Pharmacological Properties of Bioactive Metabolites from Wild Mushrooms of Azad Jamu and Kashmir region

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DEDICATION

Only with the support of beloved parents and dearest brothers did I manage to make this small contribution to the field of science. Without their support, my life would not have turned out the way it did. These foundational figures have consistently served as a source of inspiration and guidance.

Shayan naeem

DECLARATION

I, **Shayan Naeem**, an MPhil student at Quaid-i-Azam University Islamabad, Pakistan, hereby declare that the work presented in this thesis titled **"Exploring Pharmacological Properties of Bioactive Metabolites from Wild Mushrooms of Azad Jamu and Kashmir Region"** is solely my own work completed under the supervision of my supervisor, **Dr. Aamer Ali Shah**. It is also stated that no material, visuals, or tables are copied from any other source unless properly acknowledged, and the source is listed in this dissertation in the section titled references. It is the outcome of original research and I have not previously presented this work elsewhere for any other degree.

Shayan Naeem

CERTIFICATE

This thesis submitted by ***Shayan Naeem*** is accepted in its present form by the Department of Microbiology, Quaid-i-Azam University, Islamabad, as satisfying the thesis requirements for the degree of Master of Philosophy in Microbiology.

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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

Mushrooms are considered to be the most underutilized source of nutrient-dense food. Mushrooms have numerous health benefits due to their diverse chemical makeup. The current study entails a preliminary screening of eleven wild mushrooms collected from various regions of Azad Jammu and Kashmir, Pakistan. Different assays were used to evaluate the biological potential of metabolites extracted with different solvents. Following screening, the presence or absence of a specific secondary metabolite is evaluated using a variety of qualitative and quantitative tests. The acetonitrile fractions of *Russula foetentula* showed the best activity against *E. coli, B. subtilis, P. aeruginosa, S. aureus,* and *K. pneumoniae* with the inhibition zone of 16 ± 0.5mm, 21 ± 0mm, 14 ± 0.5mm, 20 ± 0mm, and 11 ± 1.0mm. In addition, the crude metabolites of *Auricularia auricula judae* and *Macrolepiota albuminosa* extracted with ethyl acetate appeared as antibacterial against all the test pathogens (MDRs) at very low concentrations. In terms of antifungal potential, the ethyl acetate and acetonitrile fractions of *Russula foetentula* were found to be active against three fungal test strains: *Aspergillus niger, Aspergillus flavus*, and *Candida albicans* with the inhibition zones of 11 ± 0.5mm, 14 ± 0.5mm and 15 ± 0.5mm. Also, most of the extracts dissolved in ethyl acetate, acetonitrile, acetone, and ethanol outperformed the others. All fractions of *Russula cerolens, Amanita phalloides*, and *Auriculaia auricula-judae* extracts displayed strong antioxidant activity. The excellent antioxidant activity was demonstrated by ethanol fractions of *Auricularia auricula-judae* (80 ± 0.5%), ethyl acetate fraction of *Russula cerolens* (76 ± 0.5%), and acetone fraction of *Amanita phalloides* (75 ± 1.0%). Likewise, all fractions of *Russula foetentula, Russula carolens, Cantharellus phalloides*, and *Chlorophyllum cinerus* exhibited considerable cytotoxic potential with a mortality rate of 100%. Extracts of *Russula cerolens* and *Chlorophyllum molybdites* showed high hemolytic activity of 98 ± 1.0% and 96 ± 1.0%. *Lactarius deliciosus* and *Macrolepiota albuminosa* extracts had the most minor hemolytic activity. *Russula emetica, Auricularia auricula judae, Russula foetentula,* and *Russula paludosa* all had low to moderate hemolytic activity. The mycochemical analysis revealed that most of the polar extracts such as ethanol and methanol tested positive for saponins and tannins. The presence of steroids was detected in both polar and non-polar extracts. Chloroform fractions of *Laccaria laccata* and *Amanita phalloides* exhibited too much presence of phenols with the values of 192mg QE/g and 163mg QE/g.

1. INTRODUCTION

Two decades ago, the term functional foods was coined, and since then, consumers have shown an avid interest in all the bioactive compounds found in foods that are beneficial to human health and minimise illness risk (Wiseman et al., 1998). In order to label an item a functional food, an in-depth awareness of each bioactive ingredient that makes up the food is required, and distinct regulations on functional foods and their health claims have been established by different nations (Hasler, 1996). The most popular functional food available today is mushroom, which has been cultivated and used for generations in Asian countries (Zhang et al., 2007). Mushrooms are classified as a macro fungus with a unique fruiting body and do not belong to either the plant or animal kingdoms (Chang & Miles, 1989). Mushrooms are macro fungi that are sufficiently big to be harvested by hand and readily apparent to be seen with the naked eye, and they have distinct fruiting bodies. Mushrooms, such as *Agaricus bisporus*, can be eaten fresh or cooked. Not all mushrooms are capable of being used for being cooked, and some are widely recognized for their effective secondary metabolites (Niazi & Ghafoor, 2021). These bioactive chemicals extracted from mushroom such as *Ganoderma lucidum* have health-related usefulness. However, both consumable and medicinal varieties have been shown to have an advantageous effect on health. Another variety of mushroom exists that has been verified and has been suggested of being harmful and includes extremely dangerous mycotoxins such as cyclopeptides and isoxazole and is never suggested for consumption (Bulam et al., 2019)

Mushrooms have been utilized as a conventional and historical pharmaceutical product since the Neolithic period. Modern studies not only established the medical value of mushrooms, but also confirmed traditional knowledge about medicinal mushrooms (Gargano et al., 2017). Representatives of several ascomycetes and higher basidiomycetes are somewhat like plants with medicinal properties that arepreviously employed in the form of extract or a powder to treat a variety of ailments (Rai et al., 2005). Aside from effectively treating ailments, mushrooms are utilized as

nutrient-rich foods and dietary supplements, and consumer demand for mushroom-sourced supplements keeps on growing (Gründemann et al., 2020).

Mushrooms are also used as insecticides, bactericides, and fungicides, proving to be useful as organic biocontrol substances. Skincare businesses have taken notice of mushrooms, and they have started using mushroom-based ingredients to give their products antiallergic, antimicrobial, and antioxidant qualities (H. Kumar et al., 2021). Polysaccharides and numerous additional enzymes discovered in mushrooms are recognized as cosmeceuticals with features that include skin growth factor stimulation and autoimmune vitiligo suppression (Y. Wu et al., 2016). The therapeutic usage of mushrooms originated in Asian countries such as Japan, Korea, and China, while the West just lately recognized the medicinal value. Mushrooms having therapeutic properties have been shown to have more strong cell walls and secondary metabolic products with additional pharmacological properties than conventional culinary mushrooms (Lindequist et al., 2005). Polysaccharides derived from mushrooms may activate the body's immune system and have potent anticancer properties (Reshetnikov & Tan, 2001). Most of them operate as immune-modulating agents and are useful in tackling cancer. Ganopoly is frequently utilized in Asian nations for the treatment of cancer-related signs and symptoms, and it also lowers blood sugar levels in people with diabetes of the type 2 (Gao et al., 2004). Free radical scavenging capability has a connection with the mushroom cell structure because the polysaccharides in their cell walls have the capability to serve as antioxidants (Al-Fatimi et al., 2005).

To evaluate their nutritional composition, the amount of protein, vitamins and minerals, and carbs content of the mushrooms must be determined. If acquired from previously available food or food items, everything that enhances wellbeing in the sense of minimizing symptoms of disease or promoting general wellness is referred to as nutraceuticals (Colak et al., 2009). The protein level of mushrooms is relatively high, although it may vary depending on conditions such as the type of species and what stage of growth in which they are (Longvah & Deosthale, 1998). According to research, these mushrooms lack sulfur-containing amino acids and have an inadequate free amino acid content. In a comparable manner cultivated mushrooms include vitamins that include

riboflavin and niacin, and the quantity of vitamin B2 in mushrooms is extremely high when compared to veggies, and in certain circumstances, it is the same as that is found in cheeses and eggs (Cheung, 2010). Aside from that, mushrooms provide the same amount of folate as vegetables. All edible mushrooms have an elevated oligosaccharide content and a relatively small fatty acid content (Ribeiro et al., 2009). People who pursue vegetarian meals may find mushrooms to be a decent substitute because they have a greater amount of protein than vegetables. Aside from nutritional compounds, mushrooms include several bioactive compounds, the quantity of which differs based on the kind of mushroom, the substrate, culture, phase of growth, conditions of storage, processing treatments, and culinary practices (Barros et al., 2008). Polyphenols, terpenoids, steroids, and various glycoproteins and polysaccharides were discovered as bioactive substances. Some novel proteins have been discovered in mushrooms that can be utilized.

Antitumor polysaccharides can be acid or neutral in nature, with quite diverse chemical makeups. They do not immediately eliminate tumor cells, instead they stimulate the immunological response in the host organisms in presence of any type of tumor, and the stimulated immune system will battle against the illness on its own (Meng et al., 2016). Beta glucans are the principal polysaccharides found in mushrooms, and they have commercial significance since they are excreted externally and recovering them, and purifying is straightforward and inexpensive. They also have cancer-fighting, antioxidant, and neuroprotective traits (Manzi & Pizzoferrato, 2000). They are not synthesized by human beings and hence cannot be recognized as self-molecules by the body's defenses, triggering adaptive as well as innate immune responses. They safeguard humans from a wide range of microorganisms, pollutants from the environment, cancer-causing agents, and diseases that are transmissible (Zhu et al., 2015). In a similar way proteins are a component of mushrooms and have medicinal properties. Antimicrobial protein molecules, laccases as well lectins, and other ribosome deactivating proteins are produced by many mushrooms. Lectins have been identified as immunological proteins, and unsaturated fatty acids are also present in mushrooms (X. Xu et al., 2011). The main sterol identified in mushrooms is ergosterol, which has good antioxidant properties, and a diet high in ergosterol assists in avoiding

heart attacks and strokes. Linoleic acid is also found in mushrooms and has a number of physiological activities, including the reduction of cardiovascular ailments, high blood pressure, and other disorders such as arthritis (Barreira et al., 2014). Mushrooms include vitamins, proteins, and phenolic compounds, in addition to carbohydrates. Secondary metabolites containing a ring of aromatic molecules and hydroxyl groups have a simple or complicated configuration. They have antibacterial, anticoagulant protein, antiallergic, and anti-inflammatory qualities. To treat several degenerative ailments, such as neurological disorders or heart disease, phenolic compounds can be employed and this is owing to their natural free radical scavenging property, which means they may serve as an antioxidant (Haytowitz, 2006).

The discovery and creation of antibiotics and antibacterial medicines for the cure of bacteria-related illnesses was a prominent subject of achievement in human medicine in the twentieth century (Bennett & Chung, 2001). The initial phase of discovery, the isolation of microbial compounds from nature, was set off by Flemming's discovery of a penicillin-producing fungus and swiftly followed by pioneers such as Dubo's thorough hunt for antibiotic producing microbes (Gaynes, 2017). Many of the well-known categories of antibiotics have emerged because of this method. These comprise the cephalosporin and penicillin branches of the beta-lactam family, the aromatic polyketides of the tetracycline class, the aminoglycosides, the polyketide macro lactones, and the glycopeptides of the vancomycin family. The pursuit for antimicrobial miracle drugs by medicinal scientists has yielded sulfa medicines, dihydrofolate reductase inhibiting agents, fluoroquinolones, and, most recently oxazolidinones (Diggins, 1999).

Active compounds from nature and synthesized antibacterial agents have been shown to be helpful probes for determining the identity of targets in pathogenic microbes throughout these discovery activities. Previously, this has proven to be a target-poor treatment area, with just four strong targets for commonly used antibacterial groups: the biosynthesis of bacterial cell walls, bacterial protein biosynthesis, replication of DNA and maintenance, and folate coenzyme assembly (Nathan & Cars, 2014). Following the use of antibiotics, several of the pathogenic bacteria involved with human

illness outbreaks developed into MDR (multidrug-resistant) variants. MDR MTB, for instance, is a prominent disease present in developed as well as developing nations and has evolved into the twentieth-century version of an old bacterium (Levy, 2002). The term "superbugs" implies microorganisms with a higher mortality rate and morbidity as a result of several modifications conferring a significant amount of resistance to the types of antibiotics that are suggested for their treatment. Alternative therapies for these organisms are limited, and hospitalizations are longer and more expensive. In certain situations, super resistant bacteria have also gained greater pathogenicity and transmissibility (Adegoke et al., 2016). Resistant to antibiotics is a pathogenicity factor and antibiotic resistance appears unavoidable. What measures might need to be implemented to avoid or at least slow the pace of this procedure? Ideas for action include tight restrictions on antibiotic consumption in individuals, requiring precise dosages, prohibiting the distribution of antimicrobial agents without a prescription from a physician, and asking regulated therapeutic applications in livestock husbandry and farming (Mohsin & Amin, 2023). Many medical specialists are reconsidering organic, safe alternative therapies due to the misuse of prescribed antibiotics, which may result in the creation of drug-resistant types of bacteria. There are several alternative organic antibiotics options that should always be researched and kept on hand. To combat any type of disease or ailments, medicines made with organic flowers can be used, which are very affordable and can be preserved for a long time(C.-H. Wang et al.,2020).

Organic products have been traditionally used as remedies for several generations, but it is only recently in the last century that scientists have begun to meticulously characterize both their biological and biochemical features. Mushrooms are unique outlets of powerful medications and have evolved into the new drug discovery gateway (Weber-Dąbrowska et al., 2006). Mushrooms have an extensive history of therapeutic usage in communities all over the world. It has long been recognized as herbal remedy in China as well as other countries of the region of Asia, particularly Korea and Japan. Mushrooms have been associated with therapeutic characteristics that can help with a variety of ailments. Antimicrobial, anticancer, antioxidant, immunity enhancer, antiviral, anti-hyperlipidemia, radical scavenger, anti-parasitic, and anti-inflammatory

qualities have been documented (Alves et al., 2012). The most prevalent medical qualities described in mushrooms include anti-tumor, immunostimulatory, antibacterial, anti-inflammatory, and antioxidant. Antibacterial and antioxidant abilities are extensive. Human illnesses are being treated by the secure and efficient medicinal properties of mushrooms. Extracts of *P. ostreatus, P. sajor-caju*, and *L. squarrosulus* demonstrate medium to excellent antimicrobial activity (Akyuz et al., 2010). It has been discovered that antimicrobial compounds from bracket fungus that are effective against methicillin-resistant *Staphylococcus* aureus (Shrestha et al., 2021).

