealed by using sterilized agar media. 100µl solution of each extract prepared by mixing with respective solvents at a concentration of 10mg/ml is added in respective wells. 100µl of pure solvent is also added in one of the wells as a negative control and a gentamycin disc is used as a positive control.

The plates were then placed in an aerobic incubator at 37℃ for 24 hours. After incubation, plates were analyzed for the results. The appearance of a transparent zone around wells indicates inhibition. Results were recorded by measuring the diameter of zones of inhibition in millimeters (Chavez-Esquivel et al., 2021).

3.7. Antifungal Potential of Crude Extracts of Mushrooms:

The antifungal potential of crude extracts of mushrooms was determined against three fungal strains via the agar well plate diffusion method using Sabouraud Dextrose Agar (SDA) media.

3.7.1. Test Strains

- *Candida albicans* (ATCC90028)
- *Aspergillus niger* (ATCC 16888)
- *Aspergilus flavus* (ATCC 9643)

3.7.2. Protocol

Fungal strains were refreshed on Sabouraud dextrose agar media. Fungal suspensions were made by mixing a small quantity of powdery mass of spores in 0.9% sterilized normal saline. These suspensions were then used to make homogenous lawns on SDA plates. Afterward, a 6mm sterile cork borer was used to make symmetrical wells whose base was sealed by using nutrient agar media. Dilutions of crude extracts were prepared using their respective solvents and maintaining a concentration of 10mg/ml. 100µl of metabolite solution was added in respective wells while using pure solvent as negative control and nystatin as positive control. The plates were then incubated in a fungal incubator for 48 hours at 30℃. After completion of incubation time diameter of the zone of inhibition was determined in millimeters and results were recorded.

3.8. Antioxidant potential of Crude Extracts of Mushrooms:

3.8.1. Chemicals required

- Dimethyl sulfoxide (DMSO)
- Ascorbic Acid
- 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent

3.8.2. Protocol

In order to analyze the antioxidant potential of crude extracts DPPH scavenging assay was conducted according to the protocol of Miser-Salihoglu with slight modifications. The DPPH was added to the methanol to make a DPPH reagent. Its OD was adjusted to 1 at 517nm to have the standard curves for comparison with the tested compound. Ascorbic acid was used as a positive control which was prepared by adding 0.01g ascorbic acid in 1ml methanol whereas DMSO was used as a negative control. Afterward, positive control, negative control, and tested compound were loaded into the microtiter plate in varying concentrations; 100µL, 75µL, 50µL, 25µL, and 5µL. This was followed up by the addition of 100µL, 125µL, 150µL, 175µL, and 195µL 2, 2-diphenyl-1-picrylhydrazyl (DPPH) in order to make the total volume of well 200ul. Because of its light sensitivity, the loading of DPPH was carried out in the dark. In addition, 200µL of DPPH was added in one of the wells as blank. The microtiter plate was then covered with aluminum foil and incubated at 37℃ for one hour. After incubation absorbance was measured at 517nm and DPPH scavenging activity was calculated using the following formula:

$$\text{Antioxidant activity (\%)} = (AD\text{-}AS/\ AD) \times 100$$

Where AS refers to the absorbance value of tested compounds and AD refers to the absorbance value of the DPPH reagent.

3.9. Cytotoxic activity of Crude Extracts of Mushrooms:

3.9.1. Required Chemicals

- Dimethyl Sulfoxide (DMSO)
- Marine salt
- Yeast extract

3.9.2. Protocol:

The cytotoxic potential of crude extracts of mushrooms was analyzed using the protocol of (Waghulde et al., 2019) with little bit modifications. Brine shrimp (*Artemia salina*) were selected as model animals for this assay. Marine water was synthesized by dissolving 34g marine salt, and 6mg yeast extract in 1L distilled water. Afterward, marine water was subjected to stirring for 30 minutes and its pH was adjusted to 8. This artificial marine water was then poured into a hatching tank comprising two chambers connected via holes. A small quantity of brine shrimp eggs was added into one of the compartments which was then covered with aluminum foil. The brine shrimps were incubated under a light lamp for 24-48 hours. This was followed up by the transfer of a calculated amount of brine shrimps into test tubes containing 5ml marine salt solution. The stock solution of crude mushroom extracts was made at a concentration of 0.01g/mL in DMSO. The cytotoxic potential of extracts was analyzed against five varying concentrations: 100µL, 80µL, 60µL, 40µL, and 20µL. The same concentrations of DMSO were used as a negative control. One of the test tubes contained only 10 brine shrimps in a salt solution and was kept as a blank. These test tubes were then incubated at 40ºC, and the number of active and dead shrimps was recorded after 24 and 48 hours. The mortality rate was calculated using the following formula:

% Mortality = No of dead shrimps / Total No of shrimps x 100

3.10. Hemolytic activity of Crude Extracts of Mushrooms:

3.10.1. Required Chemicals

- Phosphate Buffer Saline (PBS)
- Sodium Dodecyl sulfate (SDS)
- DMSO

3.10.2. Protocol:

It is crucial to ensure the safety of potential drug candidates therefore it is analyzed for its capacity to cause degradation of red blood cells. Blood from healthy donors was gathered in 3ml EDTA tubes and subjected to washing with normal saline three times. 45µl of PBS was added in 5ml blood and stored for later usage. The stock solution of test samples was

prepared by dissolving 0.01g of crude extract in 1 ml DMSO. 0.01g/ml solution of SDS in distilled water was prepared for use as a positive control. DMSO was used as a negative control and 200µl PBS was used as a blank. The hemolytic activity of test samples was checked out at five varying concentrations (20µl, 40µl, 60µl, 80µ,100µl). Samples were added to the Eppendorf in respective concentrations and mixed with a 300µl solution of RBCs. The total volume of the reaction mixture was adjusted up to 1ml by adding 600 µl, 620 µl, 640µl, and 680µl of PBS. The reaction mixture was incubated for 1h at 37°C. Afterward, it was subjected to centrifugation at 2500 rpm for 15 minutes. 200µl of supernatant was added in wells of microtiter plate and OD was determined at 517nm. The hemolytic activity was calculated using the formula:

Percent Hemolysis (%) = OD (positive control)-OD(Sample)/OD (Positive control) ×100

3.11. Mycochemical Profiling of Crude Extracts of Mushrooms:

The hexane, chloroform, ethyl acetate, acetone, acetonitrile, ethanol, and methanolic extracts of *Amanita phalloides, Trametes versicolor,* and *Phellinus glivus* were screened for the presence of mycoconstituents such as phenolic compounds, flavonoids, tannins, saponins, and steroids according to standard mycochemical screening (Anita Margret et al., 2022).

3.11.1. Test for Tannins (ferric chloride test):

2ml of each mushroom extract dissolved in DMSO was added in test tubes followed by the addition of ferric chloride reagent. The appearance of blue-black, green, or blue-green precipitates is an indicator of the positive test for tannins.

3.11.2. Test for Saponins:

2ml extract from a pre-prepared stock solution of crude extracts of mushrooms in DMSO was added in test tubes and mixed vigorously. The appearance of foam indicates the presence of saponins.

3.11.3. Test for Steroids:

3ml of chloroform and concentrated H2SO4 were mixed with 1ml of each crude extract of mushrooms. The appearance of a red-brownish layer on the lower chloroform layer is a positive test for the presence of steroids.

3.11.4. Estimation of Total Flavonoid Content:

The $AlCl_3$ colorimetric method was used to determine the total flavonoid content of the extracts. 30µl extract was mixed with 10µl of 10% AlC3 solution and incubated for 5 minutes at room temperature followed by the addition of 10µl of I molar potassium acetate solution and 150µl distill water. The absorbance was measured at 510 nm after incubation for 30 minutes in a shaker incubator. Total flavonoid content was determined by using a standard quercetin calibration curve. Results were expressed as mg of Quercetin Equivalents (QE) per gram of the extract.

3.11.5. Estimation of Total Phenolic Content:

50ul crude extracts of mushrooms were mixed with 90µl Fc reagent,10µl 6% sodium carbonate solution, 70µl distill water, and incubated for 90 minutes in a shaker incubator. The absorbance value was determined at 630 nm using a spectrophotometer. Gallic acid in varying concentrations (20µl, 40µl, 60µl, 80µl, 100µl) was employed as a positive control which was further used to construct a standard calibration curve. The results were noted as mg of gallic acid equivalent (GAE) per gram extract.

3.12. Primary purification of metabolites of mushrooms via Column Chromatography:

The strains of mushrooms with the most potent antibacterial activity were selected for further purification. Firstly, the column was washed, air dried, and packed by shoving a small chunk of cotton at the base followed up by the insertion of silica gel in a 6-inch long and 30mm wide column. The crude extract was injected at the top of the silica gel followed by another layering of silica. Silica gel is polar in nature which allows efficient adsorption of polar compounds. After packing the column elution is carried out by using solvents in a chronological order of non-polar to polar. Approximately 15 to 20 fractions were obtained by using mobile phases with varying polarity (Table 3.2).

Table 3.2. Mobile phases and fractions of crude extracts of Mushrooms.

S. No	Solvents (Eluents)	Composition	Volume	Fractions
1.	n-Hexane	100%	(50mL)	H1, H2, H3
2.	Chloroform	100%	(50mL)	C1,
3.	Ethyl acetate	100%	(50mL)	EA1, EA2, EA3
4.	Ethanol	100%	(50mL)	E1, E2, E3
5.	Methanol	100%	(50mL)	M1, M2, M3
6.	Acetones	100%	(50mL)	A1
7.	Acetonitrile	100%	(50mL)	AN1
8.	Water	100%	(50mL)	W1, W2

3.13. Antibacterial activity of Purified Fractions:

All purified fractions were analyzed for antibacterial activity using the same well diffusion method as was done for the crude extracts.

3.14. Thin-Layer Chromatography (TLC):

TLC involves the separation of mixture constituents between a fixed stationary phase and a liquid mobile phase on the basis of varying affinities between the two phases. Silica gel coated on an inert material, for instance, alumina was used as a stationary phase. First of all, the plate was developed by cutting the plate with appropriate dimensions and a line was drawn at a distance of 1 cm from the bottom of the plate. A small amount of respective solvent was added to the active fractions and a 2ul spot was placed on the TLC plate. An appropriate combination of solvents needs to be selected that would allow efficient separation. For non-polar fractions, numerous solvent combinations were tried typically starting from the combination of Hexane and Ethyl acetate in varying ratios of 20:80, 30:70, 50:50, 70:30, and 90:10. For highly polar fraction, the combination of Chloroform and Methanol was also used as mobile phase with the ratio of 20:80, 30:70, 50:50, 70:30, and 90:10. Make sure that the solvent was well below the spotted line. The solvent was allowed to move up via capillary action until it reached the marked top line (solvent front).

Afterward, the plate was taken out of the chamber and allowed to dry. The plate was analyzed under a UV illuminator with a short wavelength of 254nm and a long wavelength of 365nm(Poole, 2003).. A spectrum of bands was spotted and their Rf value was calculated using the formula:

$$\text{Rf value} = \frac{\text{Distance traveled by the sample}}{\text{Distance traveled by the solvent front}}$$

3.15. Antibacterial Activity of Bioactive TLC bands

The antibacterial activity of bioactive bands of TLC was performed by well-diffusion methods on MHA plates (Choma & Grzelak, 2011). Each of the bands on silica was marked under UV illuminator. This was followed up by scratching of bands alongside silica. Each of the acquired powdered mass was dissolved in methanol and subjected to rigorous stirring on a vortex machine. The antibacterial activity of purified extracts was then analyses via meaning the diameter of zone of inhibition.

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4. Results

4.1. Extraction of Bioactive Metabolites from Mushrooms

Each mushroom strain's powdered biomass was treated with seven solvents with varying polarity starting from n-hexane, chloroform, ethyl acetate, acetone, acetonitrile, to ethanol and methanol. (Table 4.1).

Table 4.1. Crude extracts of Mushrooms and their acquired concentrations

S. No	Macrofungi	Solvents	Code	Crude Extract (mg)
1.	*Amanita phalloides*	Hexane	K5H	360
2.		Chloroform	K5C	410
3.		Ethyl acetate	K53EA	100
4.		Acetone	K5A	250
5.		Acetonitrile	K5AN	100
6.		Ethanol	K5E	720
7.		Methanol	K5M	300
8.	*Trametes versicolor*	Hexane	K17H	480
9.		Chloroform	K17C	950
10.		Ethyl acetate	K17EA	360
11.		Acetone	K17A	350
12.		Acetonitrile	K17AN	280
13.		Ethanol	K17E	100
14.		Methanol	K17M	450
15.	*Phellinus gilvus*	Hexane	K20H	270
16.		Chloroform	K20C	410
17.		Ethyl acetate	K20EA	360
18.		Acetone	K20A	80
19.		Acetonitrile	K20AN	380
20.		Ethanol	K20E	250

21.		Methanol	K20M	270

4.2. Antibacterial Activity of Crude Extracts of Mushrooms:

Antibacterial activity of varying levels was recorded against five multi-drug resistant strains via agar well plate diffusion method (Fig 4.1). *Trametes versicolor* has proven to be the most potent mushroom as a very substantial amount of activity has been reported by all of its extracts against all five strains in the range of 11 to 22 ± 1.52mm. However, ethyl acetate, acetone and ethanol, methanol extracts have demonstrated the strongest activity against *K. pneumoniae* and *Staphylococcus aureus* with zones of inhibition of 20 ± 1.15mm, 22 ± 0.57mm, 21 ± 1.57mm, 21 ± 1.57mm respectively. In addition, the best activity was reported by acetonitrile extracts of *Phellinus gilvus* against *P. aeurignosa, S. aureus, and Bacillus subtilis* with zones of inhibition of 18 ± 1mm, 21 ± 1.57mm, and 18 ± 2mm respectively. Chloroform and Ethanolic extracts of *Trametes versicolor* have demonstrated the strongest activity against *K. pneumoniae* and *B. subtilis* with an inhibition zone of 17 ± 1.54mm and 18 ± 0.57mm respectively. Overall negligible activity was reported by hexane and methanolic extracts across all extracts of mushrooms. In contrast with other gram-negative bacteria, *E. coli* was the most resistant to the inhibitory effects of macrofungal crude extracts. (Fig 4.2, 4.3, 4.4)