Several investigators have discovered significant biologically active compounds from mushrooms that are contributing to their medicinal qualities. But the efficacy of a number of these components has yet to be proven in clinical studies (Pandey et al., 2020). Despite the fact that numerous therapeutic characteristics of mushrooms have been previously recognized, there is a paucity of knowledge on the identification of their bioactive constituents and the fundamental processes associated with promoting health (Badalyan et al., 2023). In addition, mushroom-derived prebiotics have recently piqued the interest of academics worldwide, notably for the association between the management of the intestinal microbiota and the health of the host, which includes intestinal-immune function, inflammation in the gut, weight gain, cancer of the colon, and disorders of the brain (Rai et al., 2005).

AIM AND OBJECTIVES

Aim

The main aim of the current study is to unravel the pharmacological potential, with the goal of uncovering possible therapeutic applications in medicine, hidden within the bioactive metabolites of Azad Jammu and Kashmir wild mushrooms.

Objectives

The significant objectives of the present study are:

- Perform morphological examination of the eleven distinct mushrooms collected from Azad Jammu and Kashmir, including an in-depth examination of their physical features and structures.
- Use a variety of seven different solvents to extract bioactive compounds from mushrooms.
- Perform preliminary screening to assess the biological potential of crude extract metabolites with the goal of identifying any promising medicinal qualities.
- Use qualitative and quantitative mycochemical screening methods to further analyze the specific chemical ingredients contained in the extracts.

2. LITERATURE REVIEW

From the launch of sulfonamide and penicillin into healthcare settings in the 1930s and the 1940s, respectively, people have been duped into believing that antibiotics could completely eradicate bacterial infections. The extensive use of antimicrobial agents, on the other hand, creates a tremendous selection pressure for the emergence of antibiotic resistance, which is currently a major threat to the public's health (Palumbi, 2001). We have to currently believe that the emergence of antimicrobial resistance is unavoidable; it is simply a matter of time (Davies, 1994). As the extent of antibiotic usage rises, so does the rate of resistance emergence, lowering the efficiency of antibiotics. When a novel antibiotic becomes available for medicinal consumption, clinically noticeable resistance occurs months to several decades later (Walsh, 2000). Penicillin resistance, for instance, was discovered just a few years following its commercial appearance in 1942, and streptomycin resistance was identified one year following being discovered in 1944. In the unusual situation of vancomycin, resistance emerged about three decades following it was initially introduced (Murray, 1997). The lengthy wait was most likely caused by the restricted availability of vancomycin in the initial two decades, as other highly effective antibiotics were prevalent during the "antibiotic age" of the years 1950 to 1960. However, unrestricted antibiotic usage contributed to the rise of methicillin-resistant *Staphylococcus aureus*, leading to multi antibiotic resistance to multiple structurally distinct drugs (Neu, 1992). Consequently, vancomycin-resistant Enterococci emerged in 1986 as a result of extensive use of vancomycin, which was considered an antibiotic of final resort. Then identification of moderately vancomycin resistant strains of *Staphylococcus aureus* in 1997 resulted in a pioneering outbreak of totally vancomycin-resistant strains. More specifically, what complicates matters is that around half of all medications are used in livestock as preventive measures, for treatment, and growth enhancement (Coates et al., 2002).

There is now confirmation that antimicrobial use in animals contributes to the emergence of antibiotic resistance in human illnesses. Based on what is known as the 'Precautionary Principle,' the European Union banned the administration of avoparcin for promoting growth (Casewell et al., 2003), Continued attempts to produce novel

antibiotics are plainly required to address these serious medical conditions. Following the launch of nalidixic acid in the year 1962, an entirely novel category of antibiotics, linezolid and daptomycin, have been authorized for use in clinical trials in the United States of America in the years 2000 and 2003, respectively (Walsh, 2000). Despite the fact that the newly developed antibiotics have been successful against vancomycin-resistant MRSA as well as Enterococci, it is evident that the detrimental pathogens will ultimately acquire resistance to these substances as well, which means the curative lifetime of these novel antibiotics will be reduced if they are used indiscriminately. Clinically isolated strains of *Staphylococcus aureus* and *E. faecium* with linezolid resistance first came to light in 2001, just one year after the drug was introduced (Tsiodras et al., 2001).

2.1. Antibiotic-resistant bugs

Microbes are evolution leaders, and some microbes have evolved to a stage where they offer major clinical difficulties to mankind. MRSA and *E. faecium*, both of which are the most difficult gram-positive pathogens to treat. MRSA's evolution with time indicates the genetic adaptability of an organism into a superior multidrug-resistant disease (Diep et al., 2006). Following the advent of penicillin, *S. aureus* swiftly developed resistance against these beta-lactam compounds, and by the year 2003, over fifty percent of isolates of *S. aureus* collected in hospitals were MRSA. The pathogen then started to evolve resistance to glycopeptides, initially establishing minimal resistance to vancomycin. Following that, variants of MRSA with actual, the highest-level vancomycin resistance appeared (Schwartz et al., 2008). This resistance is caused by a gain of the vanA gene cluster, which was first reported in enterococci. Luckily, just a few hundred such isolates have been identified and their spread seems to be confined. VRSA, similar to other types of MRSA related to health care, is frequently resistant to numerous medications, including sulfamethoxazole, rifampin, and fluoroquinolones. Although less harmful than MRSA, enterococci have for years posed treatment challenges, owing to their tolerance to penicillin and vancomycin. Enterococci are the third-leading cause of infectious endocarditis (Arias & Murray, 2009).

When it involves nosocomial gram-negative infections, the scenario is much worse because currently available antibiotics targeting these multidrug-resistant bacteria have yet reached the final stages of clinical testing. Resistance to among the most powerful antibiotics has lately expanded to members of the Enterobacteriaceae family. Similarly concerning is the discovery of multidrug-resistant organisms in healthy individuals not confined to hospitals, such as infections of the urinary tract that result from E. coli (Pitout & Laupland, 2008). Considering this bleak picture, 21st century clinicians must either explore substances produced years ago and formerly rejected due to safety concerns or analyze everything they may think of and make use of whatever is effective. It is more challenging than ever to completely eliminate illnesses caused by antibiotic-resistant superbugs, and the situation is aggravated by a lack of newly developed antimicrobials with bactericidal properties against gram-negative microbes and enterococci (Huycke et al., 1998). A united front from the combined forces of academic researchers and their organizations, industry, and state is required if humans are to keep the leading role in their fight with microbes.

2.2. Natural antibacterial compounds

Infectious diseases are a leading source of death and disability worldwide. Numerous illnesses have been brought about by multi-resistant microbes, leading to difficult-to-treat diseases and, as a consequence, significant rises in healthcare expenses (Strong et al., 2005). The relatively simple availability of antimicrobials, in addition to the widespread use of these compounds for commercial reasons, including food manufacturing, have both contributed significantly to the consistent growth of resistant microbes. As a consequence, these multi-resistant microbes are re-establishing themselves as global hazards. The study of natural products has made tremendous progress in the identification of novel substances with antimicrobial action (Wise et al., 1998). In truth, nature offers a rich supply of molecules with the ability for alleviating diseases, particularly infectious disorders. Medicinal plants, aquatic and land creatures, including bacteria and fungi are emphasized as identified sources of natural chemicals with valuable antibacterial potential. Nonetheless, there is a wide range of organisms that, if thoroughly researched, could give more antibacterial discoveries and novel medications (Wink, 2003).

Mother Nature has given us outstanding and amazing ways to produce naturally occurring antibacterial substances in every kind of organism that range from tiny prokaryotic beings to colossal eukaryotes, though their propensity and potential may vary depending on their contact with pathogens in their natural surroundings (Butler, 2008).

2.3. Plants-based antimicrobial research

Plants have been found to have a diverse range of naturally occurring compounds that are capable of helping cure a number of ailments. For ages, plant products such as herbs, fruits and vegetables have been the most commonly used medicines (Jimenez-Arellanes et al., 2003). Plant-based foods, such as vegetables, fruits, herbs, and spices have had a substantial function in the healing process of a variety of diseases. turmeric, which is produced from the *Curcuma longa* plant, has been traditionally used to cure a variety of diseases including wound repair, inflammatory disorders, and for the management of infections caused by microbes, discomfort in the stomach, arthritic conditions and metabolic problems (Negi et al., 1999). In a similar manner the curative properties of honey, particularly its antimicrobial and wound healing capabilities, have been widely recognized for centuries. Honey exhibits effective antibacterial properties against *Escherichia coli, Salmonella typhimurium, Staphylococcus aureus,* and *vancomycin-resistant Enterococci* (Eteraf-Oskouei & Najafi, 2013).

2.4. Animal based antimicrobial discovery

Despite numerous studies into the antimicrobial qualities of natural products, especially those derived from bacterial and plant-based sources, the enormous animal world, which comprises a wide range of land and aquatic creatures, has only been scratched the surface. Only a small percentage of the 7.77 million species of animals reported in diverse ecological niches are being studied for antibacterial efficacy. Since millennia, the wonderful ability of varied fauna to thrive in difficult conditions has created a route to find their reasons of survival on the earth (Khan et al., 2008). Several kinds of animals, including fishes, reptiles, amphibians, mammals, birds, and invertebrates are regularly subjected to different environmental circumstances in their ecosystems and are hence regarded to have strong immune systems. Insects, for instance, account for

over eighty percent of all fauna, are extensively dispersed around the world, and provide a bountiful and unexplored opportunity of new antibacterial medication discovery. Worms, cockroaches, and bugs flourish in dirty and germ-infested settings, and have fast metabolisms and immune systems, as well as the ability to manufacture antibacterial peptides that include cecropin, moricin, apidaecin, and gloverin. likewise, bioactive compounds found in cockroach include sulfonamides, furanones, and flavanones, which have antimicrobial activity against Gram-positive and Gram-negative bacteria (Yi et al., 2014). House fly larvae are being utilised in a maggot therapy for relieving wound-related infections (Bonn, 2000). In general, a wide variety of antimicrobial compounds have been produced from animal sources, primarily from their tissues, sera, and vegetation found at various locations.

2.5. Fungal sources

The fungus known as Penicillium notatum was responsible for the development of the first antibiotic, Penicillin, which is a beta-lactam ring that attacks the cell wall of microorganisms. likewise, culinary fungi like mushrooms have antioxidant and antibacterial effects against Gram-positive as well as Gram-negative bacteria (Fleming, 1929). For instance, therapeutic lignicolous fungi which include *Laetiporus sulphureus, Flammulina velutipes, Pleurotus ostreatus* and *Panus tigrinus* displayed antimicrobial properties especially against gram-positive organisms such as *Staphylococcus aureus, B. subtilis*, and *Micrococcus luteus* among others (Karaman et al., 2010). Yeast-like fungi including *Cryptococcus laurentii, Rhodotorula glutinis,* and *Sporobolomyces roseus*, on the other hand, have been demonstrated to have antibacterial activity against *P. fluorescens* and *S. aureus*. Several species of fungi were recently obtained from the marine environment. Several fungi collected from the marine environment have been found to produce secondary compounds having antibacterial capabilities against diverse microbes such as *Escherichia coli, B. subtilis*, and *S. aureus* (McCormack et al., 1994). Cephalosporins, a type of beta-lactam antibiotic generated by the aquatic fungus Acremonium, are effective against an extensive range of bacteria, both Gram-positive and Gram-negative (Abraham et al., 1953). Fungi obtained from marine sources, comprising sponges, sea sediments, fallen branches, marsh fruit, and sea

squids, from the genus Aspergillus, Penicillium, and Pestalotiopsis, have antibacterial activity against *Bacillus subtilis* (Masuma et al., 2001).

Gliotoxin, called epipoly-thiadioxopiperazine having antibacterial properties against *Staphylococcus aureus* and *Bacillus subtilis*, has been found to be released by a variety of Aspergillus obtained from the marine mud. Further studies revealed that diketo piperazine-related chemicals are also made up by other kinds of fungi in marine and terrestrial settings and operate by preferentially attaching to thiol groups that are found in the cytoplasmic walls of plant fungal pathogens such as *Rhizoctonia solani* (奥谷康一, 1977). Indanonaftol A produced by Aureobasidium species, was the primary antibiotic chemical identified from marine fungus exhibiting antimicrobial activity against Gram-positive bacteria (Silber et al., 2016).

2.6. Mushrooms as a potential source

Mushrooms are widely regarded as the most underutilized source of nutritional foods. Their production is now the most affordable biotechnology for converting lignocellulose debris into high in protein meals, while also significantly reducing harmful emissions (Kakon et al., 2012). There are over 1600 varieties of mushrooms, but only 100 of them have been identified as edible. Around 30 edible mushroom species are cultivated worldwide, but just three of them are widely grown: white button mushrooms, oyster mushrooms, and paddy straw mushrooms (Erbiai et al., 2021). These are high in fiber and components like proteins, minerals, and vitamins while being low in calories and fats (Roncero-Ramos et al., 2017). Edible mushrooms are commonly employed in the production of nutritional supplements and therapeutics with anti-tumor, antioxidant, and antibacterial effects. Mushrooms, additionally to their pharmacological characteristics, are vital in our daily meals due to their low level of lipids, abundant protein, and relatively little energy contents. The mushroom proteins contain all of the necessary amino compounds that humans require. Furthermore, these contain a variety of food elements, including phosphorus, iron, and vitamins such as ascorbic acid, vitamin C, niacin, riboflavin and ergosterol (H. Kumar et al., 2021). Mushrooms are valued based on appearance, taste, and some medicinal properties. Mushrooms that are palatable have grown increasingly recognized as health promoters,

which has led to breakthroughs in study on many varieties of mushrooms. Because of their capacity to boost the level of protein as well as the valued beneficial effects of bioactive chemicals, they can have numerous uses to augment many staple food items.

Globally, macrofungal productivity and value to economies are on the rise (Lu et al., 2020). Because of rising mushroom consumption, the total yearly revenue of the worldwide mushroom industry is expected to approach 50 billion US dollars in the future years. In the year 2023, the worldwide market value of edible mushroom products is expected to surpass USD 62.19 billion (Zhang et al., 2007). Because of their delightful and distinct flavors, in addition to their positive health effects, macro fungi have grown in favor among people. Demand for food has risen and consumption criteria have gotten more severe over the twentieth century. Meals should not only offer nutrition and alleviate hunger, but it additionally ought to assist people enhance their mental and physical well-being.