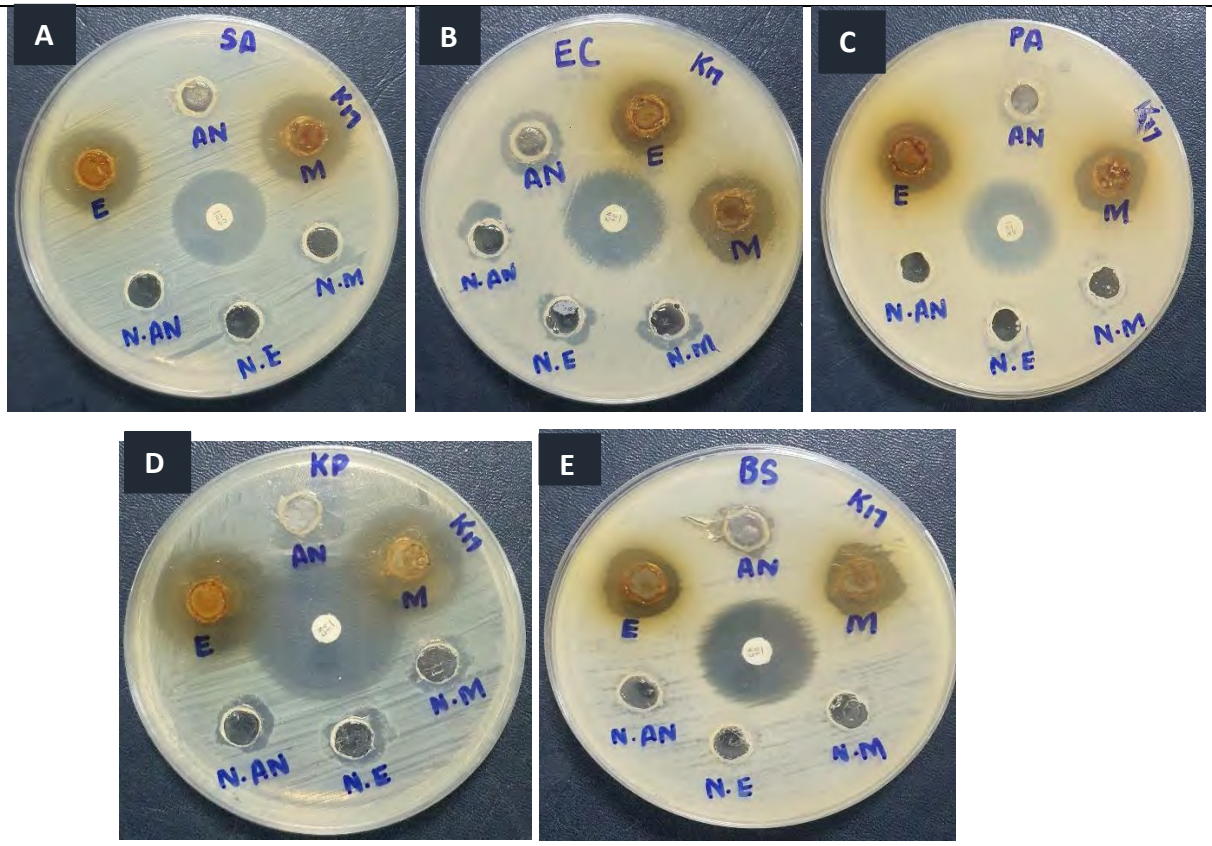

Fig 4.1. Antibacterial activity of the macrofungal crude extract against **A)** *S. aureus,* **B)** *E. coli,* **C)** *P. aeruginosa,* **D)** *K. pneumoniae,* and **E)** *B. subtilis*

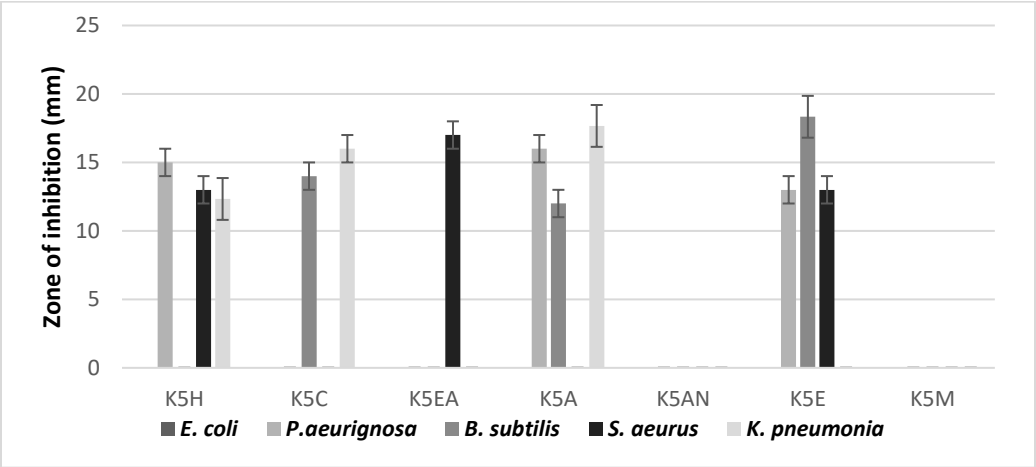

Fig 4.2. Inhibition zone (mm) of bacterial strains against the crude extracts of *Amanita phalloides.*

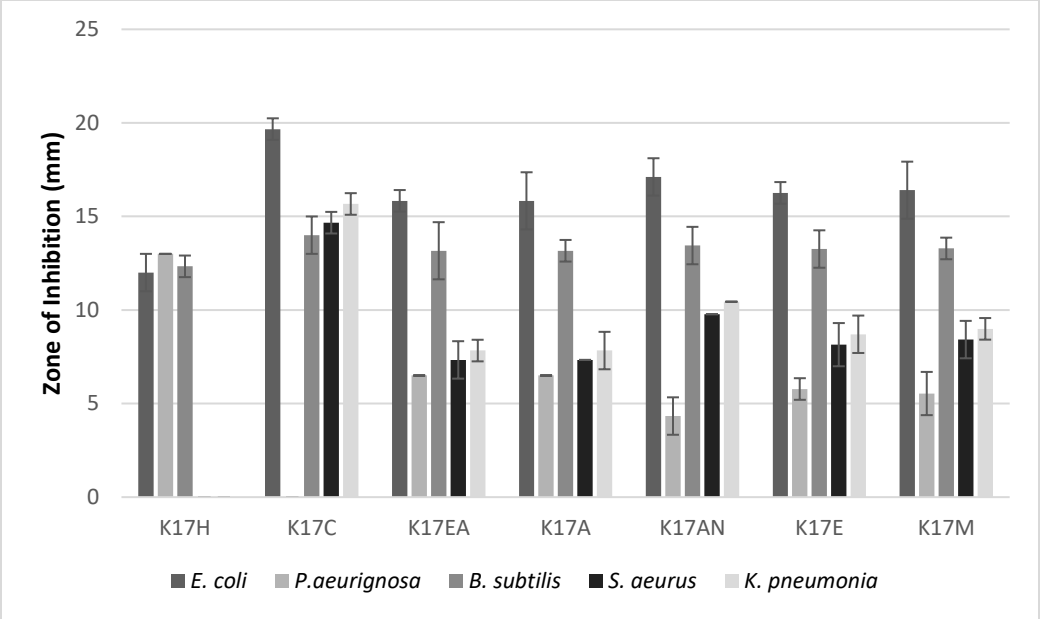

Fig 4.3. Inhibition zone (mm) of bacterial strains against the crude extracts of *Trametes versicolor.*

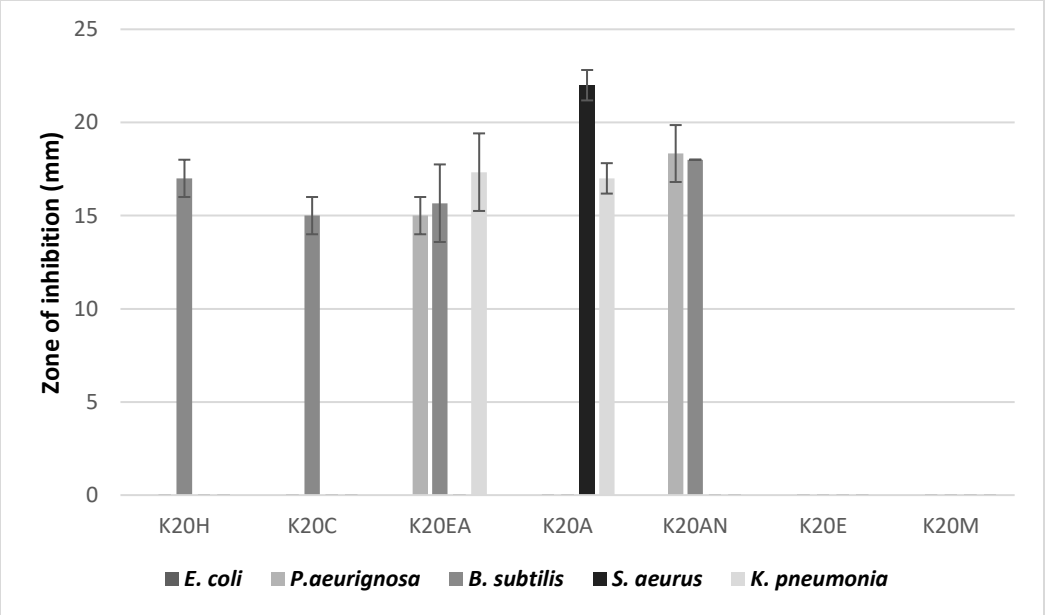

Fig 4.4. Inhibition zone (mm) of bacterial strains against the crude extracts of *Phellinus glivus.*

4.3. Antifungal Activity of Mushroom Extracts

The antifungal activity of macrofungal crude extracts was checked against three strains of fungi; *Candida albicans*, *Aspergillus niger,* and *Aspergillus flavus* (Fig 4.5.) The overall results of the antifungal activity of crude extracts of mushrooms were not very promising. A few extracts had a very minimal inhibitory effect however, most extracts have demonstrated zero activity. Overall ethanolic extracts have exhibited trivial activity against *Candida albicans.* A very negligible amount of activity was recorded by acetone and acetonitrile extracts. *Aspergillus niger* was the most resistant among all tested strains of fungi (Fig 4.6, 4.7, 4.8).

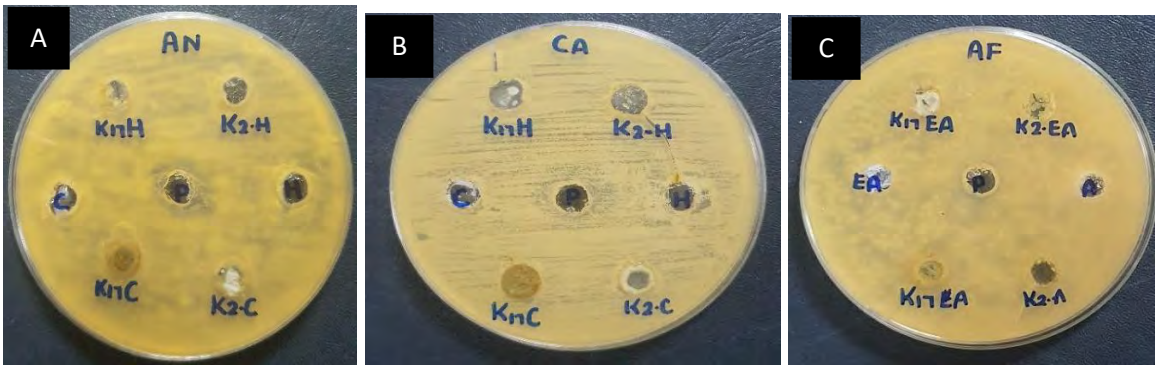

Fig 4.5. Antifungal activity of crude macrofungal extracts against **A)** *A. niger,* **B)** *C. albicans* and C) *A. flavus*

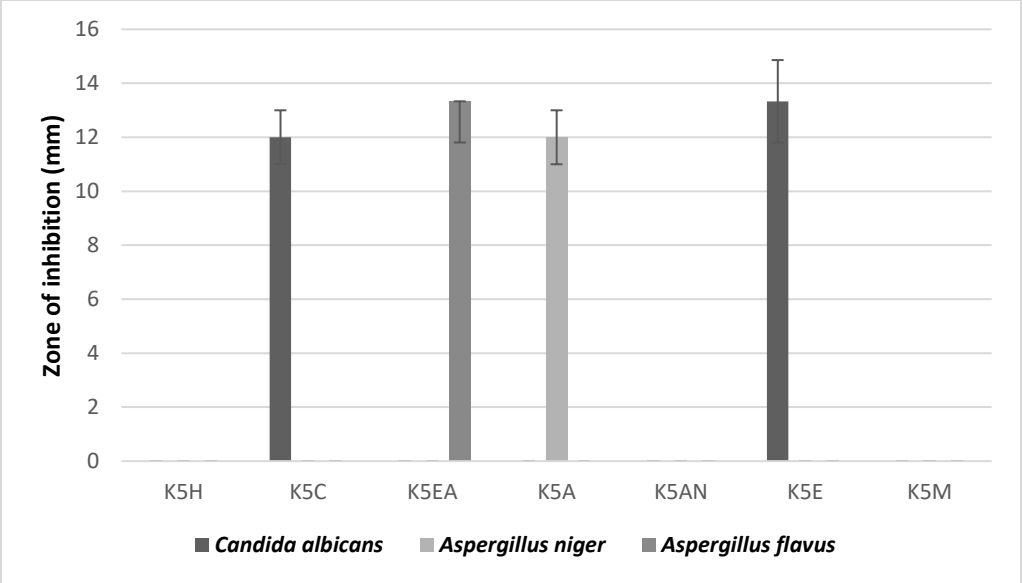

Fig 4.6. Inhibition zone (mm) of fungal strains against the crude extracts of *Amanita phalloides.*

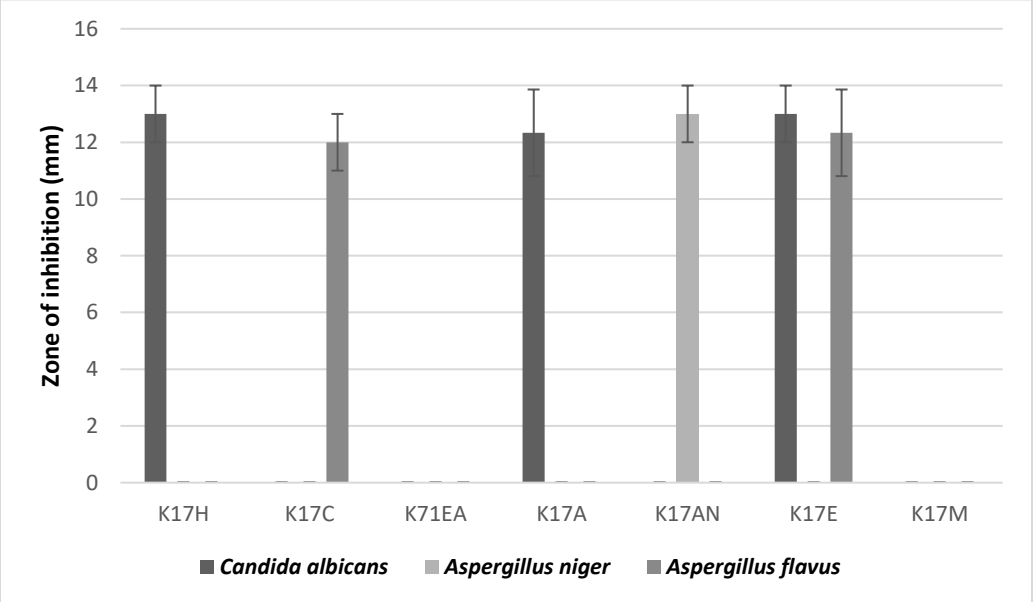

Fig 4.7. Inhibition zone (mm) of fungal strains against the crude extracts of *Trametes versicolor.*