Many cancer-preventing, antidiabetic, antihypertensive, antibacterial, antioxidant, anti-inflammatory, immunomodulatory, cholesterol reducing, neurotrophic, and neuroprotective effects of macro fungi have been examined (D. Wu et al., 2010). Macro fungi, both natural and created, can be considered beneficial functional foods. Fresh growing bodies of macro fungi have a humidity level of 70-95%. Carbs (50-65%), proteins (19-35%), and essential fatty acids (2-6%), with small quantities of nutrients such as vitamins and minerals, make up the dry matter. Many distinct nutritional supplements, which include lectins, unsaturated fats, phenolic substances, tocopherols, vitamin C, and carotenoids, can be found in edible mushrooms. Thus, eating edible macro fungi benefits health by taking benefit of the cumulative and synergistic benefits of all the beneficial substances present (J.-W. Xu et al., 2010). The most extensively farmed edible mushrooms include *Auricularia auricula, F. velutipes, Pleurotus spp.* and *Volvariella volvacea* (Walton, 2014). Such practices have motivated recent scientific research of fungal therapeutic characteristics, including anticancer bioactivities. *Antrodia cinnamomea, Phellinus linteus,* and *Xylaria nigripes* have been the most studied for their therapeutic usefulness (Ngai & Ng, 2003). Several biologically active substances have been discovered in their fruiting organs or

cultivated mycelium, including alkaloid compounds, carotenoids, proteins, lipids, folic acid, glycosides, lectins, mineral substances, acidic substances, phenol compounds, polysaccharides, amino acids, terpenoids, tocopherols, and volatile substances as a whole (Ganeshpurkar et al., 2015). Many polymers, actively functioning proteins, unsaturated fats such as oleic and linoleic, phenolic substances such as phenolic acids, vitamins (A, B complex, C), and food fiber are found in the fruiting structures of mushrooms.

2.7. Bioactive Components in Edible Mushrooms

Mushrooms produce a variety of substances that are bioactive, including phenolics, terpenoids, complex sugars, glucans, and lectins, which have been linked to over 126 medical benefits, comprising antibacterial, immune-modulating, antioxidant, antiviral, and hypocholesterolemic properties (Badalyan et al., 2023). Biomolecules with immunomodulatory and antibacterial properties, like terpenoids and glucans, can be isolated from mushrooms belonging to the genera Fomes, Inonotus, and Schyzophillum (Wasser, 2010). Proteins found in different kinds of mushrooms have a variety of biological properties, such as lectins, proteases, ribosome-inactivating protein molecules, inhibitors of protease, and hydrophobins, which have the ability to be used in various fields of biotechnology for the development of new drugs (Erjavec et al., 2012).

Erbiai et al. studied the chemical arrangement, bioactive elements, and antioxidant capacity of two natural edible mushrooms that were obtained in North Morocco and Spain, the honey fungus, and the parasol mushroom. Extracts derived from methanol demonstrated significant DPPH activity in eliminating free radicals. The North Moroccan mushroom had a particularly high antioxidant content. Vanillic acid as well as cinnamic acid were the primary compounds identified in *A. mellea*, while protocatechuic acid and its derivatives prevailed in *M. procera* samples.

Mushrooms possess both primary as well as secondary metabolites. The primary metabolites supply energy, whereas the secondary metabolites have therapeutic effects. Primary metabolites found in mushrooms that are edible, such as lipids, carbohydrates, and proteins, are used to supply sustenance to humans (Thirumurugan et al., 2018). On

the contrary, edible mushrooms possess a variety of other secondary metabolites such as terpenoids, alkaloid compounds, metal chelating molecules, lactones, sterols, sesquiterpenes, and others that are utilized in therapy to treat various ailments. Water-soluble and lipid-soluble substances including D, E, C, E, B1, B2, and B12 are abundant in mushrooms that are consumed (Schügerl, 2005).

2.8. Classes of secondary metabolites and their biosynthesis

2.8.1. The MVA and MEP pathway: Terpenoids

Terpenoids can be discovered in almost every kind of life and include a wide collection of secondary metabolic products with approximately 40 000 structures (Bohlmann & Keeling, 2008). They are composed of five-carbon units known as "isoprene units," which can come together to produce several types of terpenoids such as C5, C10, C15, C30, and tetraterpenes (C40). Isopentenyl diphosphate and its isomer dimethylallyl diphosphate are the fundamental components of all terpenes and terpenoids. IPP and DMAPP can be synthesized via the mevalonic acid (MVA) or methylerythritol phosphate (MEP) pathways, based on the generating organism. Fungi and mammals can exclusively use the MVA route, whereas phytoplankton and most bacteria employ the MEP pathway. Crops and certain bacteria can use either pathway (Gräwert et al., 2011). The MVA pathway begins with the formation of the 6-carbon molecule MVA from three acetyl-coenzyme A. Acetoacetyl-CoA is formed when one acetyl-CoA attaches to the enzyme in question and is joined with another acetyl-CoA via a Claisen condensation. An aldol insertion then attaches an additional enzyme-bound acetyl group, resulting in the synthesis of 3-hydroxy-3-methylglutaryl-CoA. A sequence of processes, involving phosphorylation, decarboxylation, and dehydration, eventually converts MVA to the 5-carbon unit, IPP. The inclusion of the proton at carbon number 4 and the stereospecific disappearance of another proton at C-2 allow IPP to form an isomer to DMAPP.

The initial ingredients for IPP and DMAPP through the MEP route are pyruvic acid and glyceraldehyde 3-phosphate. Following decarboxylation of pyruvic acid and nucleophilic attack on the aldehyde of G-3-P, deoxyxylulose 5-phosphate is produced, an action that requires a cofactor called thiamine diphosphate. Following that, a

reorganization event converts deoxyxylulose phosphate to MEP. Following numerous reaction stages, involving two reductions, the primary final product IPP and the secondary product DMAPP are formed. These fundamental distinctions in reactivities between IPP and DMAPP serve as the foundation for the development of bigger terpenoids. After losing a proton, the allylic resonance stabilized carbocation generated from DMAPP can combine with IPP to generate geranyl diphosphate (GPP). GPP is the progenitor of monoterpenes, but it can also be expanded by adding one more IPP to generate farnesyl diphosphate (FPP), the predecessor of sesquiterpenes. Comparable addition processes of IPP to FPP and GGPP create diterpene and sesterterpene precursors, geranylgeranyl diphosphate (GGPP) and geranylfarnesyl diphosphate (GFPP), respectively.

2.8.2. The shikimic acid pathway: Aromatic amino acids andphenylpropanoids

The shikimic acid pathway is a key metabolic process in plants, fungi, and other microbes for the formation of aromatic chemicals, specifically aromatic amino acids. However, because the shikimic acid pathway does not exist in animals, these amino acids are vital for humans. Phenylalanine and tyrosine, in turn, are significant precursors for numerous alkaloids as well as phenylpropanoids, which are distinguished by their C6C3 carbon skeleton that occurs in a wide range of structurally varied secondary metabolic products.

The combination of PEP and E4P to yield shikimic acid via two aldol-type reactions, one between molecules and one intramolecular, is the first stage in the shikimic acid pathway subsequently followed by water removal and a reduction process. The 5-hydroxy group of shikimic acid 3-phosphate is subsequently phosphorylated, and a second PEP molecule is linked to it. Two phosphoric acid reductions come next, leading to the ultimate outcome of the shikimic acid pathway, chorismic acid (Çitoğlu & Acıkara, 2012).

Chorismic acid is a significant intermediary and beginning point in the synthesis of numerous significant substances. A Claisen rearrangement mechanism converts it to prephenic acid. Decarboxylation, aromatization, and the elimination of prephenic acid's hydroxy group lead to phenylpyruvic acid, which is transaminated to form

phenylalanine. Cinnamic acid and 4-coumaric acid are formed as an outcome of ammonia removal via E2 processes from phenylalanine and tyrosine, respectively. These two molecules are intermediates for phenylpropanoids, which, as previously stated, are found in numerous secondary metabolite forms such as lignans, phenylpropenes, and coumarins.

2.8.3. The acetate pathway: Polyketides

Polyketides are a diverse group of secondary metabolic products that are produced by bacteria, fungi, and plants. As the most prevalent fungal secondary compounds, they are particularly significant for fungi, and several of them possess significant biological activity. A well-known instance is the cholesterol-lowering chemical the cholesterol-lowering drug lovastatin, which is formed by the fungi *Monascus ruber* and *Aspergillus terreus*. It was the initial statin to be commercialised and functions by acting as a blocker of the enzyme HMG-CoA reductase, which is involved in the synthesis of MVA (Kalam & Sylaja, n.d.).

Polyketides are generated by the sequential condensation of primarily acetyl-CoA and malonyl-CoA chains. Polyketide synthase, a class of multidomain proteins or enzyme complexes, catalyzes the processes that lead to the formation of the poly-keto chain. There are numerous varieties of PKSs observed in different animals, but the basic principles for forming the poly-keto chain are alike. The first step is to bring both the initial and extender units to their appropriate sites on the PKS. Acetyl-CoA is associated with a ketoacyl-CoA synthase domain, whilst the extender unit is associated with an acyl carrier domain. A Claisen-type reaction then happens between malonyl-ACP and acetyl-KS, followed by decarboxylation of the ACP-bound extender unit. The thereby produced beta-ketothioester linked to the ACP site can subsequently be moved to a KS site and expanded by another malonyl-ACP. This procedure is repeated until the forming beta-keto chain reaches the correct length. Once the chain has been assembled, it can be coiled and ignited at appropriate points to make way for intramolecular interactions. Other more complex aromatic molecules, such as anthraquinones, can be synthesized in the identical manner, but using a more lengthy beta-keto chain as an a foundation (Widhalm & Dudareva, 2015). The carbon-containing skeleton of

macrolides, another type of polyketide distinguished by massive lactone rings, is generated by the same basic method but with some changes.

2.8.4. Alkaloids

Alkaloids are cyclic organic molecules with several nitrogen-containing atoms that are commonly basic. They typically have major impacts on the neurological systems of humans as well as other animals. Here are a few renowned alkaloids. Alkaloids are typically formed from the amino acids such as ornithine, lysine, tryptophan, and tyrosine, but additional structural blocks, such as terpenes or acetate pathway related molecules, are also frequently included into alkaloids structures.

Ergolines are a class of alkaloids with the indole ring structure that have been discovered in numerous fungal taxa, including Claviceps. These fungi cause ergot, a fungal infection that affects planted grasses that include wheat and rye and can be deadly to both people and pets when eaten. D- (+)-lysergic acid is one of over 50 alkaloids identified among these fungi, and it is generated from tryptophan and DMAPP, which are derived through the pathway of shikimic acid and the MVA/MEP mechanism, respectively (Singh & Desgagné-Penix, 2014).

2.9. Primary metabolites

2.9.1. Carbohydrates

On the basis of dry weight, carbohydrates make up approximately 50-65 g of the contents of mushrooms (Rathore et al., 2017). Mushrooms are rich in both carbohydrates that are digestible as well as non-digestible sugars, and the most widespread carbohydrates found in mushrooms are fructose, galactose, glucose, mannose, xylose, arabinose, and mannitol (Samsudin & Abdullah, 2019). Mannitol, commonly known as mushroom sugar, accounts for approximately eighty percent of total sugars that are free. The makeup of carbohydrates, on the other hand, is determined by the diversity, the substrate, and the conditions that prevail during production. Mushroom polysaccharides have been shown to have cancer prevention, anti-diabetic, anti-inflammatory, and other medicinal uses. Ingesting Beta-glucan-enriched diets boosts protection against infections of the upper respiratory tract, allergies during the wintertime, arthritis, obesity-related complications, and helps those with cancer heal

from radiation therapy or mastectomy (Vlassopoulou et al., 2021). B -glucan obtained from mushrooms is harmless for consumption, with a no detectable adverse reaction. The microbiota of the gut decomposes mushroom polysaccharides to create compounds such as short-chain fatty acids, which are key nutritional elements that helps preserve the balance of the gut.

2.9.2. Proteins

Peptides and proteins are important biologically active substances found in mushrooms because of their various therapeutic actions. The dietary protein content of mushrooms varies according to the organisms, substrate composition, pileus magnitude, and harvesting period (Samsudin & Abdullah, 2019). Mushroom protein content ranges from 19-35g based on dry weight and is easily digested (Rathore et al., 2017). Furthermore, mushrooms contain an adequate quantity of all nine important amino acids required by human beings, (Rathore et al., 2017). Fungal immune-modulating proteins are a unique category of biologically active proteins derived from mushrooms *F. velutipes* and a few Ganoderma species with remarkable therapeutic potential (Bao et al., 2018). Ribosome deactivating proteins, a class of enzymes designed for deactivating ribosomes by eliminating any number of adenosine residues from rRNA, are additionally discovered in mushrooms. Mushroom proteins are thought to be beneficial for the alleviation of a variety of gut-related illnesses (Jayachandran et al., 2017). Mushroom proteins influence microbiota composition, bacterial metabolites manufacturing, and have a major impact on the human gut epithelium by fueling a shift in the metabolism of bacteria to the process of amino acid breakdown, resulting in different profiles of metabolites.

2.9.3. Lipids

Mushrooms have only a tiny quantity of fat, amounting to roughly 2-6g by dry weight. They have a higher amount of unsaturated fatty acids than saturated fatty acids, notably oleic acid and linoleic acid, with a low level of linolenic acid. Linoleic acid is an intermediate to 1-octen-3-ol, an important aromatic molecule that contributes to the flavor of fungi (Rathore et al., 2017). Their lipid component additionally includes

ergosterol and tocopherol, both which are believed to act as antioxidants (Valverde et al., 2015).

2.9.4. Vitamins

Because mushrooms that are edible are an outstanding source of vitamins, they can be employed as a model component in nutraceutical compositions. Mushrooms consist of the following vitamins: riboflavin, thiamine, ascorbic acid, niacin, folate and vitamin D2 (Muszyńska et al., 2020). S. commune contains more vitamin A and vitamin E than other species (Chye et al., 2008)The amount of vitamin B12 in mushrooms can be compared to that found in meat, liver, and seafood, and it has a great deal of bioavailability, which may be important for those who follow a vegetarian or vegan diet for the rest of their lives. Vitamin D levels in grown mushrooms are minimal (in some cases, non-existent). However, they have been found to have quite high quantities of the provitamin ergocalciferol (Mattila et al., 2002). The photo-conversion of a ergosterol to the form of vitamin D2 occurs during the vitamin D2 the creation process, and UV-B exposed grown mushrooms, including the ones such as Agaricus and Pleurotus species may yield significant quantities of vitamin D2 (Taofiq et al., 2017).

2.9.5. Minerals

Mushrooms have an elevated phosphorus, potassium, and magnesium content, an adequate calcium level, and relatively lower in sodium (Kalač, 2013), making them an appropriate option for those with hypertension when compared to other veggies. *L. edodes* has a low salt content, making it ideal for diabetics. Copper, iron, magnesium, zinc, and folic acid are all abundant in *Marasmius oreades*. The mineral potassium, the major ingredient in edible mushrooms, is distributed unevenly throughout the fruiting bodies, with the largest amount in the cap, next by the stipe and the spore-producing area, and lowest in spores (Kalač, 2013). Mg is the 2nd most abundant mineral discovered spontaneously in wild edible fungi (Kalač, 2013). Mushrooms are a good source of dietary selenium, particularly *Boletus edulis* containing the greatest concentration (Falandysz & Borovička, 2013).