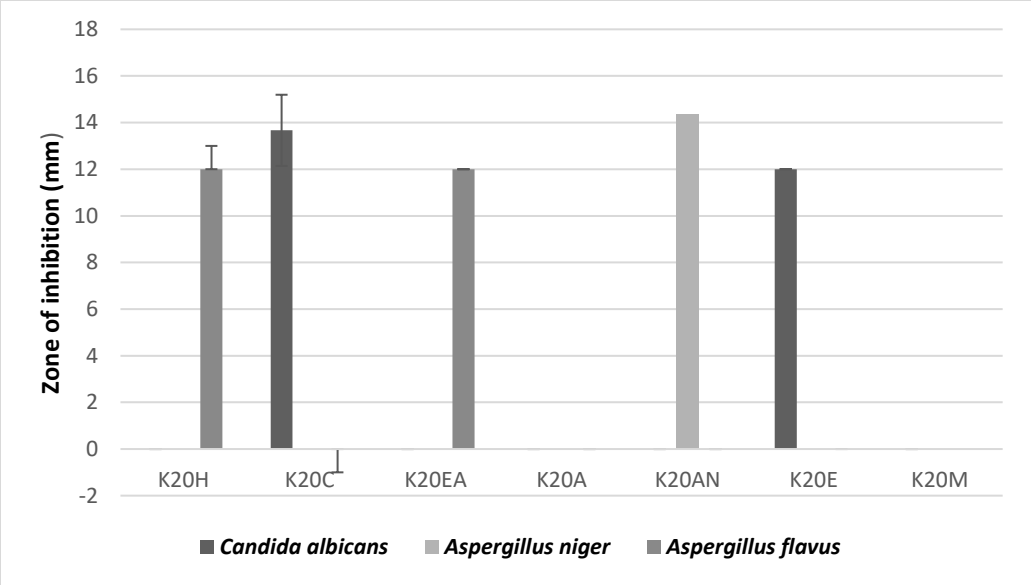

Fig 4.8. Inhibition zone (mm) of fungal strains against the crude extracts of *Phellinus gilvus.*

4.4. Antioxidant Potential (Free Radical Scavenging Assay) of Mushroom Extracts

The free radical scavenging potential of the extracts depends on the concentration of the extracts. A consistent increase in antioxidant activity was observed with increasing concentrations of the extracts (Fig 4.9). *Amanita phalloides* has exhibited very weak antioxidant activity in the range of 20 to 40 ± 1.5%. The results of the antioxidant activity of *Trametes versicolor* were fairly good, the extract of hexane has demonstrated antioxidant activity of 75 ± 1%. *Phellinu glivus* has demonstrated the best antioxidant activity; 78 ± 1.5%, 72 ± 0.57%, 75 ± 1.57%, 75 ± 1.25% antioxidant activity was exhibited by the ethyl acetate, acetonitrile, ethan,ol and methanol extracts respectively (Figure 4.10, 4.11, 4.12)

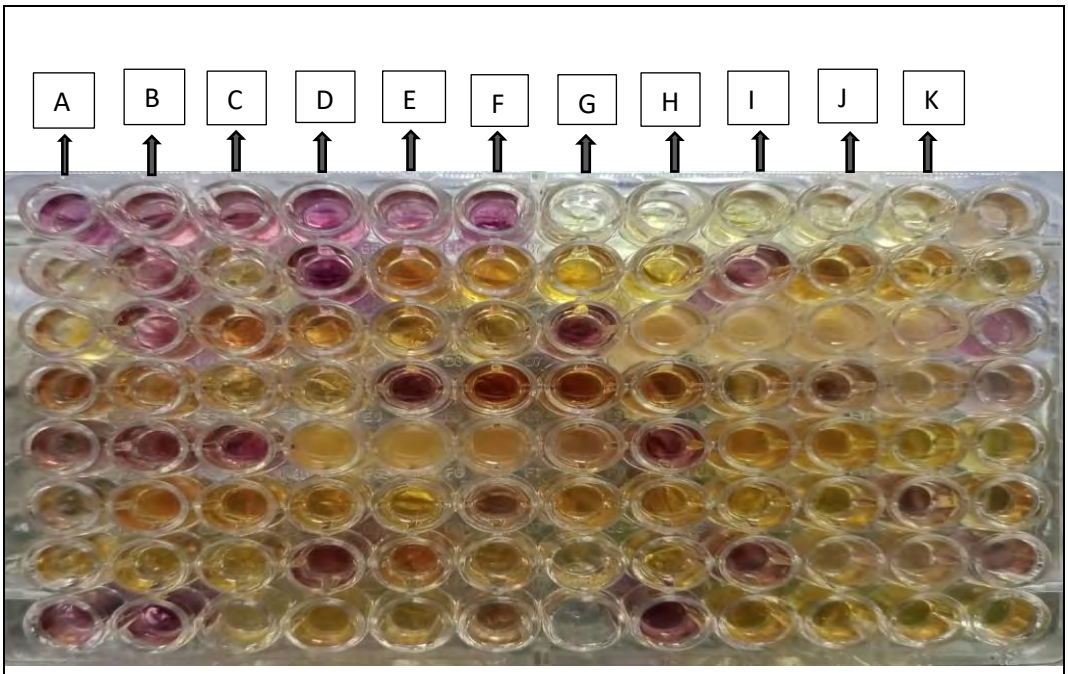

Fig 4.9. Antioxidant Activity of macrofungal crude extracts at different concentrations with **A** = Blank (DPPH); **B, C, D, E, and F**= Negative control (0.05, 0.25, 0.5, 0.75 and 1mg/mL); **G, H, I, J, and K**= Positive control (0.05, 0.25, 0.5, 0.75 and 1mg/mL).

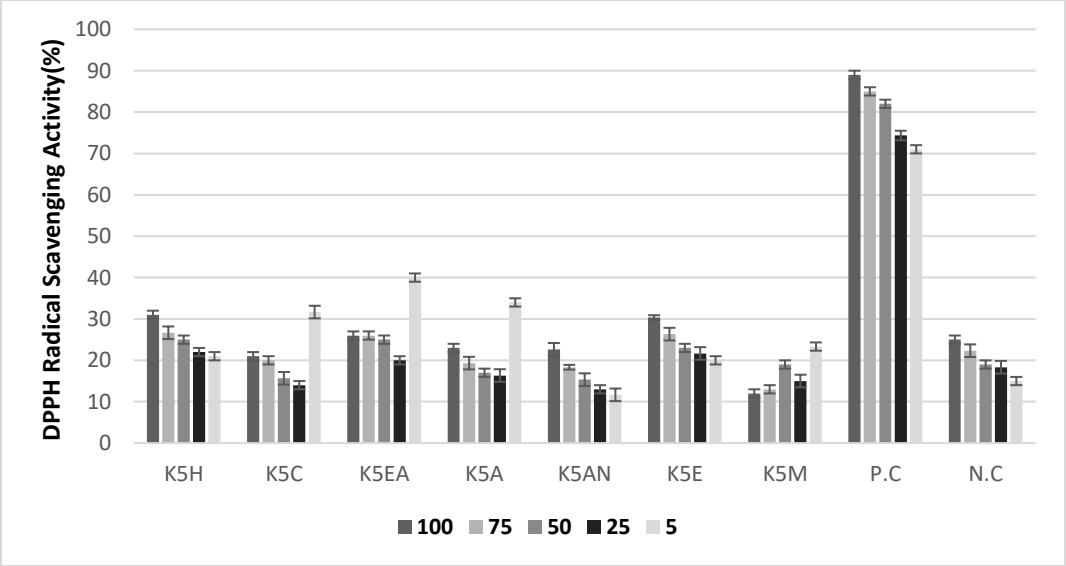

Fig 4.10. DPPH Scavenging Activity (%) of *Amanita phalloides* crude extracts.

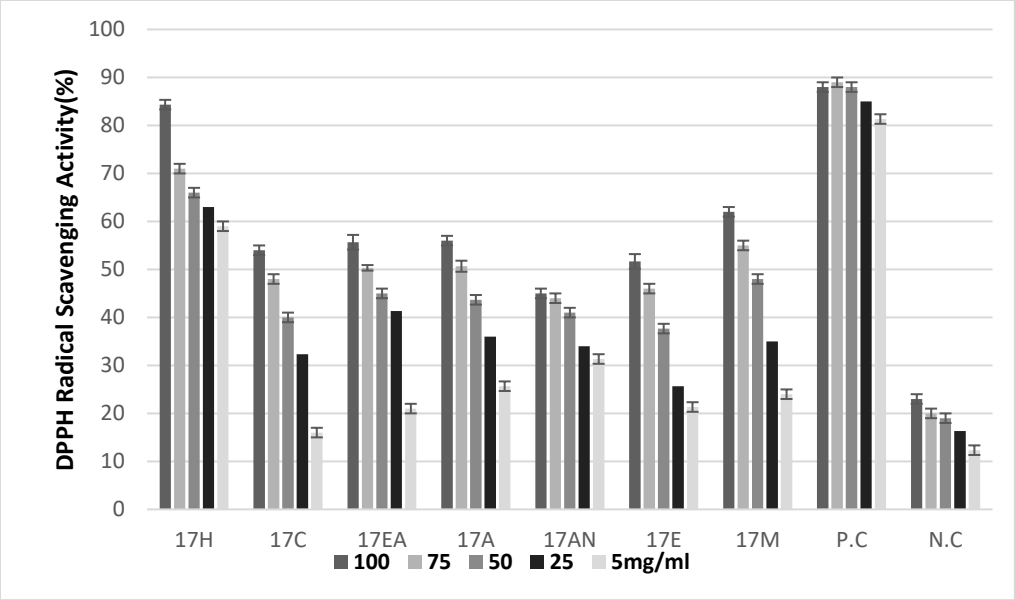

Fig 4.11. DPPH Scavenging Activity (%) of *Trametes versicolor* crude extracts.

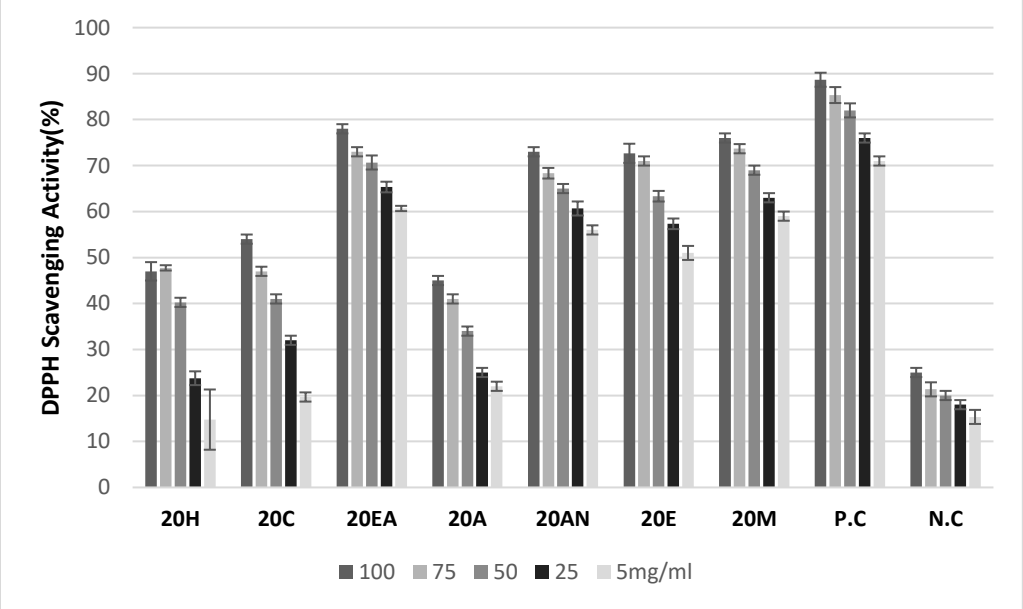

Fig 4.12. DPPH Scavenging Activity (%) of *Phellinus glivus* crude extracts.

4.5. Cytotoxicity Activity (Brine shrimp lethality assay) of Mushroom Extracts

The analysis of cytotoxic activity is a critical dimension of screening mushrooms for their therapeutic potential. The results of brine shrimp lethality assays offer a general analysis of the potential of mushrooms as a candidate for cancer therapy. The results of cytotoxic activity were recorded at various concentrations of extract. A consistent increase in survival was observed with increasing concentrations. The best cytotoxic activity was reported by the Amanita phalloides with maximum activity demonstrated by the ethyl acetate, acetone, acetonitrile, and ethanol extracts with 80 ± 1.57 % mortality at the concentration of 1mg/ml. A moderate level of the cytotoxic potential was observed in Trametes versicolor with chloroform extracts exhibiting the maximum mortality of 80 ± 2.0% after 24 hours. The cytotoxic potential of *Phellinus glivus* was fairly low with the maximum mortality of 70 ± 0.57% exhibited by the chloroform extracts at 24 hours. A progressive decline of roughly 30-40 ± 1.57% was observed in all extracts after 48 hours. DMSO used as a negative control has caused approximately 10 ± 1.57% mortality. No death of brine shrimps was recorded in test tubes used as blank (Fig 4.13. 4.14, 4.15).

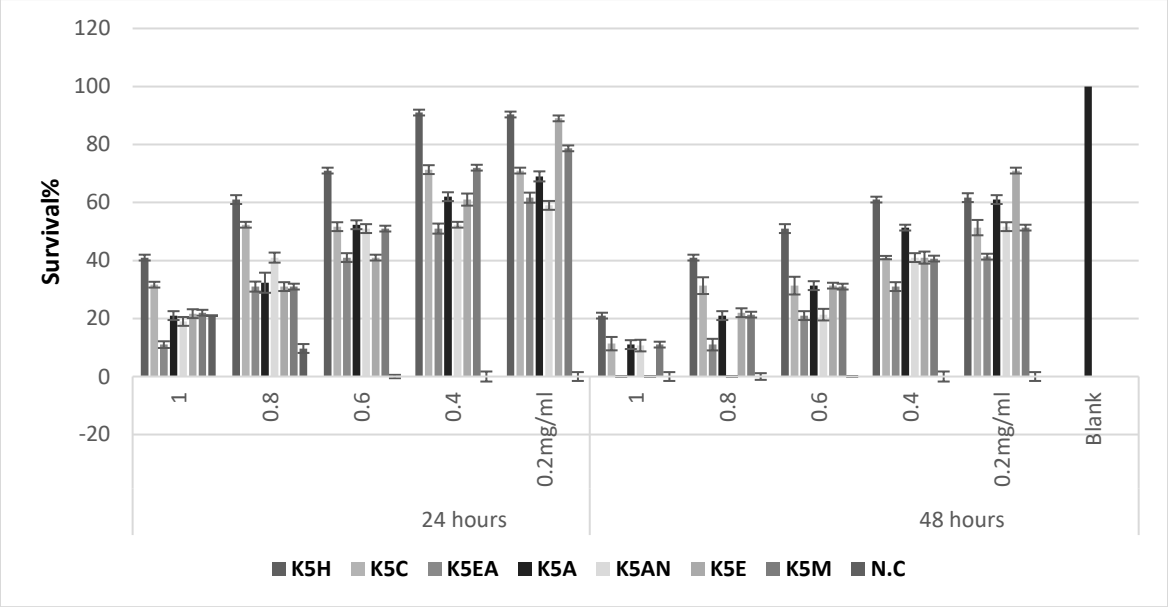

Fig 4.13. Cytotoxicity Assay of different extracts obtained from *Amanita phalloides.*