2.10. Secondary metabolites

2.10.1. Phenolic compounds

The word 'phenolic substances' refers to a wide variety of mycochemicals that have an aromatic ring with one or more hydroxyl groups in it. Because they are most commonly found in conjunction with carbohydrates as glycosides, but also as esters and polymers in general, phenolic compounds are soluble in water. On the basis of the amount of phenol rings and functional groups that are attached to these moieties, these compounds are classified into multiple categories. Therefore, simple phenols, phenolic acid, phenylpropanoids, flavonoids, flavanols, flavones, and lignans are included in the categorization. Flavonoids, hydroxybenzoic acids, stilbenes, tannins, and oxidized polyphenols are chemical compounds found in mushrooms that serve as free radical blockers, metal-inactivators, peroxide decomposing agents, or scavengers of oxygen (Rashmi & Negi, 2020). Mushroom phenolic compounds are believed to have anti-cancer, antimicrobial, and anti-inflammatory activities, as well as the ability to safeguard against a variety of degenerative conditions, such as neurological disorders, heart disease, and ageing (Yadav & Negi, 2021). Furthermore, phenol compounds extracted from mushrooms, such as chlorogenic acids, are antiangiogenic and promote blood vessels development by inhibiting. Chlorogenic, gallic, and protocatechuic acids are the major compounds related with the high antioxidant capacity of mushroom species such as *Lycoperdon utriforme, Chlorophyllum agaricoides*, and *Tricholoma populinum* (Sezgin et al., 2020). *L. edodes* had the most significant quantities of vanillic and syringic acids), indicating potential advantages in the prevention of osteoporosis. A great deal of the polyphenols in *G. lucidum* ethanol extract are flavonoids such as myricetin, quercetin, morin, and hesperetin, which are attributed for its anti-proliferative action (Saltarelli et al., 2019).

2.10.2. Phenolic acids

The principal phenolic compounds present in mushrooms are phenolic acids, which are divided into two distinct categories: hydroxybenzoic acid and hydroxycinnamic acid. The bound form of HBA derivatives is found in structures with greater complexity such as tannins, lignins, carbohydrates, and organic acids, among others. HCA equivalents are additionally found, primarily in coupled form, coupled to cell-wall structural

components including cellulose, lignin, and other proteins, or linked to natural acids via ester linkages like quinic or tartaric acids (Manach et al., 2004). The most common are the HCAs, which are beneficial not only to deliver lignin structural components but also for resistance to disease as well as growth regulation. Ferulic, caffeic, and p-/o-coumaric acids are among the most frequent HCAs found in mushrooms. HCAs are typically found in mushrooms in the form of esters, and the maximum output can be achieved through moderate alkaline hydrolysis. Gallic, protocatechuic, gentisic, 5-sulphosalicylic, syringic, veratric, and vanillic constitute the most common HBAs derivatives found in mushrooms (Ferreira et al., 2009). The shikimate route is used to synthesize HBA and HCA molecules. The essential amino acids that serve as foundation blocks in this process are L-phenylalanine and -tyrosine.

2.10.3. Flavonoids

Flavonoids are a broad collection of naturally existing phenolic chemicals which are all chemically formed from flavone, which consists of two benzene rings coupled with another C type pyran ring. Flavonoids are classified into several classes, including anthocyanidins, flavanols, flavanones, and flavanols (Manach et al. 2004). Flavonoids can be found naturally as glycosides or aglycones. Although mushrooms are unable to synthesise flavonoids, the presence of flavonoids in a variety of mushrooms that are edible, such as catechin, myricetin, chrysin, hesperetin, formometin, biochanin, resveratrol, quercetin, rutin, and kaempferol, has been reported (Gil-Ramírez et al., 2016). The detection and measurement of the flavonoids and phenolic acids from a variety of mushrooms *P. ostreatus, P. eryngii., Agaricus bisporus, Cyclocybe aegerita, Russula cyanoxantha, R. virescens, Boletus edulis Bull,* and *Tuber melanosporum Vittad* were examined using the technique of high-performance liquid chromatography coupled with mass spectrometer. Characterization of chemicals is based on retention durations, ultraviolet-visible absorption spectra, and mass spectra data, as well as comparing it to published data (Fogarasi et al., 2018). 4-Hydroxybenzoic acid and 5-feruloylquinic acid were discovered to be the primary chemicals in both *P. ostreatus and A. bisporus*, with values of 75.042 mg/100 g for *P. ostreatus* and 35.040 mg/100 g for *A. bisporus.*

The Folin-Ciocalteu assay is commonly used in studies to determine the total phenolic content (TPC) of mushrooms' methanol extract. But the technique has serious drawbacks because other easily oxidised molecules such as amino acids, vitamin C, and carbohydrates could interfere and overestimate the amount of phenolic compounds (Arbaayah & Umi, 2013). Phenolic compounds have antioxidant characteristics that can absorb free radicals, inhibit peroxidation of lipids, and bind ferrous ions (S. Kumar & Pandey, 2013).

2.10.4. Terpenes

Terpenes are a type of volatile hydrocarbons that are unsaturated exhibiting antioxidant, anti-cancer, anti-inflammatory, antimalarial, anticholinesterase, and anti-microbial effects in mushrooms. Mushrooms, according to Podkowa et al. (2020), are devoid of cholesterol and possess a variety of terpenoids, including carotenoids, which help mitigate atherosclerotic abrasion. Numerous mushroom mono and diterpenoids, sesquiterpenoids, and triterpenoids were found to be deadly to cancerous cell lines (Yue et al., 2021). Ganoderic acid A, a triterpenoid that comes from Ganoderma species of mushrooms, reduced the damage to the kidneys by minimizing creatinine in the blood, uric acid, and urea concentrations and lessening fibrosis of the kidneys (Tian et al., 2021).

Despite being high in nutritious and biologically active compounds, the toxicity of macrofungi and their derivatives must be thoroughly investigated due to the inclusion of harmful chemicals. Furthermore, mushrooms cultivated in unsuitable conditions could take in harmful contaminants, which have binding receptors for alkali-soluble polysaccharide ((Choma et al., 2018), which leads to decreased bioavailability and effectiveness. Yet, just a few studies have shown that eating macrofungi is harmful to human health. Nonetheless, worries about the safety and quality of food have risen as consumer interest in macrofungi has increased, and it is critical to examine safety and toxicology to promote their acceptance (Lu et al., 2020). Effective preprocessing before intake can aid in the reduction of microbial contaminants while also minimizing the safety risks associated with them.

2.10.5. Terpenoids

Terpenoid refers to all such compounds that have a shared metabolic origin. Terpenoids are formed from the isoprene molecule, and their carbon-based skeletons are formed by joining multiple of these C5 units. They are classified based on whether they have 2, 3, 4,6 or 8 such units. Terpenoids include essential oils, volatile mono- and sesquiterpenes as well as less volatile diterpenes, involatile triterpenoids, sterols, and carotenoids pigments. Each of these terpenoid groups is important in fungal development, metabolism, and habitat.

2.10.6. Essential oils

The volatile component fundamental for the unique odor and aroma present in numerous mushrooms can be identified in the terpenoid essential oils. They are highly valuable as the foundation of skincare in the cosmetics industry and flavor compounds in the food business. (Fogarasi et al., 2018) discovered alpha-pinene, beta-phellandrene, beta-pinene, and D-limonene as major terpenoids in *A. bisporus* and *B. edulis*. The volatile profiles of particular mushrooms can be obtained using (HS-ITEX/GC-MS). Each mushroom's fragrance characteristic is heavily influenced by its volatile elements.

Triterpenoids are carbon-based molecules having six isoprene molecules. They are generated biosynthetically from squalene, an acyclic C-10 hydrocarbon. They have cycled structures that are somewhat complicated, with the majority of them being aldehydes, alcohols, or carboxylic acids. Sterols are triterpenes with the ring structure cyclopentane perhydrophenantrene. A typical instance is ergosterol, which is found in abundance in mushrooms. Ergosterol is an essential part of the cell membrane of fungi that is transformed to vitamin D-2 when exposed to UV light. Furthermore, ergosterol offers antihyperlipidemic, anti-inflammatory, antioxidant capacity, as well as the ability to suppress the spread of fungus and bacteria (Koutrotsios et al., 2017).

The sterol composition of various *P. ostreatus* isolates was studied. Ergosterol predominated in all mushroom species analyzed, accounting for 51.9-87.4% of sterols and then metabolites such as ergosta-7-enol (12.7%), ergosta-5,7-dienol (7.6%), and ergosta-7,22-dienol (6%), respectively (Koutrotsios et al. 2017). *P. eryngii* has an ergosterol level of 20 mg/100 g dw, while a greater value was detected in commercially

available (Souilem et al., 2017). (Kikuchi et al., 2017) isolated and characterized ergostane-type sterols and bisabolane-type sesquiterpenes from *P. eryngii* with aromatase and nitric oxide (NO) inhibitory activities.

2.10.7. Alkaloids

Sinensine was the initial pyridine-containing alkaloid identified from *G. sinense* fruiting bodies. This chemical showed protective action against hydrogen peroxide-mediated damage in HUVEC cells. A subsequent chemical examination of *G. sinense* fruiting bodies yielded the identification of four more pyridine-containing alkaloids, sinensines B to E (C. Liu et al., 2010). The four different pyridine-containing compounds known as erinacerins M-P have been determined from a solidified culture of the Lion's Mane mushroom, *H. erinaceum*. According to a suggested biogenetic process, the manufactured substrates are amino acids, and four separate molecules of acetyl CoA engage in cascade reactions of condensation (K. Wang et al., 2015).

Orellanine is a kidney-damaging bipyridine N-dioxide poison generated by different Cortinaceae mushrooms. *Cortinarius orellanus* and *C. rubellus* are two of the world's most deadly mushrooms, with striking resemblances to the edible species , all of which have resulted in casualties (Herrmann et al., 2012). 2-[2-formyl-5-(methoxymethyl)-1H-pyrrol-1-yl]acetic acid, a novel pyrrole alkaloid, has been isolated out of the fruiting bodies of *Leccinum extremiorientale*. The valuable therapeutic fungus *Xylaria nigripesis* is known in Chinese as "Wuling Shen" and is utilised as Traditional Chinese Medication to alleviate sleeplessness and sadness (Xiong et al., 2016).

2.11. Pharmacological properties of mushrooms

2.11.1. Antioxidant activity

Aerobic microorganisms' regular metabolic process in cells generates radicals that are harmful including reactive oxygen species, or ROS, and reactive nitrogen species. To prevent oxidative damage, a balance between the defense system of antioxidants and free radicals must be established inside the body. Natural full of antioxidants supplements assist the body's own defense system in reducing damage caused by oxidation. Mushrooms have oxidative resistant elements that include phenols, flavonoids, carotenoids, and ascorbic acid, which serve as an abundant source of

antioxidants. The antioxidant mechanisms of mushroom extracts can be characterized based on their bioactive capacities to donate hydrogen, chelate metals, and absorb superoxide and free radicals (Kurutas, 2015). The primary class of phytocomponents that contribute to mushroom species' antioxidant properties is phenolic molecules, and their association was identified in Boletus, a naturally occurring edible mushroom. Boletus has been used as a dietary source for organic antioxidants due to its high amounts of phenolic components and strong antioxidant capabilities (Yeh et al., 2011). The antioxidant properties of eight therapeutic mushroom species from China was tested, with the *G. lucidum* mushroom exhibiting the most significant antioxidant activity (T. Wu & Xu, 2015).

2.11.2. Anti-inflammatory activity

Inflammation is linked to the onset of several incurable medical conditions, including plaque buildup, obesity, neurological diseases, and cancers. Because mushrooms have strong therapeutic and nutritional properties, they have been utilised for hundreds of years to relieve inflammation. Complex carbohydrates, phenol and alkaloid compounds, steroids, fats, carotenoids, vitamins, and minerals are all found in these mushrooms. Because of their antitumor, antiviral, cholesterol, and blood sugar lowering properties, edible mushrooms are employed as therapeutic foods (Garcia-Lafuentea et al., 2010).

Cordymin, an anti-inflammatory polypeptide isolated from the entomopathogenic fungus *Cordyceps sinensis*, was tested for cytokines and antioxidant capabilities (Qian et al., 2012). Another work isolated beta-D-glucan from aqueous and alkali extracts including polysaccharides derived from the medicinal fungus *Cordyceps militaris*. In addition, ethanolic extracts of *Hericium erinaceus* inhibited the buildup of myeloperoxidase induced by dextran sulphate sodium in gastrointestinal tissues, compromised histological alterations among neutrophils and lymphocytes, and safeguarded the epithelium of the mucosal layer, serving as a protective agent in the medical management of inflammatory bowel conditions (Mori et al., 2015). Agaricoglycerides, a type of fungus secondary metabolite, were given to mice to prevent inflammation of the liver and rectify hepatic glycemic metabolic malfunction.

It was discovered and established that the supplement reduced the levels of cytokines associated with inflammation and suppressed metabolic disorders by regulating the NF-B pathway (Yu et al., 2013). *Agaricus blazei Murill*, an edible mushroom, was studied for its anti-inflammatory properties in response to various treatments. This mushroom's alkaline and water-based extracts were found to moderately suppress edoema.

2.11.3. Anti-aging activity

Mushrooms are high in ergothioneine and glutathione, both of which have anti-aging benefits. The high quantities of ergothioneine and glutathione found in 13 different varieties of Italian Porcini mushroom have been studied and documented (Kalaras et al., 2017). Mycelia selenium polysaccharides dramatically reduced malonaldehyde concentration and overall cholesterol levels, according to the findings. Furthermore, in response to D-galactose-induced ageing, there was a considerable enhancement in the functioning of the superoxide dismutase and glutathione peroxidase enzymes, in addition to a greater antioxidant activity. The qualitative examination of MSPS also revealed monosaccharide composition, which included the following: glucose, galactose, mannose, arabinose, and rhamnose. These findings suggested that these polysaccharides could be useful in reducing harmful chemical-induced ageing processes and could be used in anti-aging treatment (M. Liu et al., 2016).

2.11.4. Anti-cancer activity

Mushrooms demonstrate anticancer effects through their stimulation of cancer-fighting immune cells known as lymphocytes. Different mushrooms from various genera have been investigated for antihistamine effects as well as anticancer properties in different forms, that include angiogenesis inhibitor, antimitotic, mitotic kinase inhibitor, and eventually stopping proliferation of cancer cells. A comprehensive analysis of the pharmacologically biologically active substances found in mushrooms used in chemotherapy for cancer has been published, providing insights into the molecular foundations of their anticancer efficacy (Patel & Goyal, 2012). Polysaccharides and their derived compounds are the primary chemicals found in mushrooms that are accountable for their anticancer activities. The anticancer potential of these biological

macromolecules is determined by their origin, solubility, framework, and process of isolation.