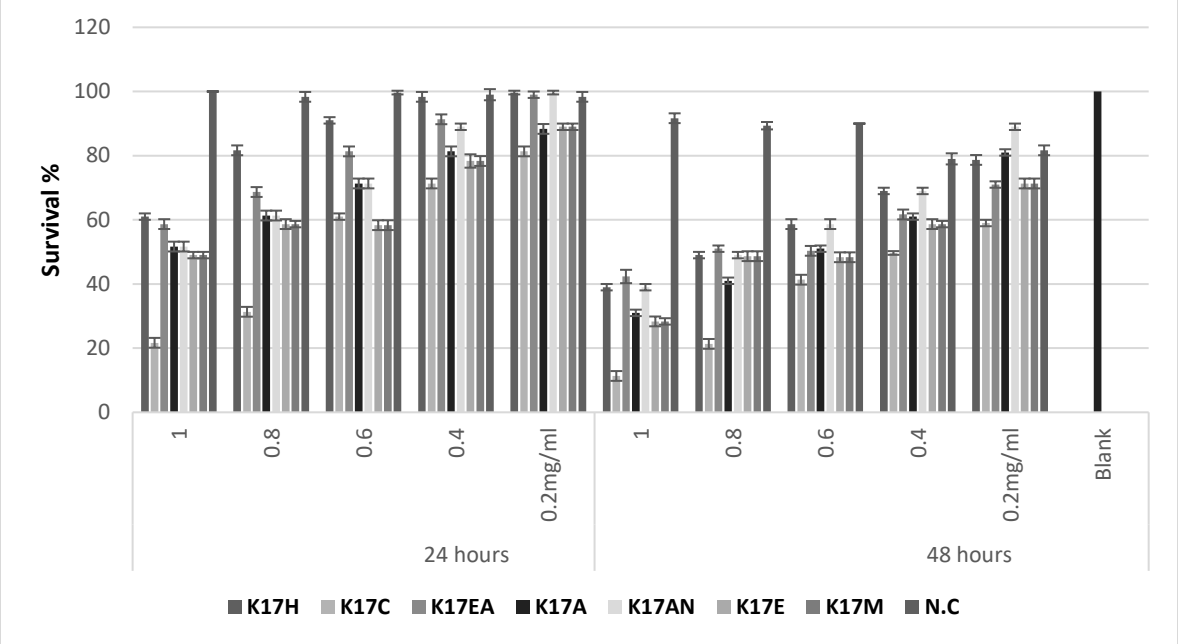

Fig 4.14. Cytotoxicity Assay of different extracts obtained from *Trametes versicolor.*

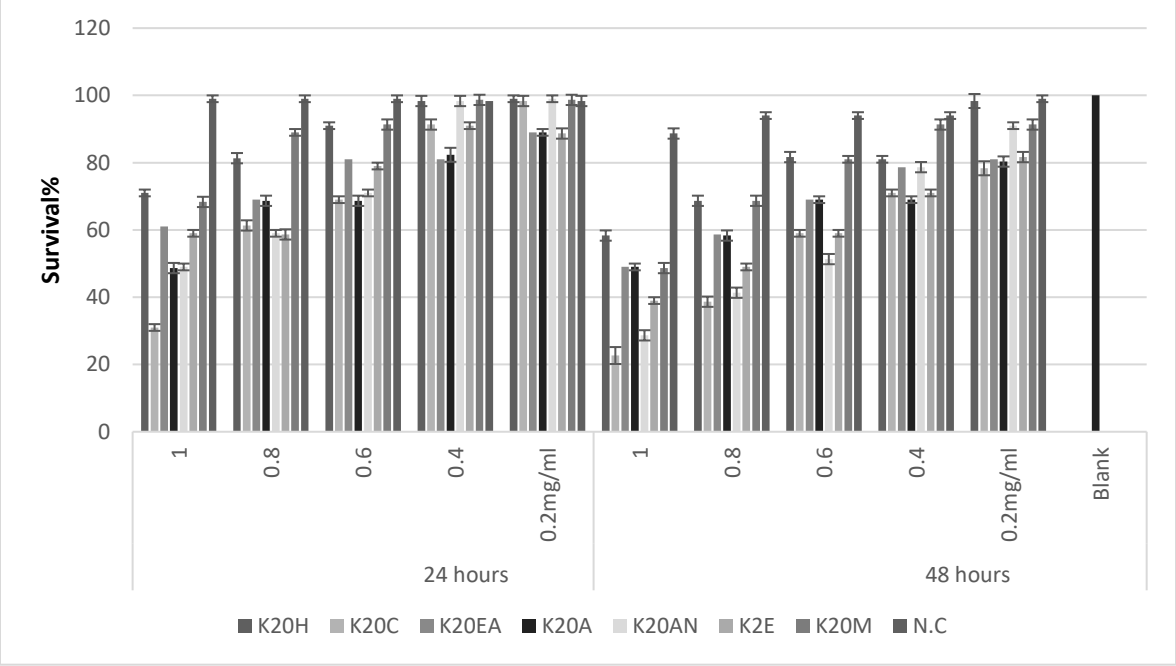

Fig 4.15. Cytotoxicity Assay of different extracts obtained from *Phellinus gilvus*..

4.6. Hemolytic Activity of Mushroom Extracts

The analysis of hemolytic activity is critical for establishing a comprehensive safety profile of mushroom extracts. The hemolytic assay involves studying the effect of extracts on the membrane and correlating it with their cytotoxic proclivity. The results of hemolytic activity can be classified into three categories; non-hemolytic, slightly hemolytic, and highly hemolytic. Non-hemolytic extracts do not impart any damage to RBCs and could be used safely for further applications. Slightly hemolytic cause minimal levels of hemolysis and are considered safe at lower concentrations however, highly hemolytic extracts can cause substantial damage to RBCs and impose a major safety risk. Therefore, mushrooms with no or minimal level of hemolytic activity are safe for human consumption.

A direct correlation between the hemolytic activity and concentration of extracts was observed. The highest level of hemolysis was observed by the strains of *Phellinus gilvus* 50 to 50 ± 1.57% hemolysis was caused by the hexane, chloroform, and ethylacetate extracts. In contrast, *Trametes versicolor* has shown comparatively low hemolysis with the majority of fractions showing hemolysis in range of 20 to 30 ± 1.57% with ethanol and methanol extracts showing the highest hemolysis of 40 ± 1% and 44 ± 0.57%. Ethyl acetate, acetone, ethanol, and methanol extracts of *Amanita phalloides* had shown slight hemolysis of 31 ± 1.57%, 19 ± 2%, 26 ± 1.57%, and 15 ± 1.57% whereas acetonitrile fractions have demonstrated the highest hemolysis of 46% (Figure 4.16, 4.17, 4.18, 4.19, 4.20)

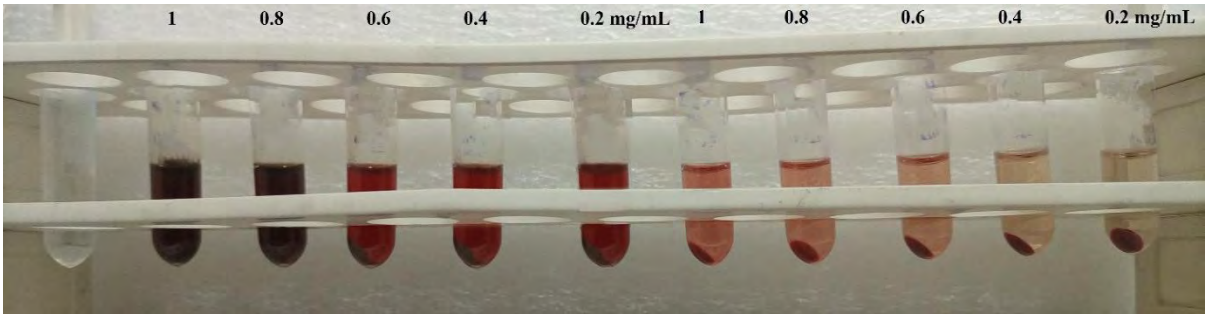

Fig 4.16. Showing blank, and positive control of hemolytic activity on the right side and negative control on the left side.

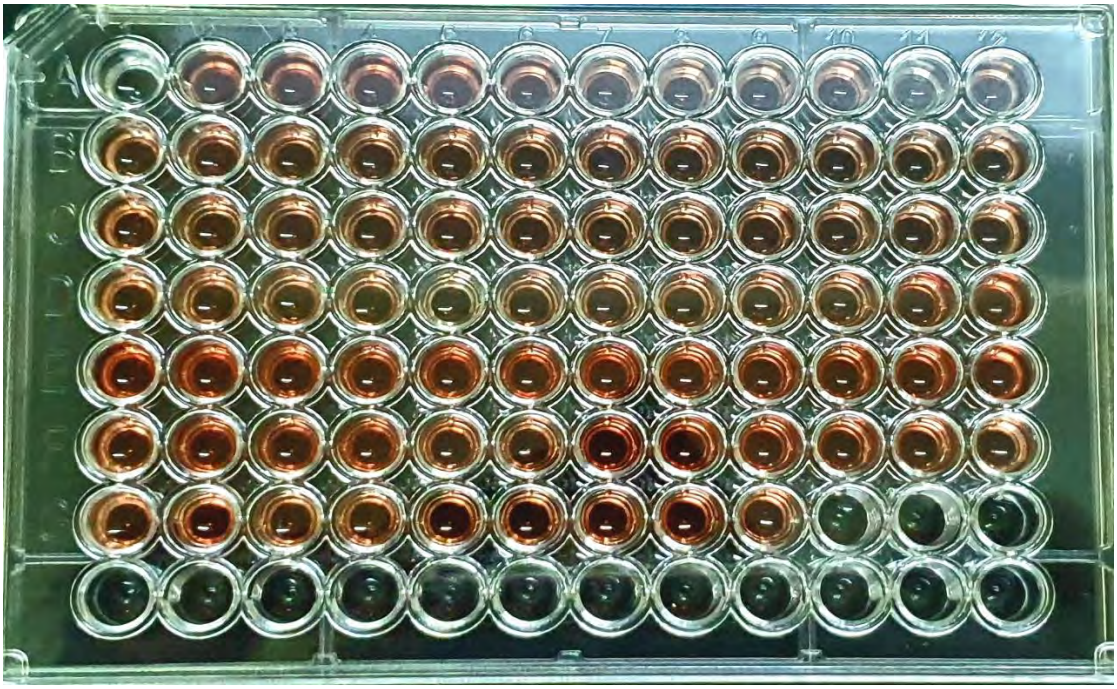

Fig 4.17 Hemolytic Activity of macrofungal crude extracts at different concentrations with **A1** = Blank (DPPH); **A2, A3, A4, A5, A6**= Positive control (0.05, 0.25, 0.5, 0.75 and 1mg/mL); **A7, A8, A9, A 10, A11**= Positive control (0.05, 0.25, 0.5, 0.75 and 1mg/mL).

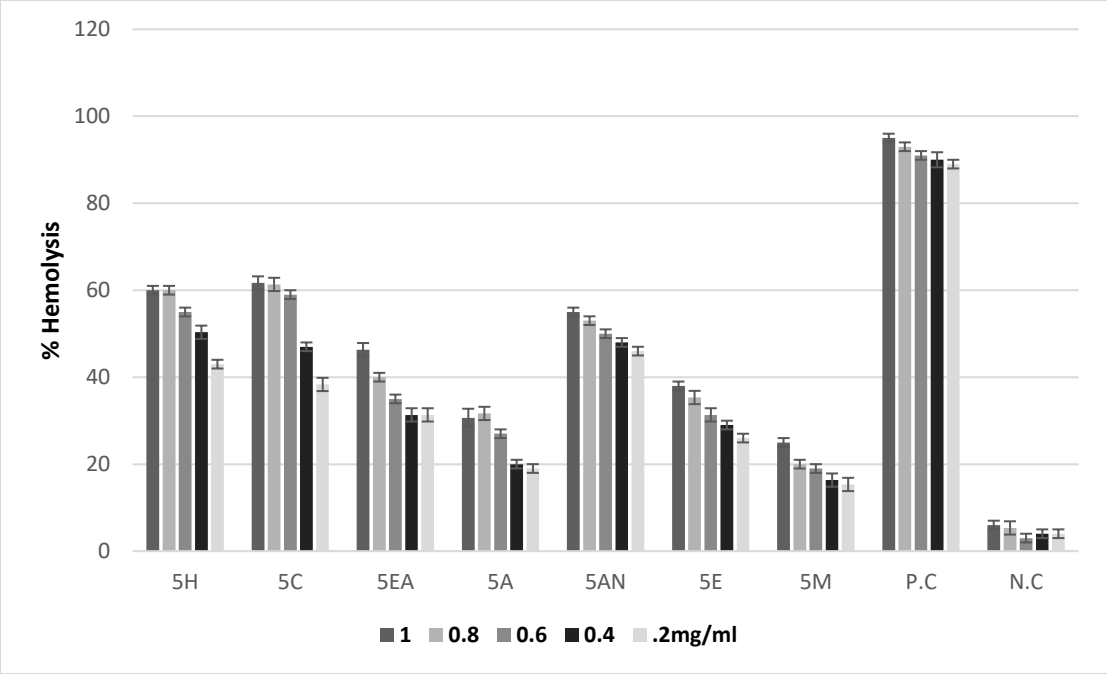

Fig 4.18. The dose-dependent hemolytic activity of crude extracts of *Amanita phalloides.*

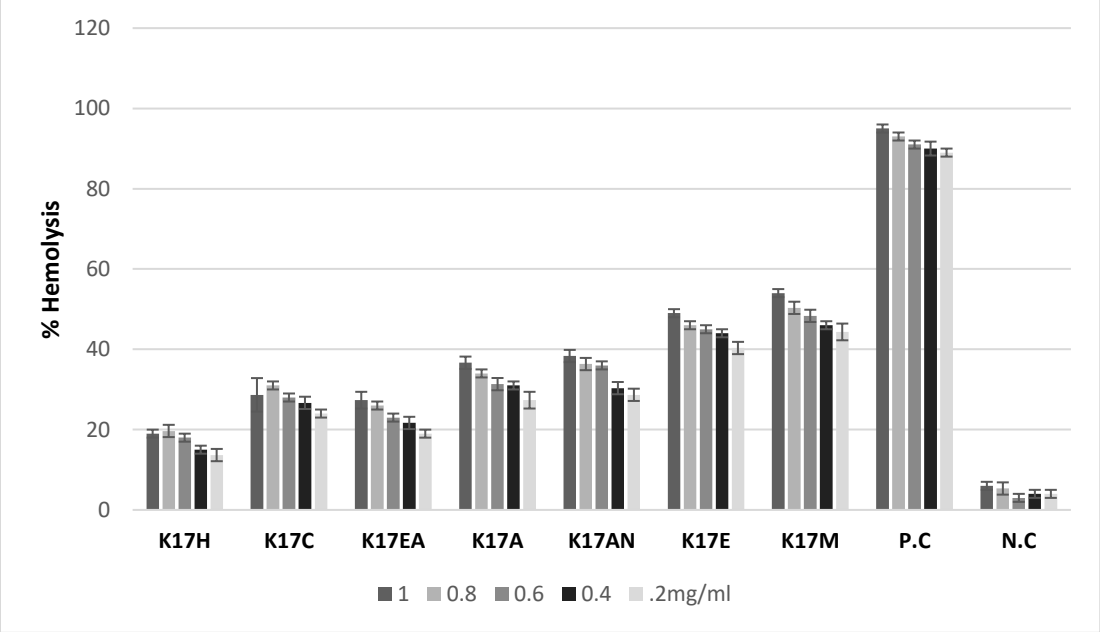

Fig 4.19. The dose-dependent anti-hemolytic activity of crude extracts of *Trametes versicolor.*

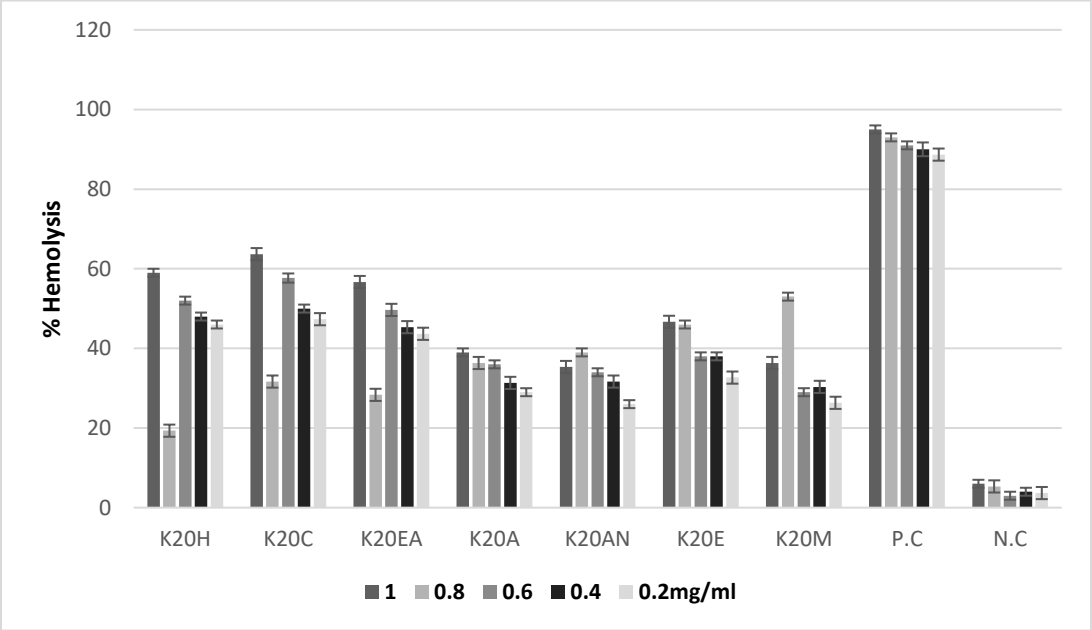

Fig 4.20. The dose-dependent anti-hemolytic activity of crude extracts of *Phellinus gilvus.*

4.7. Mycochemical profiling of Mushroom extracts:

Mycochemical tests provide a peek into the chemical complexity of mushrooms and offer indistinct clues about the potential therapeutic applications. This lays the foundation for the analysis of prospective bioactivities and the identity of individual compounds. These tests were performed initially to have a rough estimation of the presence of secondary metabolites. This assessment gave a crude idea about the potential bioactivity and chemical composition of mushrooms. Compounds like flavonoids, phenolic compounds, saponins, and tannins often exhibit strong therapeutic potential. The majority of the extracts of *Amanita phalloides, Trametes versicolor,* and *Phellinus gilvus* have demonstrated positive results for the presence of tannins and saponins (Figure 4.21, 4.23). None of the extracts of all three species have given positive results for the presence of steroids. (Figure 4.22)

The total flavonoid content in all mushroom species was fairly high with *Phellinus gilvus* showing the highest concentration in the range of 110 to 174mg QE/g of all seven fractions. Chloroform extracts of *Trametes versicolor* have shown the highest total phenolic content of 98mg GAE/g whereas hexane and methanol extracts have shown moderate presence in the range of 70%. The total flavonoid content of *Amanita phalloides* was also quite good with methanolic extract showing the highest concentration of 102mg QE/g, 81mg QE/g, and 89mg QE/g total flavonoid content noted in hexane and acetone extracts. (Fig 4.25, 4.27)

The highest total phenolic content was reported in Amanita phalloides; 107mg GAE/g, 94mg GAE/g, and 85mg GAE/g was recorded in methanol, hexane, and acetone extracts. A very minimal level of total phenolic content in the range of 8 to 20mg GAE/g was recorded in the *Trametes versicolor* and *Phellinus glivus*. (Fig 4.24, 4.29)

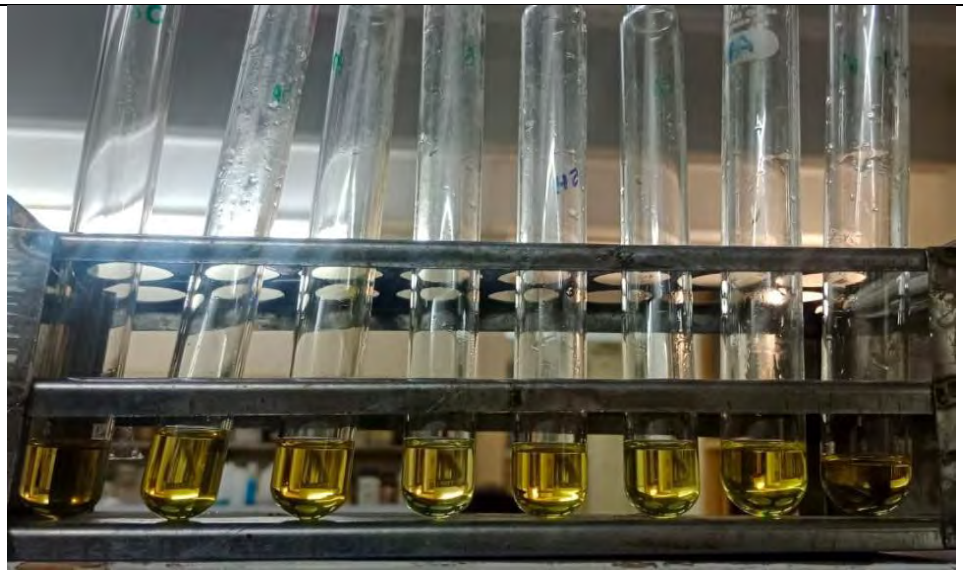

Fig 4.21. Test for the presence of tannins.

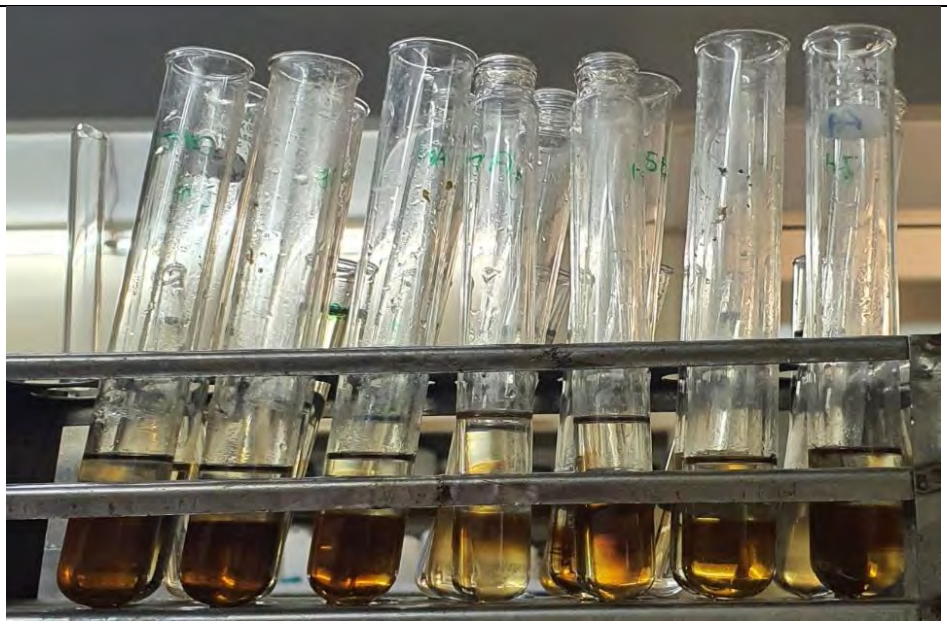

Fig 4.22. Test for the presence of steroids

Fig 4.23. Test for presence of saponins

Table 4.2. Results of Qualitative tests for the presence of saponins, tannins and steroids.

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Extracts	Tannins	Saponins	Steroids
K5H	-	+	-
K5C	+	+	-
K5EA	-	-	-
K5A	+	-	-
K5AN	+	-	-
K5E	-	+	-
K5M	+	+	-
K17H	+	+	-
K17C	-	+	-
K17EA	+	+	-
K17A	-	+	-
K17AN	+	+	-
K17E	+	+	-
K17M	+	+	-
K20H	-	+	-
K20C	-	+	-
K20EA	+	-	-
K20A	-	-	-
K20AN	+	-	-
K20E	+	-	-
K20M	+	+	-

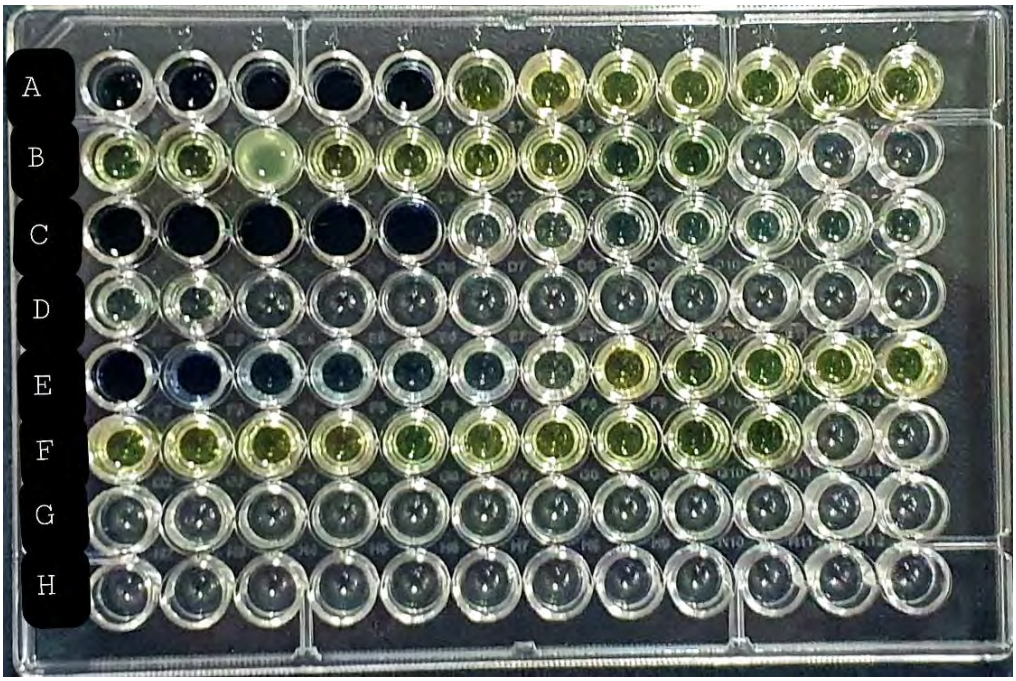

Fig 4.24. Tests for the analysis of Total Phenolic Content

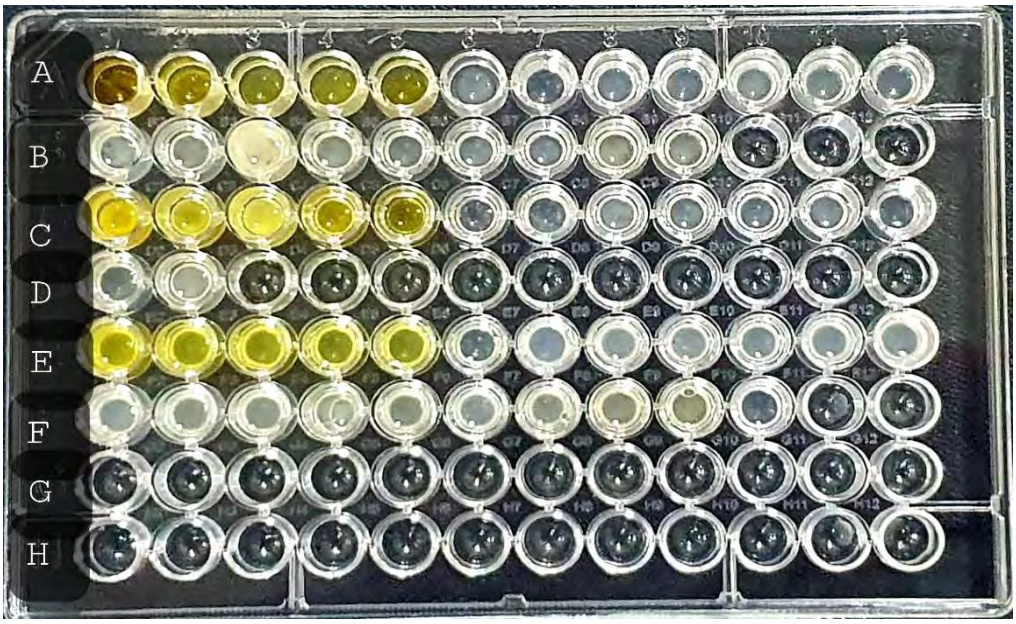

Fig 4.25. Test for the analysis of Total Flavonoid Content.

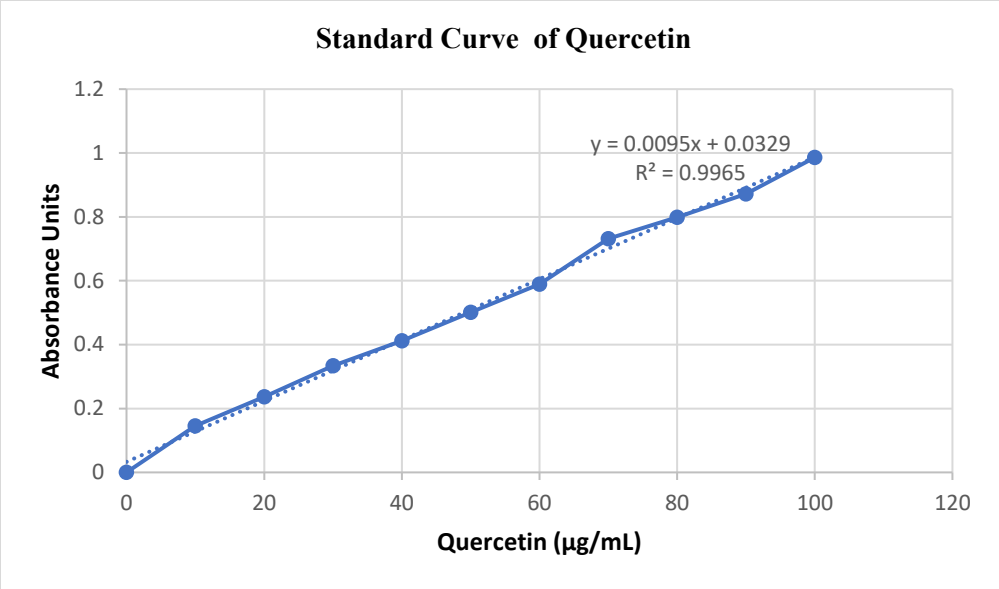

Fig 4.26. Standard curve of Quercetin.