Polysaccharide Krestin, isolated from *Trametes versicolor*, was composed of beta-glucan, a sugar component, and a peptide linked by glycosidic linkages. It, like lentinan, is a very common medicine in Japan for a variety of diseases. Several clinical trials have established its efficacy as a chemotherapeutic medication equivalent. It is utilized in veterinary pharmaceuticals alone as an anticancer therapy against sarcoma, carcinoma, colon illness, and malignant growth in the lungs (Ye et al., 2012). Two *Phellinus linteus* extracts, PL-ES and PL-I-ES, were evaluated against ten different cell lines from humans. PL-ES demonstrated anti-cancer action by inhibiting proliferation in every cell type of cancer after 72 hours, but PL-I-ES at the same dose has been shown to be beneficial in four types of cancer cells.

2.11.5. Antimicrobial activity

Mushrooms could be used as an efficient antibacterial against harmful germs (Kick et al., 2017). *Lentinus edodes* was a well-studied species exhibiting antimicrobial properties against Gram-positive as well as Gram-negative bacteria. The antifungal activity is attributed to high- and low-molecular-weight components in the fungus extract, which range from peptides and proteins to steroids and organic acids. Grifolin from *Albatrellus dispansus* was discovered to be efficacious against harmful fungus, with more powerful positive inhibition (Alves et al., 2012). Ganodermin isolated from *Ganoderma lucidum* actively reduced the spread of hazardous Botrytis cinerea. Agrocybin isolated from *Ganoderma lucidum* inhibited HIV-1 reverse transcriptase activity while inhibiting various fungus species. The N-terminal sequence of Eryngin showed commonalities with the antifungal protein obtained from the *Lyophyllum shimeiji* mushroom. A deadly antifungal enzyme with a molecular mass of 12 kDa was identified and refined from *Cordyceps militaris* and this protein had antifungal properties.

Preclinical investigations revealed that Ganoderma mushrooms had antibacterial and antiviral properties. Clinical investigations in animals showed that *Ganoderma lucidum* polysaccharides might considerably reduce the blood levels of Hepatitis B virus DNA

and hepatitis B e-antigen (HbeAg), but human models are still needed (Gao et al., 2005). The antibacterial properties of 20 basidiomycete extracts were investigated, and it was discovered that the chloroform extract of *Hygrophorus agathosmus* are more powerful against both bacteria and yeast (Yamaç & Bilgili, 2006). Furthermore, the antibacterial activity of *Inonotus hispidus* extracts against pathogenic bacteria such as *Bacillus cereus, P. aeruginosa*, and *Candida albicans*, among other pathogens was assessed and reported (Smolskaitė et al., 2015).

2.11.6. Immunomodulatory effects

The majority of the low molecular weight secondary compounds generated from mushrooms has immunomodulatory properties. Polysaccharides derived from mushrooms, notably those separated from the fungus sclerotia, are well-known immunological potentiators for boosting the body's defences. The activation of death in tumour cells is one of the most dramatic immunomodulatory activities of mushroom polysaccharides. According to one study, *Agaricus blazei* has immunomodulatory properties that involve macrophage and neutrophil stimulation, which results in tumor regression (Ishii et al., 2011).

Mushrooms' immunostimulatory action is also accomplished through the coupling of polysaccharide-protein aggregates via a complicated method. The water-soluble polysaccharide-protein combination derived from *Ganoderma lucidum* spore demonstrated immune-modulating action. Many clinical research have shown that alcohol or water-soluble extracts of medicinal mushrooms can be utilised to counteract negative immunomodulatory impacts (Martel et al., 2017). The immune-regulating properties of *Inonotus obliquus* aqueous extract were studied in immunocompromised mice. The extract, when taken orally, elevated serum IL-6 levels. Control animals were treated with chemicals and tested for higher TNF- levels. As a consequence, extract-treated animals produced more favorable immunomodulatory benefits than control mice (Kim, 2005). In human monocytic cells, ergosterol peroxide, a bioactive molecule found in mushroom lipopolysaccharide, caused immunomodulatory activity. The EPO complex suppressed the production of cytokines by inhibiting the expression of MyD88 and VCAM-1 (S.-J. Wu et al., 2013).

2.11.7. Antidiabetic activity

Overall, the homopolysaccharides found in mushrooms influence the metabolism of insulin by influencing the release of insulin via the hormone signaling route. These health-promoting polysaccharides have been demonstrated in fat cells, rats, and humans to have anti-obesity, anti-diabetic, anticarcinogenic, antibacterial, and antiviral properties (Friedman, 2016). *Ganoderma lucidum* proteoglycan inhibited hyperglycemia after a meal by inhibiting tyrosine phosphatase, 1B enzyme, and triterpenoids on aldose reductase and alpha glucosidase in vitro. As a result, the hypoglycemic properties of *Ganoderma lucidum* phytoconstituents are useful in diabetic therapies (Ma et al., 2015). A research investigation on the anti-diabetic impact of *Agaricus bisporus* was also conducted in male rats treated with streptozotocin for diabetes of type 2. Rats fed with mushroom enriched meal exhibited a substantial decrease in TG content with a significant amount of antioxidants, dietary fibers, and vitamins including C, D, and B12 that modulated the insulin system and blocked the diabetes disease effects (Jeong et al., 2010). Similarly, the antihyperglycemic effect of polysaccharides in *Pleurotus florida* and *Lignosus rhinocerotis* in hyperglycemic rats was investigated. Polysaccharides from *Pleurotus florida* dramatically lowered glucose, blood cholesterol levels, fatty acids, and ketones in mice.

2.11.8. Cardioprotective effect

Heart disease is one of the main health concerns and one of the most common causes of death. The levels of fatty acids, cholesterol, lipoproteins, and triacylglycerols are all controlled by mushroom bioactive compounds, which effectively decrease the risk of heart diseases (Guillamón et al., 2010). Mushrooms' cardioprotective properties are demonstrated by biologically active substances on metabolic indicators such as lower-density lipoprotein, high-density lipoprotein, and homocysteine levels, which are linked to heart disease and stroke (Agrawal et al., 2017). *Pleurotus cystidiosus* had the most potent antihypertensive action in an investigation of nine edible mushrooms because it contains a protein with a molecular mass of 8300 Da. It is demonstrated that the mushroom extract has a cardioprotective action due to its antioxidant characteristics, which lowered necrotic demise of heart cells and reperfusion contracture (Lasukova et al., 2015).

2.11.9. Antihemolytic activity

Reactive species of oxygen can induce oxidative stress and hemolysis, which are key factors in the development of illnesses such as a condition called glucose-6-phosphate dehydrogenase deficiency and sickle cell anemia. Due to the elevated levels of polyunsaturated fats in the cell membrane and functional molecules of hemoglobin linked to transporting oxygen, free radicals predominantly attack red blood cells. Oxidation lowers the protein content of the membrane, breaks blood cells, and impairs microcirculation (Khalili et al., 2014). Hemolysis has historically been used as proof of damage caused by free radicals and to operate against antioxidants. It is useful for evaluating oxidizing or anti-oxidizing substances. Several macrofungal physiologically active compounds like phenols, flavonoids, and alkaloids shield cells from damage caused by free radicals. These chemicals aid in the stabilization of the membranes of erythrocytes by absorbing free radicals and lowering the peroxidation of lipids (Kakoti et al., 2021). A hemolytic substance, such as H2O2, can promote the oxidation of lipids in blood cell membranes, resulting in hemoglobin discharge into the matrix of cells. Only a few research have reported mushrooms' anti-hemolytic potential.

3. MATERIALS AND METHODS

3.1. Study area

The present research is an experimental form of study that was carried out at the Applied and Environmental Laboratory (AEG), Department of Microbiology, Quaid-I-Azam University, Islamabad.

3.2. Sample site

The naturally occurring mushrooms were obtained from specific regions of the Azad Jammu and Kashmir (AJ&K), Pakistan, between April to September 2021

Serial number	Common name	Scientific name	Edibility
1	Fetid russula	Russula foetentula	inedible
2	No common name	*Russula carolens*	inedible
3	Brittlegill mushroom	*Russula paludosa*	edible
4	The deceiver or waxy laccaria	*Laccaria laccata*	edible
5	Death cap	*Amanita phalloides*	inedible
6	Theashen chanterelle	*Canthrellus cinereus*	edible
7	Green spored parasol	*Chlorophyllum molybdites*	inedible
8	The sickener	*Russula emetica*	inedible

9	Judas ear	*Auricularia auricula judae*	edible
10	Saffron milk cap	*Lactarius deliciosus*	edible
11	Termite mushroom	*Macrolepiota albuminosa*	edible

Table 3.1. List of macrofungi collected from distinct locations of Azad Jammu and Kashmir, Pakistan during the year 2021.

3.3. Collection and Identification of Macrofungi

Fresh macrofungi were collected from various places of Azad Jammu and Kashmir in Pakistan. The samples of mushrooms were obtained throughout June to September since the temperatures are ideal for mushroom growth during these times of year. In their native environment, both ecological and anatomical aspects were documented. The recognition of materials is based on their physical shape, including color, appearance, size, and furthermore, as well as the accessible linked literature (Alexopoulos et al., 1996).

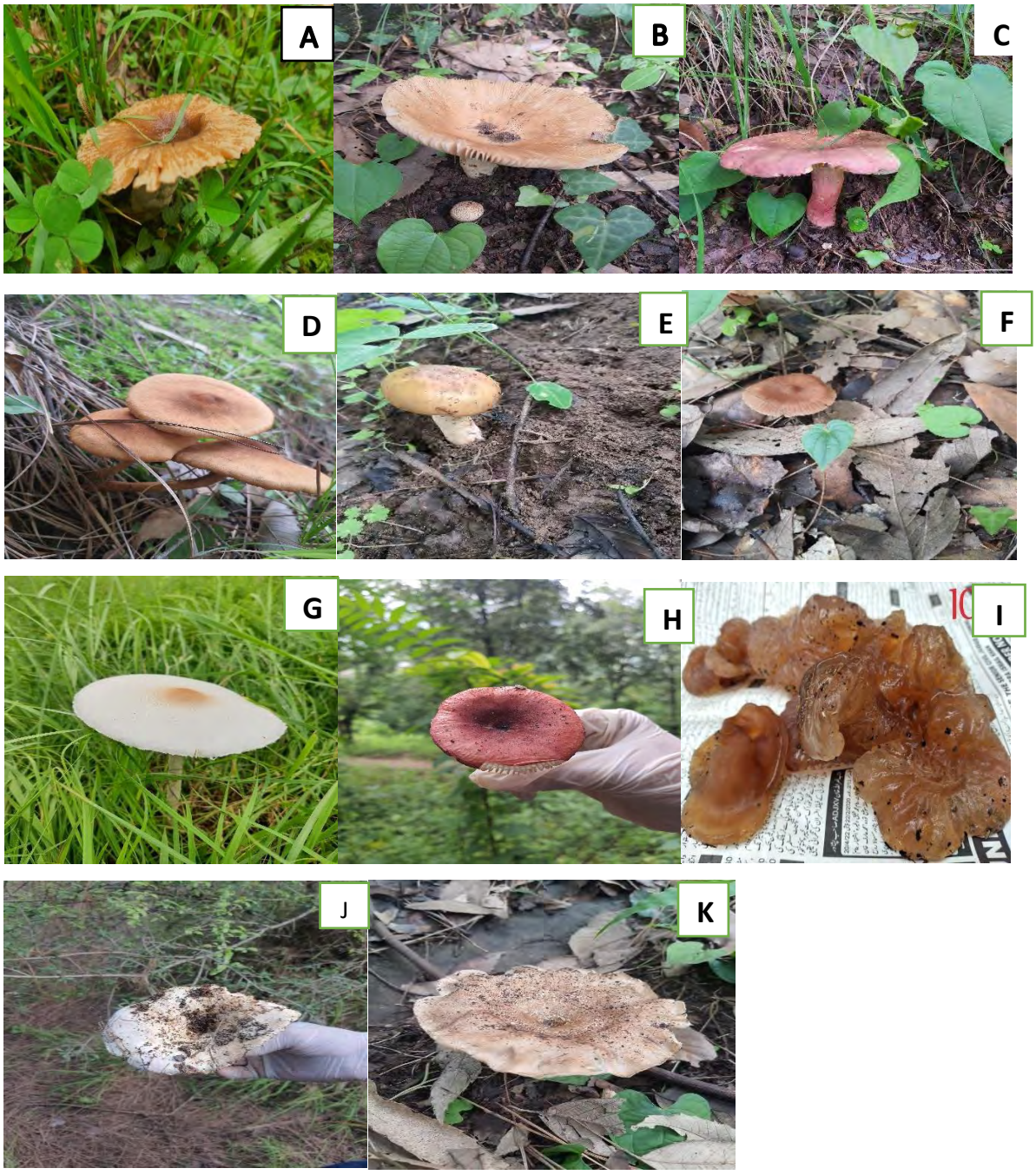

Figure3.1. Collection of different mushrooms from Azad Jammu and Kashmir region.

3.4. Processing of Macrofungal Samples

The mushrooms were dried out in the direct sunlight either outdoors or in an oven at temperatures ranging from 40 to 45°C. Utilizing the mortar and pestle or a mixer, the dried mushrooms were ground to an extremely fine powder.

Figure 3.2. Powder of dried macrofungal samples

3.5. Extraction of Bioactive Compounds from Macro fungi

The crushed mushrooms samples (40g) were subjected to treatment with several solvents (500mL) in a sequence of non-polar to polar, such as n-hexane, chloroform, ethyl acetate, acetone, acetonitrile, ethanol, and methanol, and left at room temperature for a period of 24 hours. The resulting extracts were passed through Whatman filter paper no.4 and processed in a rotary evaporator with the temperature set to 45 degrees Celsius to 50 °C. The separated fractions have been kept at room temperature until they were examined.

3.6. Antibacterial Potential of Macrofungal Crude Extracts

The antibacterial potential of macrofungal extracts was determined by the process of agar well plate diffusion (Das et al., 2010). Mueller Hinton Agar (MHA) is used as media.

3.6.1. Test Strain

- **Gram Positive**
  Bacillus subtilis
  Staphylococcus aureus
- **Gram Negative**
  Escherichia coli
  Pseudomonas aeruginosa
  Klebsiella pneumoniae

3.6.2. Procedure

Bacterial cultures were refreshed on nutrient agar media. The bacterial suspension was prepared through taking out the inoculum from a new culture plate and combining it in a 0.9% solution of normal saline; the turbidity was the same as that of a 0.5% solution. Using sterilised cotton swabs, bacterial suspensions (50L) were distributed over MHA plates. Following the setting up of the lawn, appropriate wells were created using a 6 mm sterile cork borer. After creating the wells, they were filled with sterilised agar material. The well was then filled with 100L of each macrofungal extract at a concentration of 8mg/mL of respective solvents. Each solvent (100L) served as a negative control, with ciprofloxacin/ceftriaxone serving as a positive control for gram-negative microbes and vancomycin serving as a positive control for gram-positive bacteria. The plates were left to incubate at 37°C for 24 hours. Following the

incubation time, the zone of inhibition in millimeters was measured to demonstrate the antibacterial activity of the crude bioactive substances.