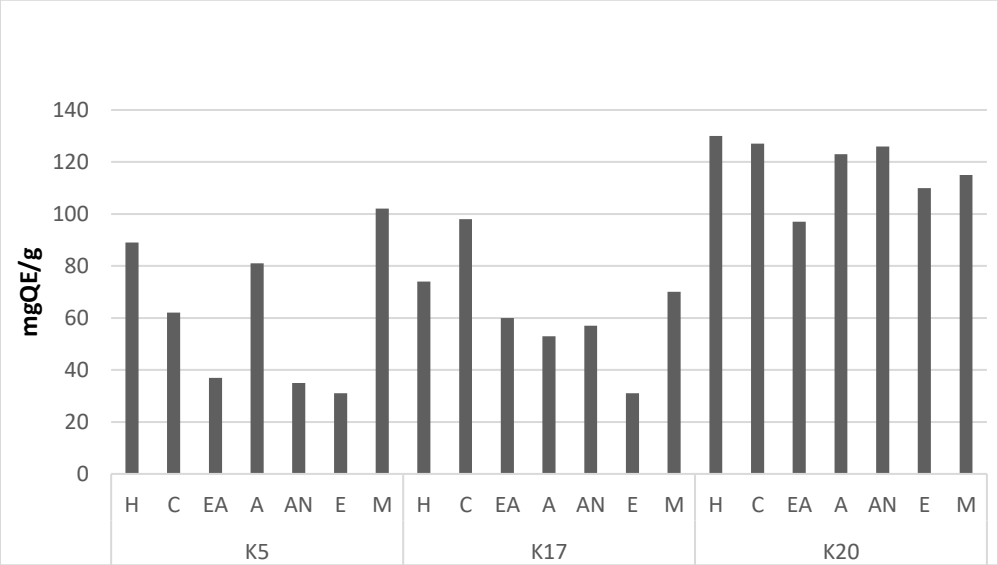

Fig 4.27. Result of test for the analysis of Total Flavonoid Content.

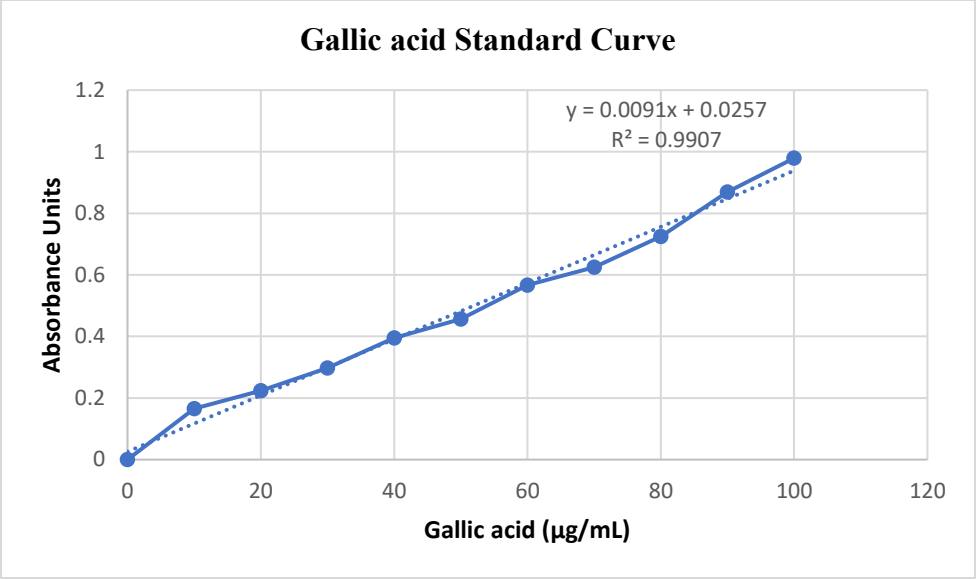

Fig 4.28. Standard Curve of Gallic acid.

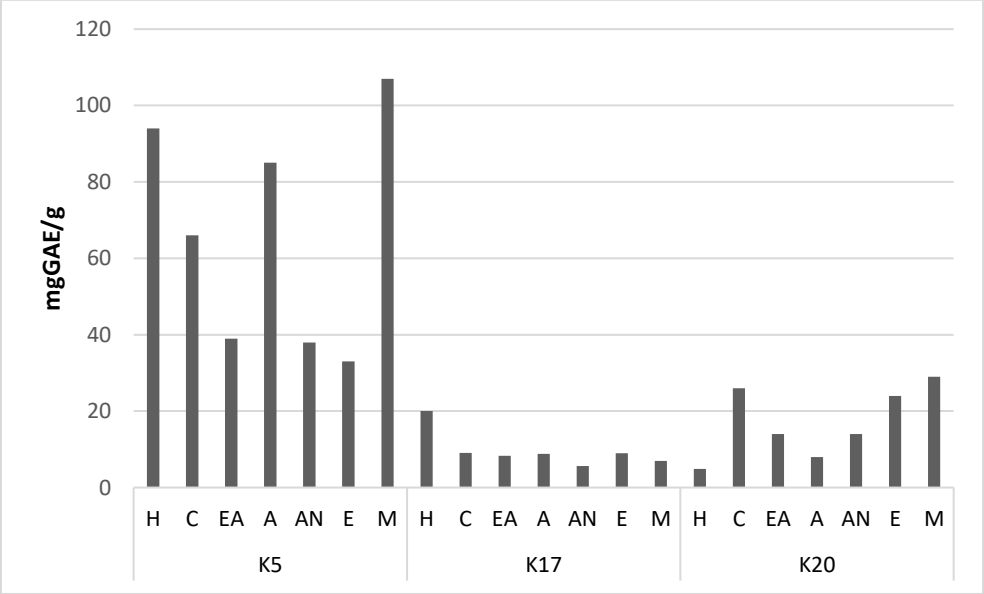

Fig 4.29. Test for the analysis of Total Phenolic Content.

4.8. Purification of Macrofungal Crude Extracts by Column Chromatography

Elution Column Chromatography was performed for each fraction of *Amanita Phalloides, Trametes versicolor,* and *Phellinus glivus.* The extracted metabolites were dissolved in respective solvents. Elution was performed by using different solvents such as hexane, chloroform, ethyl acetate, ethanol, methanol, acetone, acetonitrile, and water. A total of 10-15 fractions were obtained after running the column of each extract. The fractions containing metabolites are mentioned below (Table 4.30).

Figure 4.30. Purified Fractions of Mushrooms obtained via elution column chromatography.

4.9. Biological Activity of Purified Fractions

Active fractions of all three species of mushrooms were subjected to purification via elution column chromatography and antibacterial activity of purified fractions was observed further. Trametes versicolor has demonstrated the best antibacterial activity. Maximum activity in the range of 15 to 16mm was recorded by the fractions of Ethylacetate, acetone,

acetonitrile, and ethanol mostly against *S. aureus, B.subtilis, and P. aeurignosa. S.aureus* was the most susceptible to the antibacterial action of purified extracts. *Phellinus gilvus* demonstrated fairly good activity. Zone of inhibition in the range of 13 to 19mm was manifested by the hexane, chloroform, ethyl acetate, and acetonitrile fractions (Figure 4.31). A moderate level of activity was recorded by the *Amanita phalloides*. Hexane, ethyl acetate, acetone, and ethanol extracts have demonstrated inhibition zones in the range of 13 to 15mm. (Figure 4.32, 4.33, 4.44).

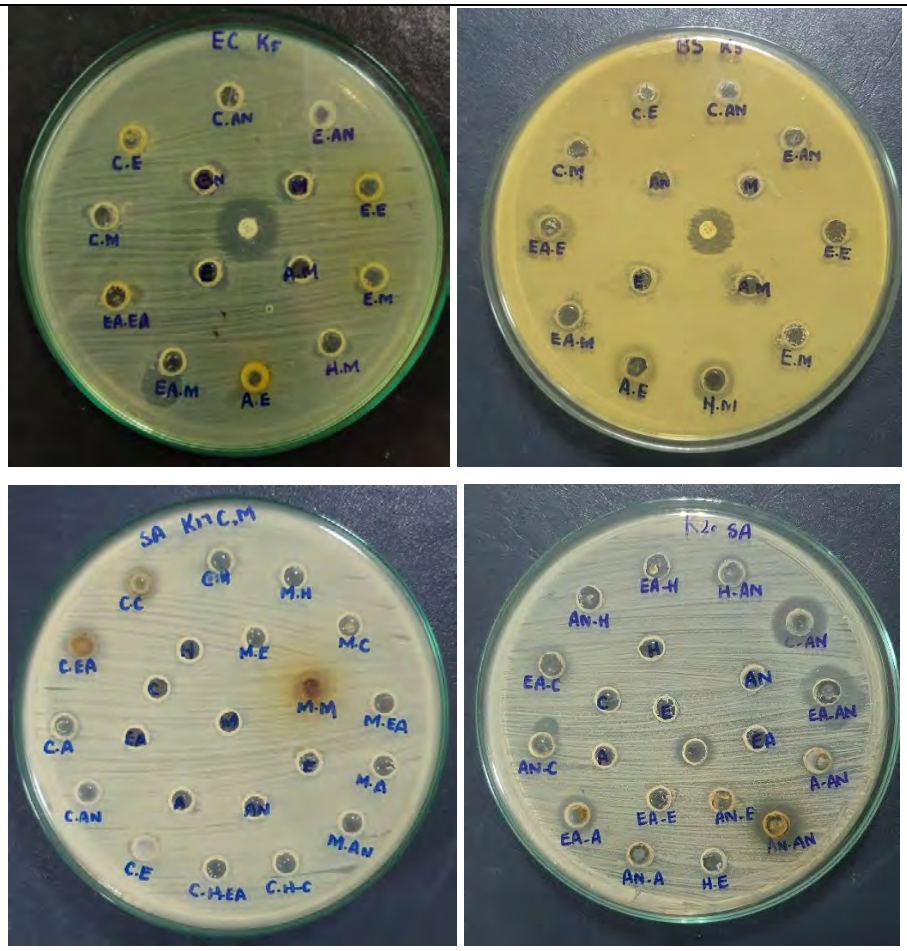

Fig 4.31. antibacterial activity of purified fractions against bacterial strains such as **A)** *E. coli,* **B)** *B. subtilis,* **C) and D)** *S. aureus.*

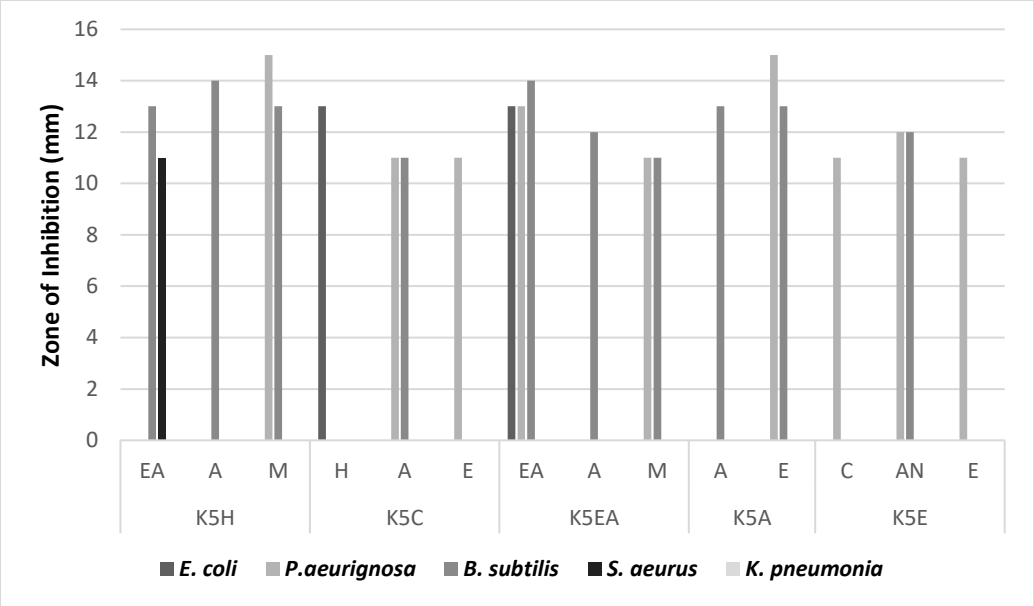

Fig 4.32. Inhibition zone (mm) of bacterial strains against the purified extracts of *Amanita phalloides.*

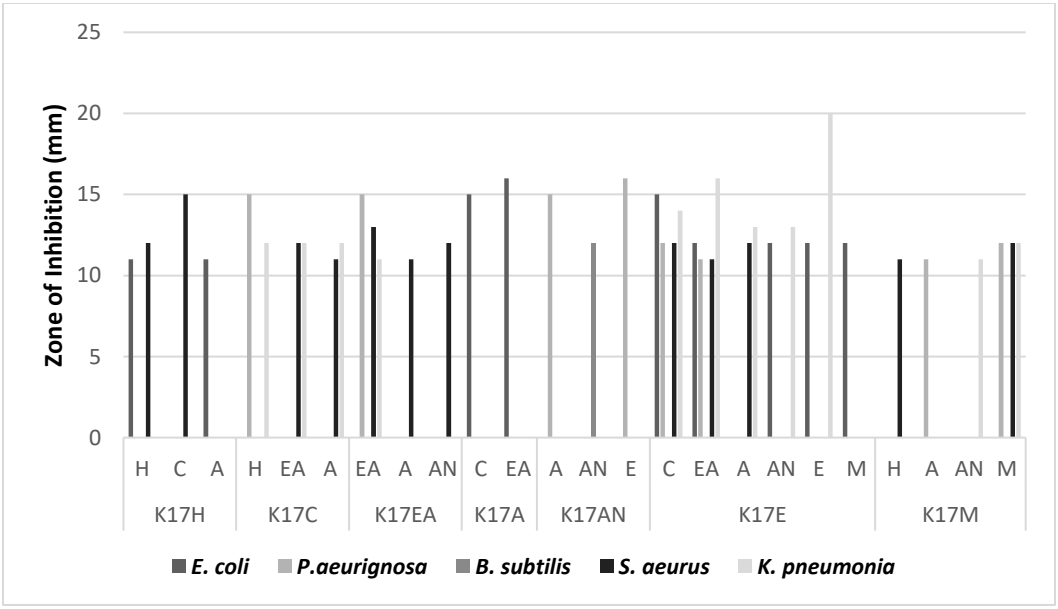

Fig 4.33. Inhibition zone (mm) of bacterial strains against the purified extracts of *Trametes versicolor.*

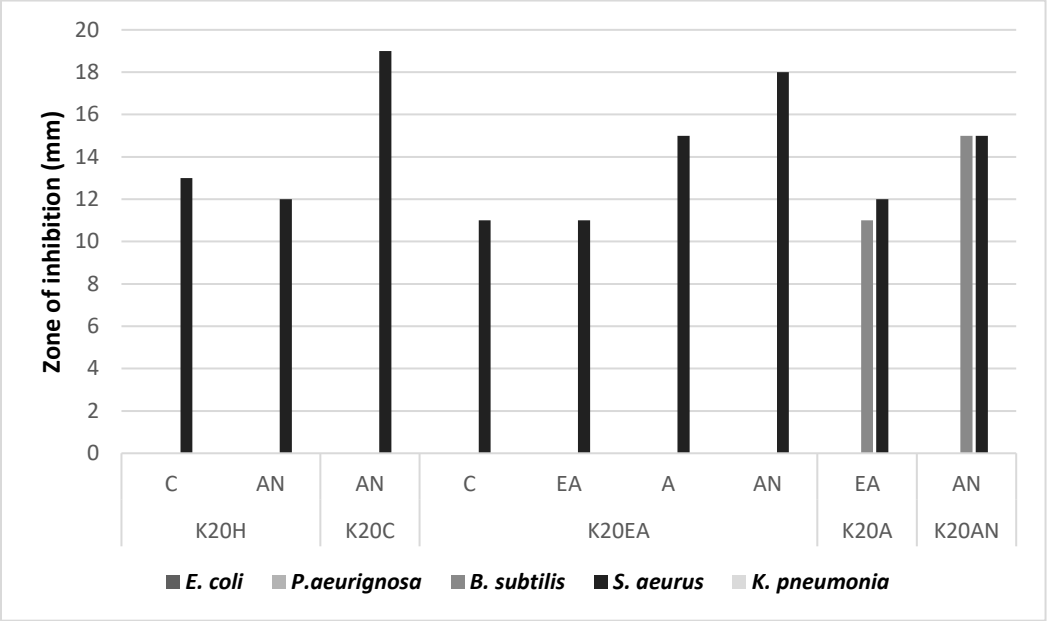

Fig 4.34. Inhibition zone (mm) of bacterial strains against the purified extracts of *Phellinus gilvus.*

4.10. Thin-Layer Chromatography of Purified Bioactive Fractions

TLC was done to check the purity of bioactive ethyl acetate fractions of all extracts by using different mobile phases such as ethyl acetate (100%), ethyl acetate: hexane (70:30), ethyl acetate: hexane (50:50), ethyl acetate: hexane (30: 70) and ethyl acetate: hexane (10:90). The clear bands were visualized in the mobile phase of ethyl acetate: hexane (10:90). Three bands were visualized in ethyl acetate fraction of hexane, chloroform, and ethanol with Rf values of 0.42, 0.57, and 0.64 while the band was obtained in ethyl acetate fractions of ethyl acetate, acetone, acetonitrile, and methanol with Rf value of 0.42. (Fig 4.35). Likewise, clear bands of ethanol and methanol fractions were observed in the mobile phase of chloroform: methanol (9:1).

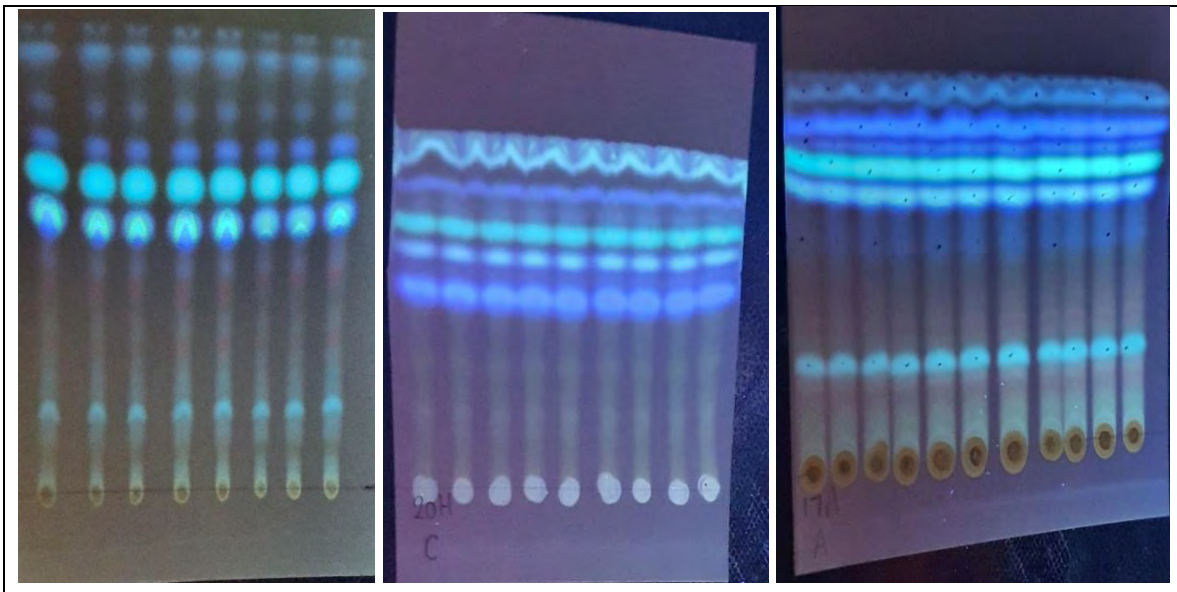

Fig 4.35. TLC results of purified fractions of A) Hexane, B) Chloroform, C) Ethyl acetate

4.11. Antibacterial activity of TLC bands:

The antibacterial activity of acetone and ethyl acetate fractions of *Trametes versicolor* and hexane fractions of *Phellinus Glivus* was analyzed by scratching bands of TLC. Minute level of activity was reported by the h band of acetone. A zone of inhibition of 12mm was spotted by c and d bands of ethyl acetate TLC. 17mm zone of inhibition was demonstrated by the d band of hexane extracts (Figure 4.36).

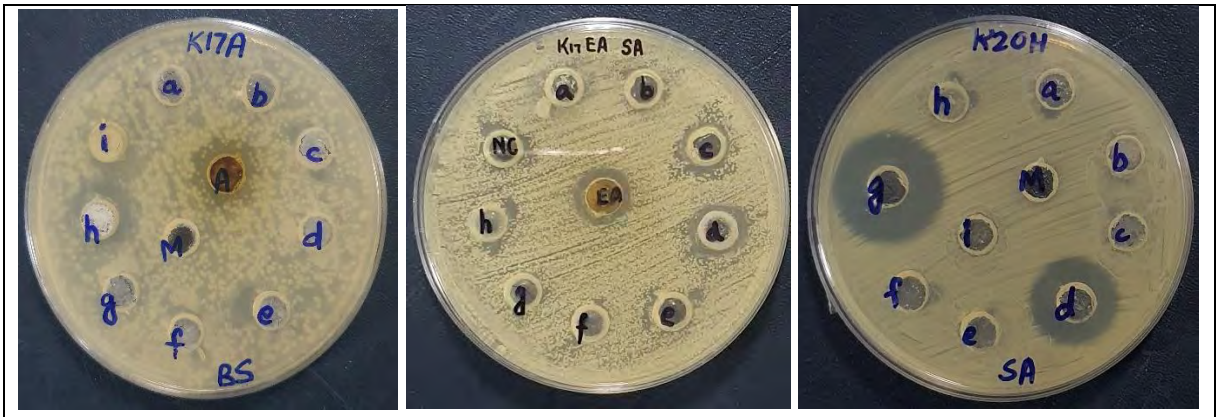

Fig 4.36. Antibacterial activity of bands of TLC

5. Discussion:

The revolution in the field of medicine caused by the discovery of antibiotics has come to an end. Antimicrobial resistance has imposed a serious health concern worldwide. An alarming increase of 70% has been reported in South Asia. A continuous rise in the incidence of multi-drug resistant infection has been reported in Pakistan. The rise of antibiotic resistance is a complicated and multidimensional issue caused by a coalition of several factors. A comprehensive understanding of these factors is crucial for developing strategies to control AMR. One of the core reasons for antibiotic resistance is the misuse and overuse of antibiotics in both animals and humans. Failure to complete antibiotic courses, misplaced use of antibiotics such as for viral infections, and imprecise prescriptions are some of the main reasons. The ability of bacteria to transfer genes via horizontal gene transfer is another reason for the widespread of resistance genes within bacterial populations. Due to high cost, the research for the discovery of novel antibiotics has declined over the years resulting in limited treatment options for curing infections caused by resistant bacteria. Poor infection control strategies and below-par hygiene conditions in healthcare settings are some of the main reasons for the prevalence of resistant bacteria infections among patients and healthcare workers. In addition, the lack of proper strategies to treat wastewater and antibiotic disposal leads to resistance development in environmental bacteria which is then transferred to human pathogens via the exchange of genetic material among bacteria. Antibiotic resistance can only be controlled by the coordinated efforts of healthcare professionals, policymakers, and the general public. Some of the strategies that could be employed include raising public awareness, improving the efficacy of diagnostic procedures, designing effective infection control strategies, advertising rational use of antibiotics, and most importantly discovering novel sources of antibiotics such as mushrooms. Mushrooms are the least explored treasure of nature and may act as a novel source of antibiotic agents. The presence of antibacterial activity points to the presence of bioactive compounds within mushroom extracts that can kill bacterial pathogens by interfering with their physiology. Our study involved collection and screening of diverse mushroom species collected from AJK for pharmacological properties

such as antibacterial, antifungal, antioxidant, cytotoxic, and hemolytic activities. Secondary metabolites were extracted from the powdered biomass of mushrooms through solvent extraction. Seven solvents of varying polarity; hexane, chloroform, ethylacetate, acetone, acetonitrile, ethanol and methanol were selected and each of it's extract was dried and subjected to various activity analysis assays. The antibacterial potential of each crude extract was determined against five MDR strains and activity of varying levels was recorded via the agar well plate diffusion method. *Trametes versicolor* has proven to be the most potent mushroom as a very substantial amount of activity has been reported by all of its extracts against all five strains in the range of 11 to 22 ± 1.52mm. However, ethyl acetate, acetone and ethanol, methanol extracts have demonstrated the strongest activity against *K. pneumoniae* and *Staphylococcus aureus* with zones of inhibition of 20 ± 1.15mm, 22 ± 0.57mm, 21 ± 1.57mm, 21 ± 1.57mm respectively. In addition, the best activity was reported by acetonitrile extracts of *Phellinus gilvus* against *P. aeurignosa, S. aureus, and Bacillus subtilis* with zones of inhibition of 18 ± 1mm, 21 ± 1.57mm, and 18 ± 2mm respectively. Chloroform and Ethanolic extracts of *Trametes versicolor* have demonstrated the strongest activity against *K. pneumoniae* and *B. subtilis* with an inhibition zone of 17 ± 1.54mm and 18 ± 0.57mm respectively. Overall negligible activity was reported by hexane and methanolic extracts across all extracts of mushrooms. In contrast with other gram-negative bacteria, *E. coli* was the most resistant to the inhibitory effects of macrofungal crude extracts. Dogan et al have reported the antibacterial activities of *Amanita ovoidea* in differing concentrations. The methanolic extracts have reported the strongest maximum antimicrobial activity at the concentration of 100 µg/mL against *Bacillus subtilis, S. aureus, L. monocytogenes, S. pyogenes K. pneumoniae, and P. vulgaris*. A moderate level of activity was reported by ethanolic extracts at a dose of 312.5 µg/mL. The second most effective solvent was acetone. However, in our research, the best study was reported by the chloroform, ethylacetate, acetone, and ethanol fractions while no activity was recorded by the methanolic extracts (Doğan & Arslan, 2015). . Similarly, Reis et al has reported that methanolic and ethanolic extracts of *Phellinus liteus* have shown the strongest antibacterial activity for all bacteria. However, in our study no activity was reported by methanolic extracts of *Phellinus gilvus* (Reis et al., 2014). Several studies

conducted in the past on *Amanita phalloides* have also reported similar antibacterial effects however, it must be noted that it is a highly toxic mushroom and should not be ingested.

The difference in activity among different species underscores the diverse chemical composition and mechanisms of action. The antibacterial activity of mushroom extracts is often associated with compounds like polysaccharides, terpenoids, and phenolic compounds. These compounds may act via inhibition of bacterial enzymes, disrupting the integrity of cell membranes, or interfering with cellular processes in ways that are not conceivable yet. An understanding of these mechanisms is crucial as they may provide invaluable insights into how these extracts target resistance mechanisms which can be manipulated further for in-silico drug synthesis. Albeit, further studies need to be conducted for further safety and efficacy assessment, these extracts could be used for novel drug development. In addition, toxicity assessment on animal models and clinical settings needs to be done. The intricate interplay of bioactive compounds and their mechanism of action underpins the necessity to undertake multidisciplinary research in order to fully realize the therapeutic potential of these macrofungi.

Due to increasing cases of fungal infections and the limited arsenal of antifungal drugs, discovery of novel sources of antifungal agents is exceedingly important. The evidences of traditional usage of mushrooms for medicinal applications makes them promising candidates for novel drug discovery and may help us fight against lingering issue of fungal resistance. The antifungal activity of macrofungal crude extracts was checked against three strains of fungi; *Candida albicans*, *Aspergillus niger,* and *Aspergillus flavus*. The overall results of the antifungal activity of crude extracts of mushrooms were not very promising. A few extracts had a very minimal inhibitory effect however, most extracts have demonstrated zero activity. Overall ethanolic extracts have exhibited trivial activity against *Candida albicans.* A very negligible amount of activity was recorded by acetone and acetonitrile extracts. *Aspergillus niger* was the most resistant among all tested strains of fungi. However, these results are by no means an exhaustive representative of antifungal potential of mushrooms and more research on screening of further mushroom species needs to be done followed up by the advanced studies on toxicity and efficacy assessment on

animal models and clinical settings. The screening of macrofungi for antifungal agents is important for agriculture as well. Mushrooms with potent antifungal activities can offer compelling implications in agriculture as they could be used in to control fungal pathogens of plant, thus providing natural and environment-friendly fungicides(Owaid et al., 2017).