3.7. Antifungal Potential of Macrofungal Crude Extracts

The antifungal potential of macrofungal extracts against two fungal strains was determined through the method of agar well plate diffusion (Das et al., 2010). Sabouraud Dextrose Agar (SDA) is used as media.

3.7.1. Test Strain

Aspergillus niger (ATCC 16888)

Candida albicans (ATCC90028)

Aspergillus flavus (ATCC9643)

3.7.2. Procedure

Fungi strains were renewed on Sabouraud Dextrose Agar media. To ensure that the turbidity was identical to 0.5% McFarland solution, the fungal cultures were suspended in 0.9% sterilized normal saline. Cotton swabs were used to disperse 50L of fungal suspension over SDA plates. Following the formation of the lawn, wells with adequate spacing were created using a 6 mm sterile cork borer. The wells were then filled with sterilized agar material. Crude extracts were combined in each solvent at an 8mg/mL concentration and applied to the well. Fluconazole was utilised as a positive control. Negative controls were created by using the appropriate solvents. The incubation period was 48 hours at 30°C. The incubation period was 48 hours at 30°C. Following the incubation time, the zone of inhibition in millimeters was estimated, indicating the antifungal ability of the crude bioactive compounds.

3.8. Antioxidant Potential of Macrofungal Crude Extracts

3.8.1. Chemicals Required

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

3.8.2. Procedure

The DPPH scavenging assay of macrofungal extracts was carried out with a slight modification to the Miser-Salihoglu et al., 2013 technique. 96 well microtiter plates were used for the DPPH test. To use for standard curves of the test chemical, the DPPH reagent was prepared by dissolving the DPPH in methanol and ensuring that the OD of the DPPH reagent was equal to 1 at 517nm. A stock solution of ascorbic acid was prepared as a positive control by dissolving 0.01g ascorbic acid in 1mL methanol. Dimethyl sulfoxide (DMSO) was employed as a negative control. The macrofungal extract stock solutions were made in DMSO at a concentration of 0.01g/1mL. Then, 100µL, 75µL, 50µL, 25µL, and 5µL of the tested sample, positive control, and negative control was loaded within the wells of the microtitre plate. After loading of these samples, 100µL, 125µL, 150µL, 175µL, and 195µL 2, 2-diphenyl-1-picrylhydrazyl (DPPH) reagent was put into the wells in dark to make the total concentration of 200µL in each well. In addition, 200L DPPH was exclusively utilised as a control. The plate was covered with aluminum foil and incubated for 1 hour at 37°C. After incubation, absorbance at 517 nm will be determined.

Antioxidant activity (%) = (AD-AS/ AD) × 100

where AD refers to the absorbance value of DPPH reagent (blank) and AS refers to the absorbance value of tested compounds.

3.9. Cytotoxicity Activity of Macrofungal Crude Extracts

3.9.1. Chemicals Required

- Artificial Sea Salt
- Dimethyl Sulfoxide (DMSO)

3.9.2. Procedure

The cytotoxicity potential of crude extracts of macrofungi was assessed against the brine prawn (*Artemia salina*) using the procedure of (Alowonle, 2014) with minor changes. Brine prawns (*Artemia salina*) larvae were utilised as model animals in this experiment. The artificial marine water was created by dissolving 34g of marine salt in 1L of distilled water, resulting in a final concentration of 34g/L. The salt solution was then filtered and placed into a hatching tank, which comprises of two chambers connected by small openings. In one compartment, one

teaspoon of brine prawn eggs (*Artemia salina*) was placed and covered with aluminium foil. The tank was then placed under the lamp, causing the hatched shrimps to migrate to the second chamber. The shrimp hatched in 24 to 48 hours and were taken away from the lit chamber. Ten hatched prawns were placed in a test tube with a 5mL artificial salt solution. Each mushroom extract stock solution was produced by dissolving it in DMSO at a concentration of 0.01g/mL. The activity was performed by using different concentrations from a stock solution such as 100µL, 80µL, 60µL, 40µL, and 20µL and added them into the test tubes having brine shrimps. DMSO has been used as a negative control with the same concentration of 100µL, 80µL, 60µL, 40µL, and 20µLThe test tube having only 10 brine shrimps was also utilised as a blank. These tubes were then incubated at 40ºC. The number of dead and active shrimps was recorded after 24 and 48 hours. The mortality rate was determined through the formula,

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

3.10. Hemolytic Activity of Macrofungal Crude Extracts

3.10.1. Chemicals Required

- Phosphate buffer saline (PBS)
- Sodium dodecyl sulphate
- Dimethyl Sulfoxide (DMSO)

3.10.2. Procedure

Blood from a healthy volunteer was taken in an EDTA tube to prevent it from coagulation. The blood was centrifuge at 1000 rpm for 10 minutes. The serum was thrown out and washing of remaining pellet was carried out three times through PBS (pH 7.4). The stock solutions of tested compounds were made by dissolving the 0.01g mushroom extract in 1mL DMSO. The stock solution of positive control was also made by adding 0.01g SDS in 1mL distilled water. DMSO was utilized as a negative control. A mixture of 200µL PBS was used as blank. Five different concentrations such as 100µL, 80µL, 60µL, 40µL and 20µL of a mushroom extract, positive control, and negative control were mixed with 300µL solution of RBCs and adjust the total volume of reaction mixture up to 1000µL by adding 600µL, 620µL, 640µL, 660µL, and 680µL PBS. The mixture was incubated for 1 hour at 37ºC. Afterward, the centrifugation was carried out at 2500rpm, for 15 minutes. 200µL supernatant was added to 96 well plates and absorbance was observed at 517nm. The hemolytic activity was measured through the formula,

Hercentage Hemolysis = (OD_sample - OD_negative control) / (OD_positive control - OD_negative control) × 100

3.11. Mycochemical Analysis

Using standard protocols, mycochemical analysis was performed to determine the presence of biochemical substances in wild mushroom samples.

3.11.1. Saponins Test

2 ml of mushroom extract was mixed with 20 ml of distilled water and vigorously shaken to estimate saponins. The formation of a 1 cm froth layer proved the presence of saponins.

3.11.2. Tannins Test

Tannin presence was determined by combining 2 mL of mushroom extract with a few drops of 5% ferric chloride (FeCl3). The presence of tannins is confirmed by the presence of dark brown or black colour.

3.11.3. Steroids test

2ml of mushroom extract was mixed with 2ml of chloroform and conc H2SO4. In the lower layer the presence of red color indicates the presence of steroids.

3.11.4. Total phenolic content

Total phenolic content was determined by using Folin ciocalteau (Fc) reagent, which turns blue due to redox reaction in the presence of phenols. Different concentration of extracts was added with 90µl of Fc reagent and incubated at room temperature for 5 min. 9µl of 6% Na2CO3 was added to reaction mixture and again incubated at 37°C for 90 min in shaking incubator at 150 rpm. Gallic acid (2mg/ml) was used as positive control and negative control was considered as without samples. Absorbance was measured at 630 nm. Total phenolic content was determined by using Gallic acid standard curve.

3.11.5. Determination of Total Flavonoid content (TFC)

Aluminium chloride calorimetric assay was used to determine the total flavonoid content of the extracts. Quercetin was used as standard or as a positive control 20µl of extract was added into the 96 well plate then 10% aluminium chloride µl was mixed with it and allowed to stand

for 5 minutes. After which µl solution of IM potassium acetate was added sequentially and mixed vigorously and make the final volume up to 200µl by adding distilled water. Plates was incubated for 30 mints at 37°C then absorbance of this reaction mixture was recorded at 510nm. Total flavonoid content was calculated as quercetin equivalents (mgQE/g).

4. RESULTS

4.1. Extraction of Bioactive Compounds from Macrofungi

Seventy-seven fractions of crude extract were obtained after treating mushroom samples with 7 solvents, including n-hexane, chloroform, ethyl acetate, acetone, acetonitrile, ethanol and methanol (Table 4.1).

S. No	Macrofungi	Solvents	Code
1.	*Russula foetentula*	Hexane	KIH
2.		Chloroform	K1C
3.		Ethyl acetate	K1EA
4.		Acetone	K1A
5.		Acetonitrile	K1AN
6.		Ethanol	K1E
7.		Methanol	K1M
8.	*Russula carolens*	Hexane	K2H
9.		Chloroform	K2C
10.		Ethyl acetate	K2EA
11.		Acetone	K2A
12.		Acetonitrile	K2AN
13.		Ethanol	K2E
14.		Methanol	K2M
15.	*Russula paludosa*	Hexane	K3H
16.		Chloroform	K3C
17.		Ethyl acetate	K3EA
18.		Acetone	K3A
19.		Acetonitrile	K3AN
20.		Ethanol	K3E
21.		Methanol	K3M

22.	*Laccaria laccata*	Hexane	K4H
23.		Chloroform	K4C
24.		Ethyl acetate	K4EA
25.		Acetone	K4A
26.		Acetonitrile	K4AN
27.		Ethanol	K4E
28.		Methanol	K4M
29.	*Amanita muscaria*	Hexane	K6H
30.		Chloroform	K6C
31.		Ethyl acetate	K6EA
32.		Acetone	K6A
33.		Acetonitrile	K6AN
34.		Ethanol	K6E
35.		Methanol	K6M
36.	*Canthrellus cinereus*	Hexane	K7H
37.		Chloroform	K7C
38.		Ethyl acetate	K7EA
39.		Acetone	K7A
40.		Acetonitrile	K7AN
41.		Ethanol	K7E
42.		Methanol	K7M
43.	*Chlorophyllum molybdites*	Hexane	K8H
44.		Chloroform	K8C
45.		Ethyl acetate	K8EA
46.		Acetone	K8A
47.		Acetonitrile	K8AN
48.		Ethanol	K8E
49.		Methanol	K8M
50.	*Russula emetica*	Hexane	K9H
51.		Chloroform	K9C

52.		Ethyl acetate	K9EA
53.		Acetone	K9A
54.		Acetonitrile	K9AN
55.		Ethanol	K9E
56.		Methanol	K9M
57.	*Auricularia auricula judae*	Hexane	K10H
58.		Chloroform	K10C
59.		Ethyl acetate	K10EA
60.		Acetone	K10A
61.		Acetonitrile	K10AN
62.		Ethanol	K10E
63.		Methanol	K10M
64.	*Lactarius deliciosus*	Hexane	K11H
65.		Chloroform	K11C
66.		Ethyl acetate	K11EA
67.		Acetone	K11A
68.		Acetonitrile	K11AN
69.		Ethanol	K11E
70.		Methanol	K11M
71.	*Macrolepiota albuminosa*	Hexane	K11H
72.		Chloroform	K11C
73.		Ethyl acetate	K11EA
74.		Acetone	K11A
75.		Acetonitrile	K11AN
76.		Ethanol	K11E
77.		Methanol	K11M

Table 4.1. Macrofungal crude extracts and their concentrations

4.2. Antibacterial Potential of Macrofungal Crude Extracts

Different levels of antibacterial activity have been detected in the macrofungal crude extracts against five multi-drug resistant pathogens (Fig). According to the findings of the agar well plate diffusion method, the acetonitrile fractions of *Russula foetentula* and *Laccaria laccata* and the ethyl acetate fractions of *Auricularia auricula judae* and *Macrolepiota albuminosa* demonstrated the best activity against all bacterial strains, including *Bacillus subtilis, P. aeruginosa, E. coli, S. aureus* and *Klebsiella pneumoniae*. The best activity against *K. pneumoniae* was consistently produced by hexane fractions of *Cantharellus cinerus, Chlorophyllum molybdites, Laccaria laccata,* and *Russula emetica*. *Cantharellus cinerus* and *Chlorophyllum molybdites* showed the strongest effectiveness against *K. pneumoniae* and *S. aureus* in chloroform fractions. The best effectiveness against *B. subtilus, P. aeruginosa*, and *K. pneumoniae* was demonstrated by the ethyl acetate fraction of *Russula foetentula, Russula carolens, Amanita muscaria, Cantharellus cinerus,* and *Chlorophyllum molybdites*. The highest results for *B. subtilis* were consistently obtained using the acetone fractions of *Laccaria laccata*, *Cantharellus cinerus, Chlorophyllum molybdites,* and *Lactarius deliciosus*. Ethyl acetate fractions were good for all except *S. aureus* and *E. coli*. Acetone fractions of all 11 samples cover all bacteria except *E. coli*. Among acetonitrile fractions, *Russula foetentula* and *Laccaria Laccata* were the best because they gave activity against all 5 bacterial strains. Inhibition zone of the acetonitrile fractions of *Russula foetentula* against bacterial strains in order of *E. coli, Bacillus subtilis, P. aeruginosa, S. aureus* and *Klebsiella pneumoniae* is 16 ± 0.5mm, 21 ± 0mm, 14 ± 0.5mm, 20 ± 0mm and 11 ± 1mm. Ethanol fractions of *laccaria laccata* gave activity against *P. aeruginosa* and *russula foetentula* and *Laccaria laccata* gave good activity against *Staphylococcus aureus*. Methanolic fractions of extracts gave activity against *E. coli, B. subtilis, P. aeruginosa* and *K. pneumonia*. The bulk of extracts lacked effectiveness against *E. coli*, with the exception of the ethanolic fractions of 3 extracts, and the ethyl acetate and acetonitrile fractions were the best overall. The positive control in case of antibacterial activity was gentamycin.