The analysis of antioxidant activity was the crucial step to assess the therapeutic potential of mushrooms. The results of free radical scavenging assays have elucidated the ability of mushrooms to help cope with numerous chronic diseases caused by oxidative stress. The imbalance between the antioxidant defense mechanism of the body and production of reactive oxygen species (ROS) is the primary cause of pathogenesis of numerous diseases such as cardiovascular diseases. These antioxidant assays serve to indicated the antioxidative potential of mushrooms thus indicating their capacity to minimize the impact of oxidative damage. The free radical scavenging potential of the extracts depends on the concentration of the extracts. A consistent increase in antioxidant activity was observed with increasing concentrations of the extracts. *Amanita phalloides* has exhibited very weak antioxidant activity in the range of 20 to 40 ± 1.5%. The results of the antioxidant activity of *Trametes versicolor* were fairly good, the extract of hexane has demonstrated antioxidant activity of 75 ± 1%. *Phellinus glivus* has demonstrated the best antioxidant activity; 78 ± 1.5%, 72 ± 0.57%, 75 ± 1.57%, 75 ± 1.25% antioxidant activity was exhibited by the ethyl acetate, acetonitrile, ethanol and methanol extracts respectively. The Methanolic extracts of *Amanita phalloides* have also exhibited remarkable activity of 85% however a moderate level of activity was exhibited by the *Trametes versicolor.* Samachai et al has reported The EtOAc fractions of crude extracts have exhibited the strongest antioxidant potential at an IC_{50} value of 17.73 ± 0.27 µg mL^{-1}.These results align with our findings of *Phellinus gilvus* whose ethyl acetate extracts have exhibited the highest activity of 72% (Ajith & Janardhanan, 2007). The differences in the antioxidant activity of different mushroom extracts are noteworthy. These variations may stem from differences in the type and concentration of constituent bioactive compounds such as phenolic compounds and flavonoids that are considered to possess strong antioxidant potential. These compounds act as chelating agents, antioxidants, and metal ion reducers and thus are the key players

responsible for the observed antioxidant effects. Another class of compounds, polysaccharides, is also very widespread in mushrooms and have been associated with the antioxidant activity possibly via modulation of immune response. This diversity in distribution of these compounds underpins the importance of screening diverse species of mushrooms to discover those with strongest antioxidant potential. Although, the antioxidant potential of mushrooms thus recorded suggests their prospective role in prevention and treatment of oxidative stress related diseases however, it must be borne in mind that in vitro antioxidant activity may or may not impart same effect in vivo. In order to assure the health benefit of mushrooms in a real-world context, more studies on animal models and clinical trials need to be executed (Fogarasi et al., 2020). The promising results of antioxidant activity leads to emergence of new avenues for future research. An understanding of underlying mechanisms via identification and quantification of specific bioactive compounds responsible for antioxidant affects will deepen our understanding. Furthermore, determination of the metabolism and bioavailability of these compounds in the human body is critical to give the final verdict about their actual significance for human health. The findings of this research add to growing body of literature that highlights the therapeutic potential of mushrooms. Nevertheless, in order to reap actual benefits rigorous research needs to be done that could bridge the gap between in vitro findings and in vivo effects. This research can pave the way for the development of novel dietary products directed at combating oxidative-stress related disease(Podkowa et al., 2021).

Cancer is a critical global health issue and exploration of a novel safe and effective compound is of prime importance. The potential of mushrooms as a source of novel anticancer drugs is assessed via cytotoxic assays. The observed cytotoxic activity of mushroom extracts is of paramount importance for cancer research. Identification of natural compounds in mushrooms with potential to impart cytotoxic affects is a very significant step toward advancing our quest for search of novel anticancer drugs and complementary therapies. The best cytotoxic activity was reported by the *Amanita phalloides* with maximum activity demonstrated by the ethyl acetate, acetone, acetonitrile,

and ethanol extracts with 80 ± 1.57 % mortality at the concentration of 1mg/ml. A moderate level of the cytotoxic potential was observed in *Trametes versicolor* with chloroform extracts exhibiting the maximum mortality of 80 ± 2.0% after 24 hours. The cytotoxic potential of *Phellinus glivus* was fairly low with the maximum mortality of 70 ± 0.57% exhibited by the chloroform extracts at 24 hours. A progressive decline of roughly 30-40 ± 1.57% was observed in all extracts after 48 hours. DMSO used as a negative control has caused approximately 10 ± 1.57% mortality. No death of brine shrimps was recorded in test tubes used as blank. Samachai et al has found that methanol, chloroform and ethylacetate extracts of *Phellinus ovoidae* have demonstrated cytotoxic activity against MFC7 and NCI-H187 cancer cells. Our study of cytotoxic activity analysis against brine shrimps strong has reported the best activity by the chloroform, acetone, acetonitrile and ethanol extracts (Hyder & Dutta, 2021). The correlation between difference in cytotoxic activity among different species and concentrations underscore the differences in the composition and distribution of bioactive molecules. This assessment stresses the importance of exploring wide variety of mushrooms in quest of the ones with most compelling cytotoxic activities. The cytotoxic effects of mushrooms are often attributed to the presence of compounds like phenolics, terpenoids and polysaccharides. Several mechanisms of action have been ascribed to these compounds such as programmed cell death (apoptosis), interference with the cell signaling pathway, inhibition of cell cycle progression and cell proliferation. However, these are just crude suggestions that only give a blur view of what's really going on inside and ask for further research to shed light on the dynamics of interaction between cancerous cells and these compounds. Elucidation of these mechanisms can provide invaluable insights on the novel potential targets for chemotherapeutic drugs within cancer cells. Another important aspect to note here is that in vitro cytotoxic activity may not directly translate into an in vivo setting. In order to validate cytotoxic potential further studies must be conducted on the animal models and clinical trials with concomitant analysis of safety and efficacy for human use. The results of this preliminary cytotoxic assay have paved the way for further studies. Isolation and identification of specific cytotoxic compounds from mushroom extracts is of paramount

importance in order to make this research of real value. Furthermore, the selectivity and specificity of these cytotoxic compounds must be investigated to minimize the potential side effects. Despite all the impending challenges the immense cytotoxic potential of mushrooms stands as a testament to the presence of enormously potent compounds in nature that need to be discovered (Kolundžić et al., 2016).

The analysis of hemolytic activity is critical for establishing a comprehensive safety profile of mushroom extracts. Hemolytic assay involves studying the effects of extracts on membrane and correlating it with their cytotoxic proclivity. It is necessary to assess the possible cytotoxic effects that mushrooms may have on red blood cells. These tests provide insights about the capability of mushrooms to cause hemolysis by disrupting cell membranes. The results of hemolytic activity can be classified into three categories: non-hemolytic, slightly hemolytic and highly hemolytic. Non-hemolytic extracts do not impart any damage to RBCs and could be used safely for further applications. Especially if these compounds are intended for internal use like pharmaceutical or nutraceutical synthesis it becomes mandatory to have minimum hemolytic activity to assure overall safety. Slightly hemolytic cause minimal level of hemolysis and are considered safe at lower concentration however, highly hemolytic extracts can cause substantial damage to RBCs and impose a major safety risk. Therefore, mushrooms with no or minimal level of hemolytic activity are safe for human consumption. The highest level of hemolysis was observed by the strains of *Phellinus gilvus* 50 to 50 ± 1.57% hemolysis was caused by the hexane, chloroform, and ethylacetate extracts. In contrast, *Trametes versicolor* has shown comparatively low hemolysis with the majority of fractions showing hemolysis in range of 20 to 30 ± 1.57% with ethanol and methanol extracts showing the highest hemolysis of 40 ± 1% and 44 ± 0.57%. Ethyl acetate, acetone, ethanol, and methanol extracts of *Amanita phalloides* had shown slight hemolysis of 31 ± 1.57%, 19 ± 2%, 26 ± 1.57%, and 15 ± 1.57% whereas acetonitrile fractions have demonstrated the highest hemolysis of 46%. Nevertheless, hemolytic activity tests provide an oversimplified method of assessing cytotoxicity of potential drug candidates and do not take into consideration the complicated interactions that may take place within human body. Henceforth, additional studies like cell culture

assays and application on animal models must be undertaken in order to establish a satisfactory understanding of their safety. However, these results add to the data on safety assessment of mushroom extracts and underpins the significance of rigorous safety assessment in any endeavor of exploration of bioactive compounds (O'neill et al., 1973).

Mycochemical tests provide insights into the chemical complexity of mushrooms and offer indistinct clues about the potential therapeutic applications. This lays the foundation for the analysis of prospective bioactivities and the identity of individual compounds. These tests were performed initially to have a rough estimation of the presence of secondary metabolites. This assessment gave a crude idea about the potential bioactivity and chemical composition of mushrooms. Compounds like flavonoids, phenolic compounds, saponins, and tannins often exhibit strong therapeutic potential. The majority of the extracts of *Amanita phalloides, Trametes versicolor,* and *Phellinus glivus* have demonstrated positive result for the presence of tannins and saponins. None of the extracts of all three species have given positive result for the presence of steroids.

The total flavonoid content in all mushroom species was fairly high with *Phellinus glivus* showing the highest concentration in the range of 110 to 174mg QE/g of all seven fractions. Chloroform extracts of *Trametes versicolor* have shown the highest total phenolic content of 98mg GAE/g whereas hexane and methanol extracts have shown moderate presence in the range of 70mg GAE/g. Total flavonoid content of *Amanita phalloides* was also quite good with methanol, hexane and acetone extracts showing the highest concentration of 102, 81, and 89mg QE/g. The highest total phenolic content was reported in *Amanita phalloides*; 107, 94 and 85 mg GAE/g was recorded in methanol, hexane and acetone extracts. A very minimal level of total phenolic content in the range of 8 to 20 mg GAE/g was recorded in the *Trametes versicolor* and *Phellinus glivus*. The correlation between results of mycochemical tests and potential bioactivities is pretty complicated and cannot be explained in a straightforward manner as the bioactivity of these compounds is influenced by myriad of factors like interaction with other compounds, concentration and variations in chemical structure. These tests merely give unrefined clues about the presence of certain compounds and need to be further verified by other bioactivity assays in order to

justify the functional utility of these compounds in the context of therapy. The spectrum of compounds detected because of mycochemical test in different mushroom species points to their rich chemical diversity (Hammami et al., 2021). Mushrooms may have varying secondary metabolites depending on the stages of their lifecycle, ecological niche and surrounding biodiversity. The bioactivities exhibited by the mushrooms are often the consequence of synergistic interactions of these compounds. For instance, a mushroom may manifest potent antioxidant activity because of the synergistic action of both saponins and tannins. This underscores the significance of taking into account the entire chemical profile of mushrooms instead of focusing solely on individual compounds. Microchemical tests are only a prelude to comprehending the chemical composition of the mushrooms and do not offer any precise information about chemical composition. Further purification and analysis need to be done like chromatography and spectroscopy to isolate and identify individual compounds. Additionally, linking mycochemical results with particular bioactivities demands deeper pharmacological investigations (Cateni et al., 2022).

Bioctivity analysis was followed up by purification of active fractions of all three species of mushrooms via elution column chromatography and antibacterial activity of purified fractions was observed further. *Trametes versicolor* has demonstrated the best antibacterial activity. Maximum activity in the range of 15 to 16mm was recorded by the fractions of ethyl acetate, acetone, acetonitrile and ethanol mostly against *S. aureus, B. subtilis,* and *P. aeurignosa*. *S. aureus* were the most susceptible to the antibacterial action of purified extracts. *Phellinus gilvus* as well has demonstrated fairly well activity. Zones of inhibition in the range of 13 to 19 mm were manifested by the hexane, chloroform, ethyl acetate and acetonitrile fractions. Moderate level of activity was recorded by the *Amanita phalloides*. Hexane, ethyl acetate, acetone, and ethanol extracts have demonstrated inhibition zone in the range of 13 to 15mm. Elution column chromatography permits the isolation and purification of crude extracts. This precision facilitates the identification of specific compounds responsible for the observed activity. Elution column chromatography followed up by further characterization via TLC and LC-MS can elucidate the extract compound responsible for the activity. This data provides the molecular basis of

antibacterial activity and sets the stage for isolation and synthesis of these potent compounds for further research. Moreover, identification and purification of individual compounds can not only enhance the efficacy but also minimizes the potential side effects to beneficial microbiota. Purified compounds extracted from distinct mushroom extracts could be checked for synergistic effects against the targeted pathogens. Although purified extracts enhance specificity, on the downside this may result in loss of antibacterial activity on account of loss of synergistic effects of various compounds. Additional studies on animal models followed up by clinical trial need to be done so as to translate these findings to clinical relevance (Faulstich et al., 1973).

Conclusion:

Due to the exasperating rise in antimicrobial resistance in recent years, the interest of the scientific community in exploring the untapped therapeutic potential of mushrooms has piqued. Azad Jammu and Kashmir is home to a wide variety of mushrooms whose pharmacological potential has not been explored yet. This study involved collecting mushrooms from diverse ecological niches of Azad Jammu & Kashmir and a detailed taxonomic identification and characterization was conducted in order to ensure thorough assessment of collected strains. A comprehensive mycochemical profile of each strain was synthesized by performing several tests such as tests for saponnins, tannins, steroids, total flavonoid content, and total phenolic content. Additionally, each strain was screened for pharmacological properties such as antibacterial, antifungal, anticancer, antioxidant, and hemolytic. The results of these activities have indicated that mushrooms have a remarkable antibacterial, anticancer, and antioxidant potential. The results of antibacterial activity were especially extraordinary as a strong inhibitory effect of macrofungal extracts was recorded against MDR strains of *E.coli, P. aeurignosa, S. aureus, B. subtilis,* and *K. pneumoniae*. A very strong antibacterial activity was demonstrated by all three macrofungal species. A very good antioxidant, and cytotoxic activity was also recorded that varied depending upon the concentration of extract. Macrofungal extracts with the most potent antibacterial activity were further purified using column chromatography. Purified extracts were again investigated for antibacterial activity and the most potent ones were analyzed via TLC. The results of TLC have indicated the presence of a plethora of compounds. In addition, Contact TLC bioautography was performed and the bioactive fractions acquired by scratching bands of TLC have been sent for LC-MS characterization.

The findings of this research reflect the richness of the biodiversity of mushrooms and inspire further investigations to be undertaken into the uncharted territories of natural resources. The vast array of compounds that we have encountered underscores the potential for the exploration of novel drug candidates. The implications of this research are multifaceted and have the proclivity to revolutionize the field of nutrition, medicine, and cosmetics. Although the overwhelming rise in AMR is intimidating, this research stands as a testament to the boundless treasures hidden at

obscure places across the planet, waiting to be discovered and utilized for the benefit of humanity. Hopefully, this research will act as a catalyst for further exploration, collaboration, and innovation.

Future Perspectives:

The future implications of this research are not only promising but also spectacularly diverse. Mushrooms have been used for their nutritional and therapeutic properties since ancient times. This study highlights the therapeutic potential of mushrooms which can bridge the gap between traditional medicine and modern science leading to breakthroughs in the field of medicine. Further investigations may identify novel bioactive compounds that could be developed into several pharmaceutical and nutraceutical products of great value for human health. This can inspire researchers and pharmaceutical companies to collaborate in order to harness the potency of these compounds for better health and economic gains. Screening of the biodiversity of mushrooms may yield compounds that manifest specificity toward individual genetic variations and certain diseases. This could open the door for personalized treatment approaches which entails prescribing specific medicines that are tailored to the individual's genetic makeup and health profile.

Mushrooms are very well known for their ability to sustain the balance of ecosystems and bioremediate polluted environments. An attempt to explore the properties of mushrooms can lead to the discovery of mushroom species that are adept at degrading pollutants and absorption of heavy metals. Such a discovery can contribute substantially to the restoration of the ecosystem. Certain mushroom species are a rich reservoir of essential nutrients that could be developed into novel nutritional supplements and functional foods that can be used to address nutritional deficiencies in a precise and targeted manner. The potential of mushrooms is multidimensional and extends far beyond the realms of medicine. Certain enzymes and compounds discovered in mushrooms can have far-ranging implications in the fields of agriculture, biofuel production, and biotechnology. Furthermore, the demonstration of the enormous therapeutic potential of mushrooms can help in the preservation of the biodiversity of AJK by influencing the policies related to biodiversity conservation. Additionally, this research can garner the attention of researchers across the world to collaborate and carry out interdisciplinary research endeavors. This study could act as a model for further research to be carried out at other locations harboring distinct

varieties of mushrooms. In short, this research has the potential to create a massive impact by improving the life quality of many individuals be it through the discovery of novel drug candidates, environmental restoration strategies, or the synthesis of functional foods.

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