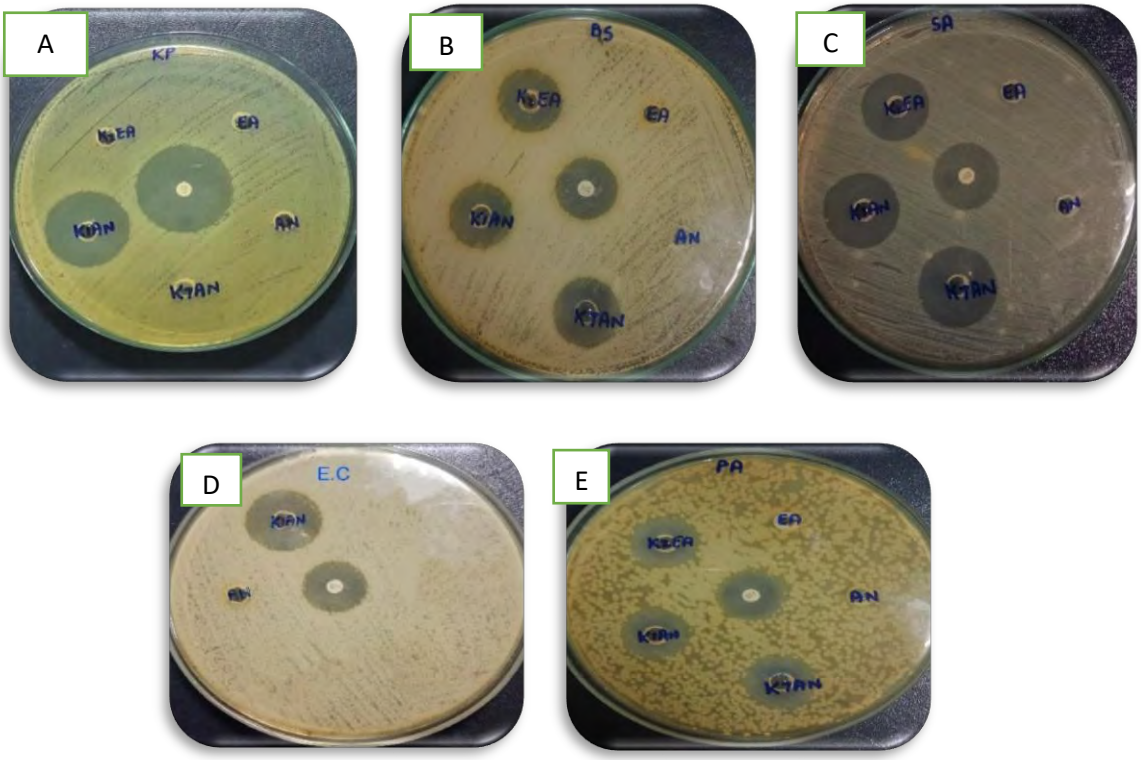

Figure 4.1. Antibacterial activity of the macrofungal crude extract against A) B. subtilis

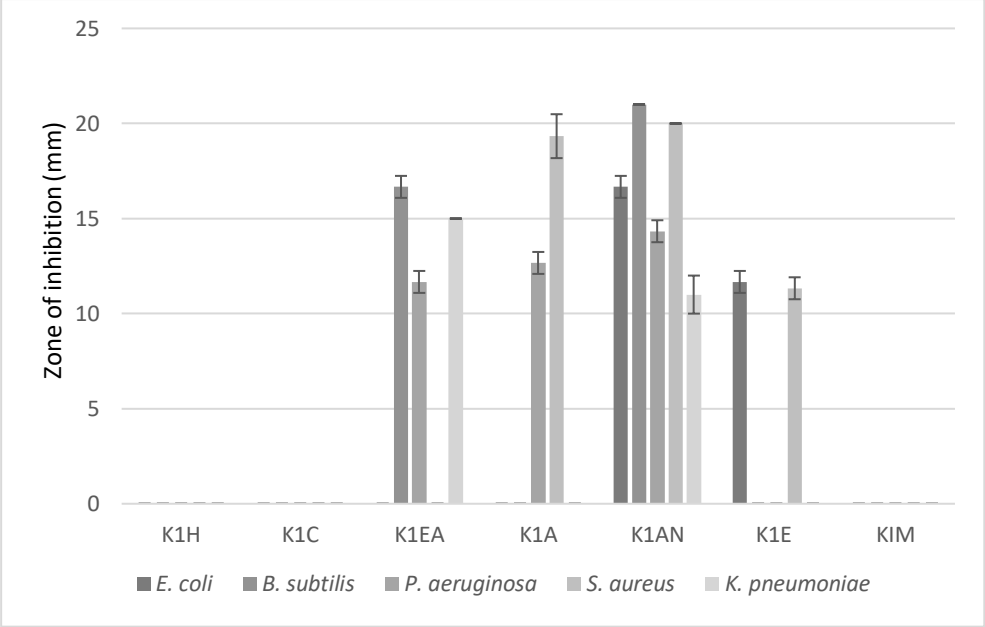

Figure4.2. Inhibition zone (mm) of bacterial strains against the crude extracts of Russula foetentula.

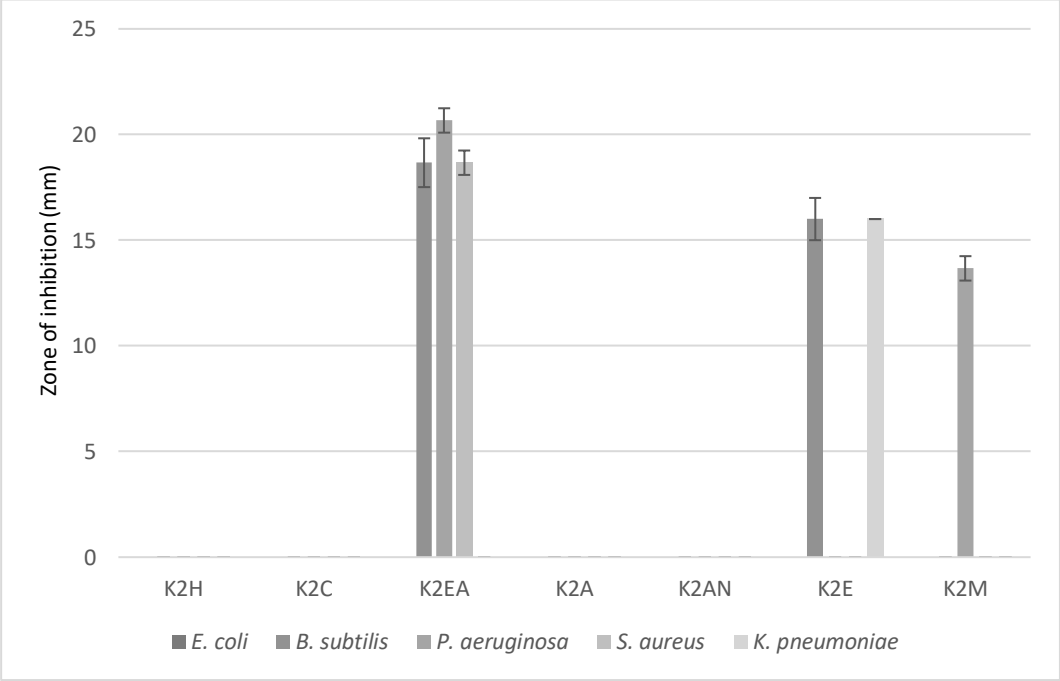

Figure 4.3. Inhibition zone (mm) of bacterial strains against the crude extracts of Russula cerolens.

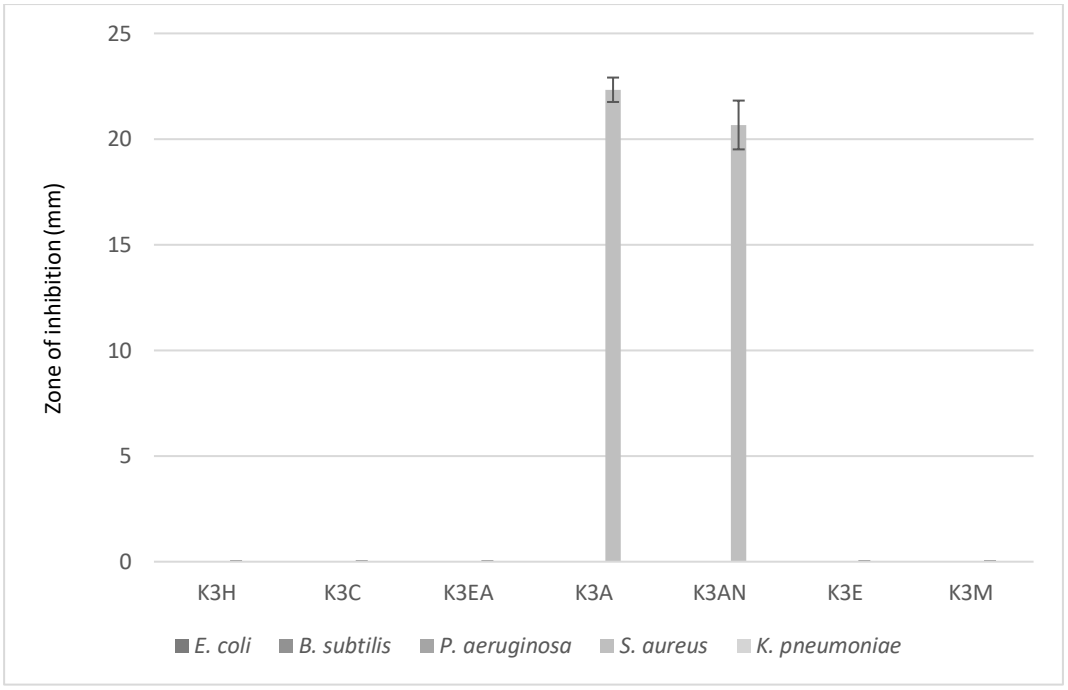

Figure 4.4. Inhibition zone (mm) of bacterial strains against the crude extracts of Russula paludosa.

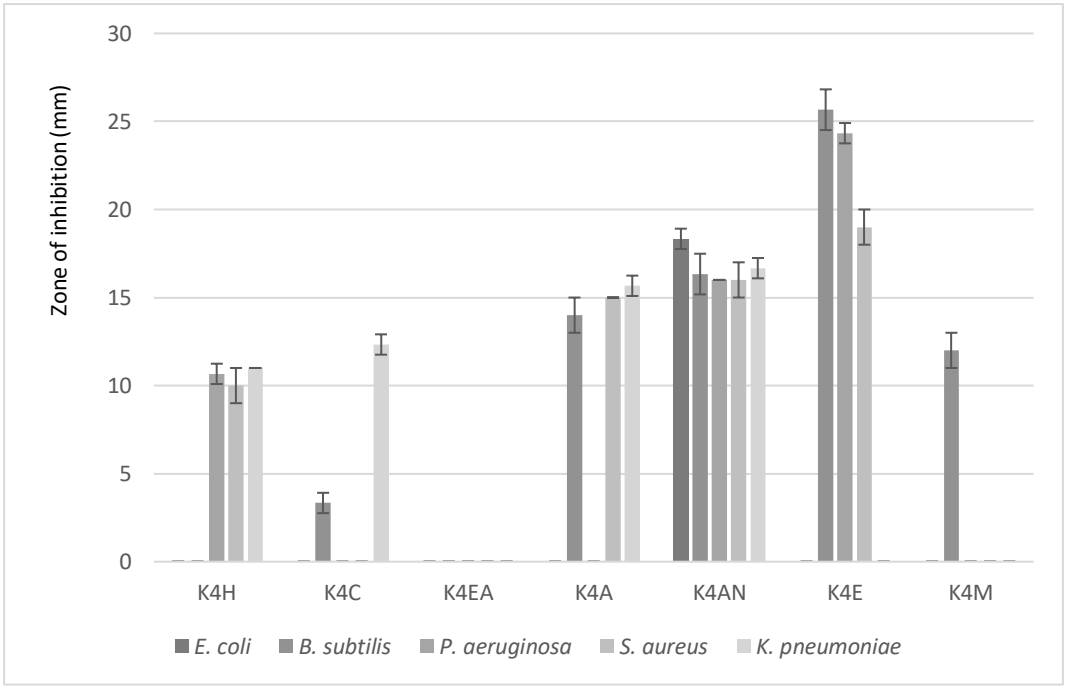

Figure 4.5. Inhibition zone (mm) of bacterial strains against the crude extracts of Laccaria Laccata.

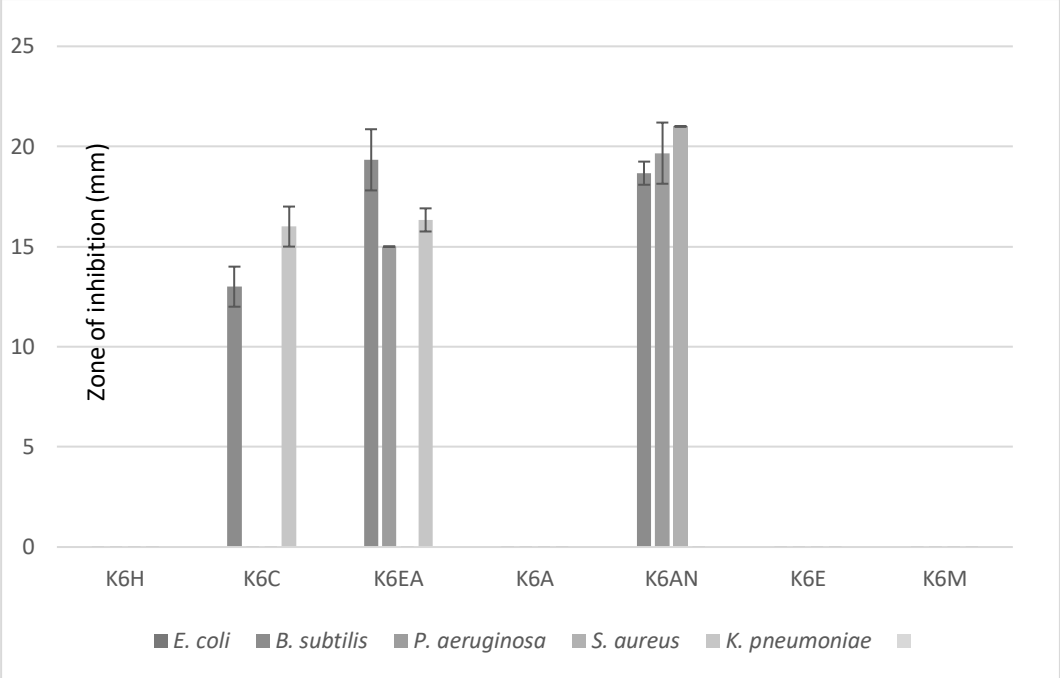

Figure 4.6. Inhibition zone (mm) of bacterial strains against the crude extracts of Amanita muscaria.

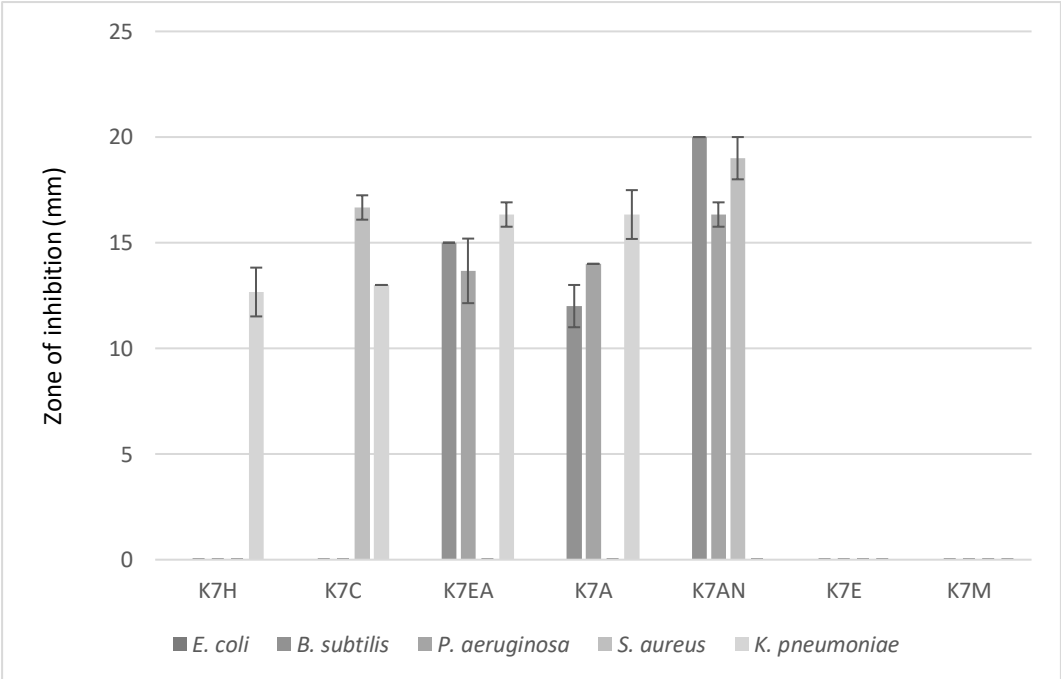

Figure 4.7. Inhibition zone (mm) of bacterial strains against the crude extracts of Cantharellus cinerus.

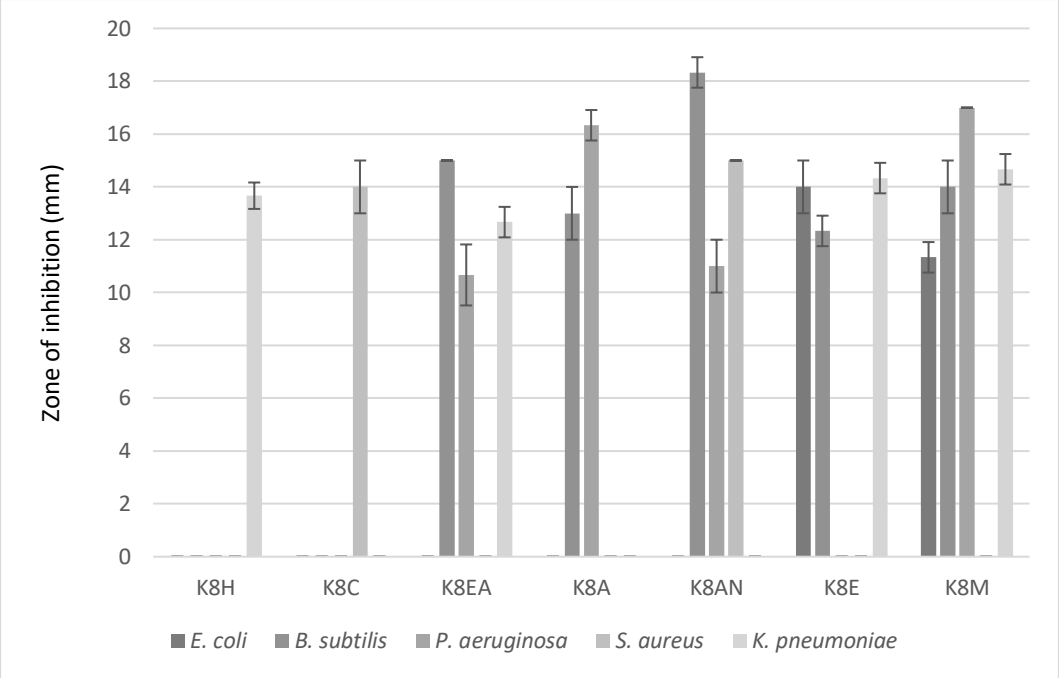

Figure 4.8. Inhibition zone (mm) of bacterial strains against the crude extracts of Chlorophyllum molybdites.

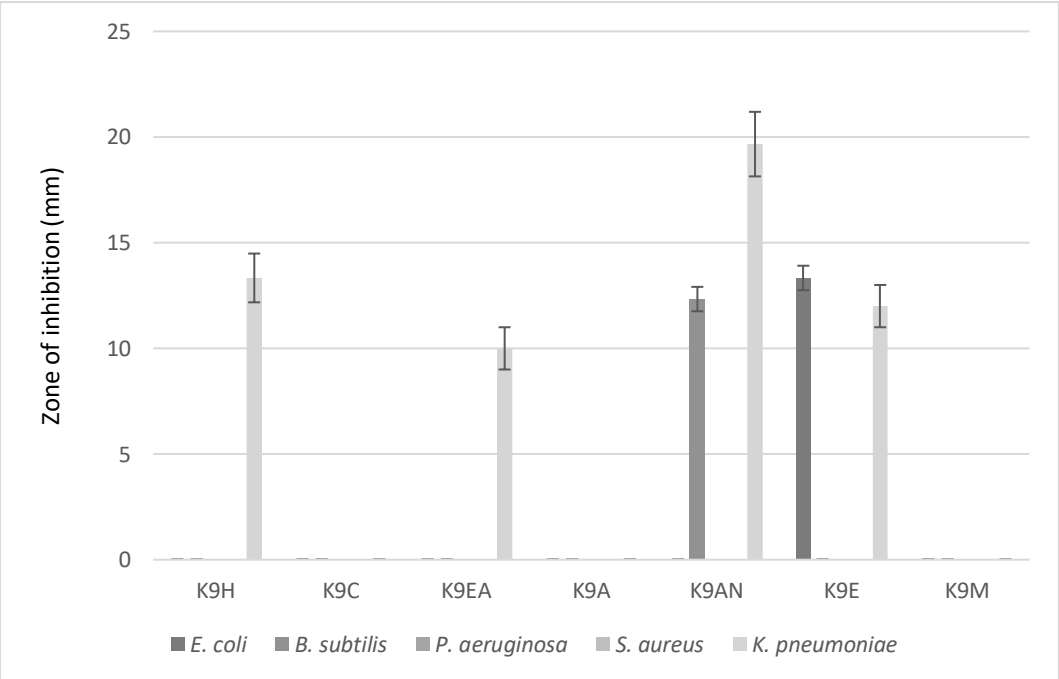

Figure4.9. Inhibition zone (mm) of bacterial strains against the crude extracts of Russula emetica.

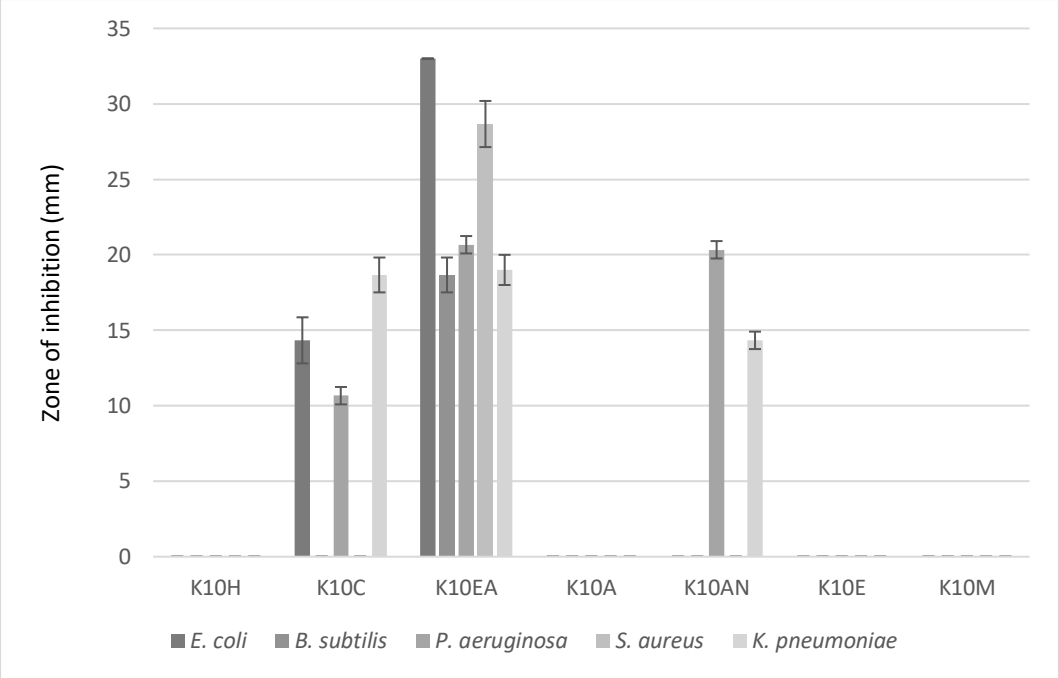

Figure 4.10. Inhibition zone (mm) of bacterial strains against the crude extracts of Auricularia auricula-judae.

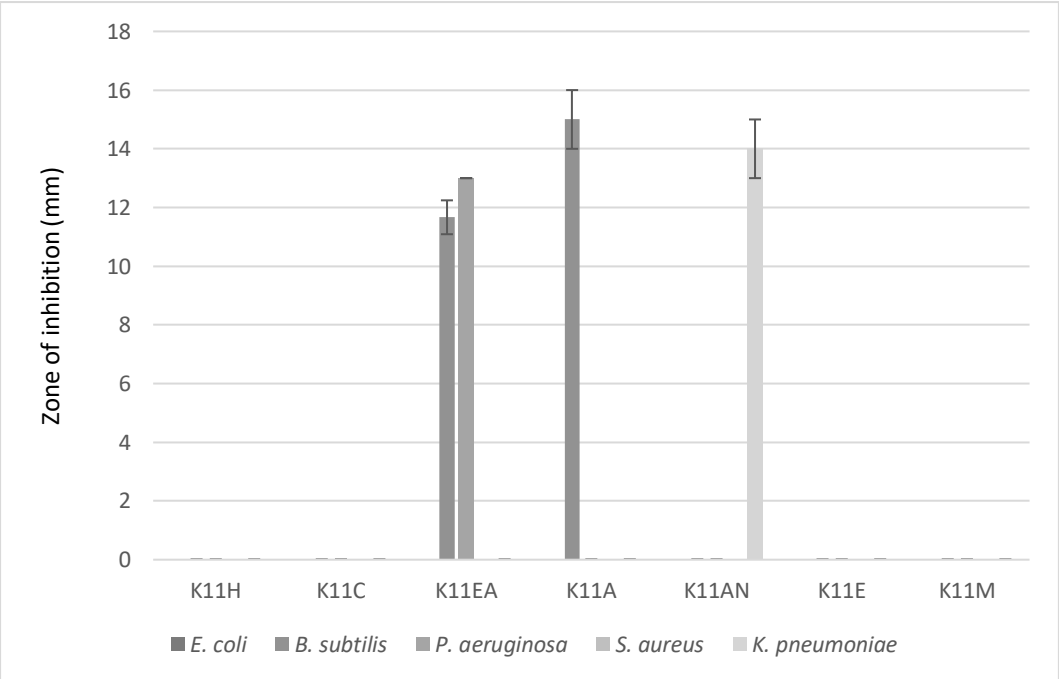

Figure 4.11. Inhibition zone (mm) of bacterial strains against the crude extracts of Lactarius deliciosus.

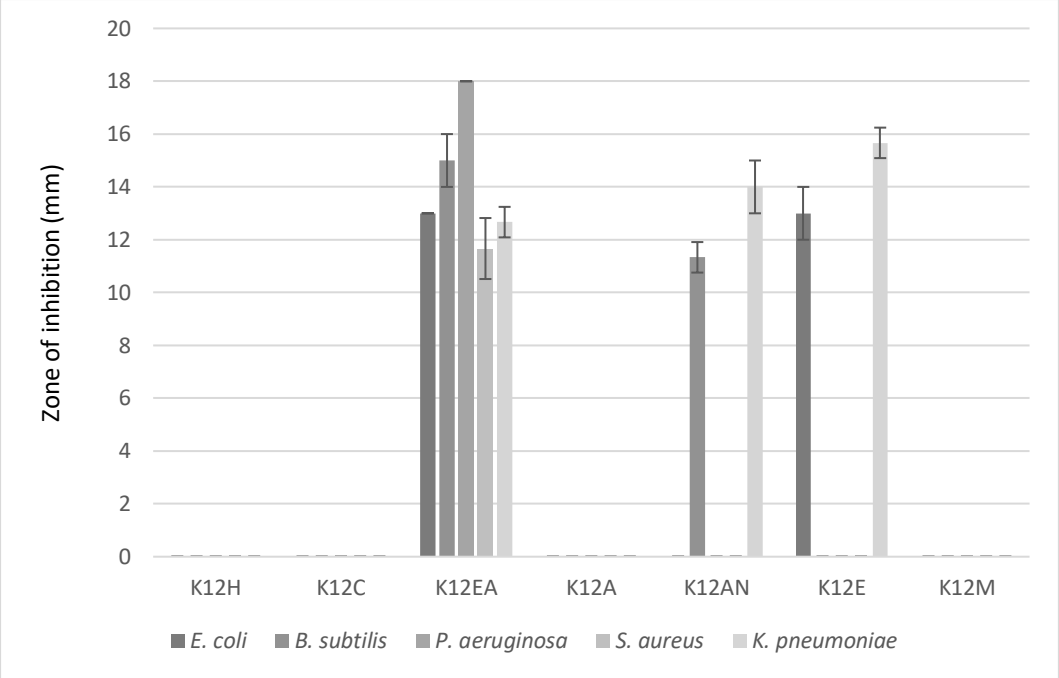

Figure 4.12. Inhibition zone (mm) of bacterial strains against the crude extracts of Macrolepiota albuminosa.

4.3. Antifungal Activity of Mushroom Extracts

The crude extracts of macrofungi also shown varying levels of antifungal activity (). Ethyl acetate and acetonitrile fractions of *Russula foetentula* were found to be active against three fungal test strains: *Aspergillus niger, Aspergillus flavus*, and *Candida albicans* and inhibition zone of 11 ± 0.5mm, 14 ± 0.5mm and 15 ± 0.5mm was recorded respectively in case of ethyl acetate extract and inhibition zone of 19 ± 1.13mm, 15 ± 0.5mm and 18 ± 0.5mm was recorden in case of acetonitrile fractions. Ethyl acetate and ethanol fractions of *Russula carolens* were active against three test strains. No fraction of *Russula paludosa*, out of seven, proved effective against any of the test strains. Hexane, acetone, acetonitrile, and ethanol fractions of *Laccaria laccata* were found to be effective against pathogenic fungal strains. *Amanita muscaria'* chloroform, acetone, and acetonitrile fractions had potent antifungal activity. *Canthrellus cinereus'* chloroform and acetonitrile fractions, as well as *Chlorophyllum molybdites* ethyl acetate, ethanol, and methanol fractions, were effective against all three strains.

Acetone and ethanol, and acetone and chloroform, were effective against *Russula emetica* and *Auricularia auricula judae*, respectively. *Lactarius deliciosus'* hexane and ethyl acetate fractions, as well as *Macrolepiota albuminosa's* chloroform, ethyl acetate, ethanol, and methanol fractions, displayed high effectiveness against fungal pathogens. In terms of antifungal potential, most of the extracts dissolved in ethyl acetate, acetonitrile, acetone, and ethanol outperformed the others.

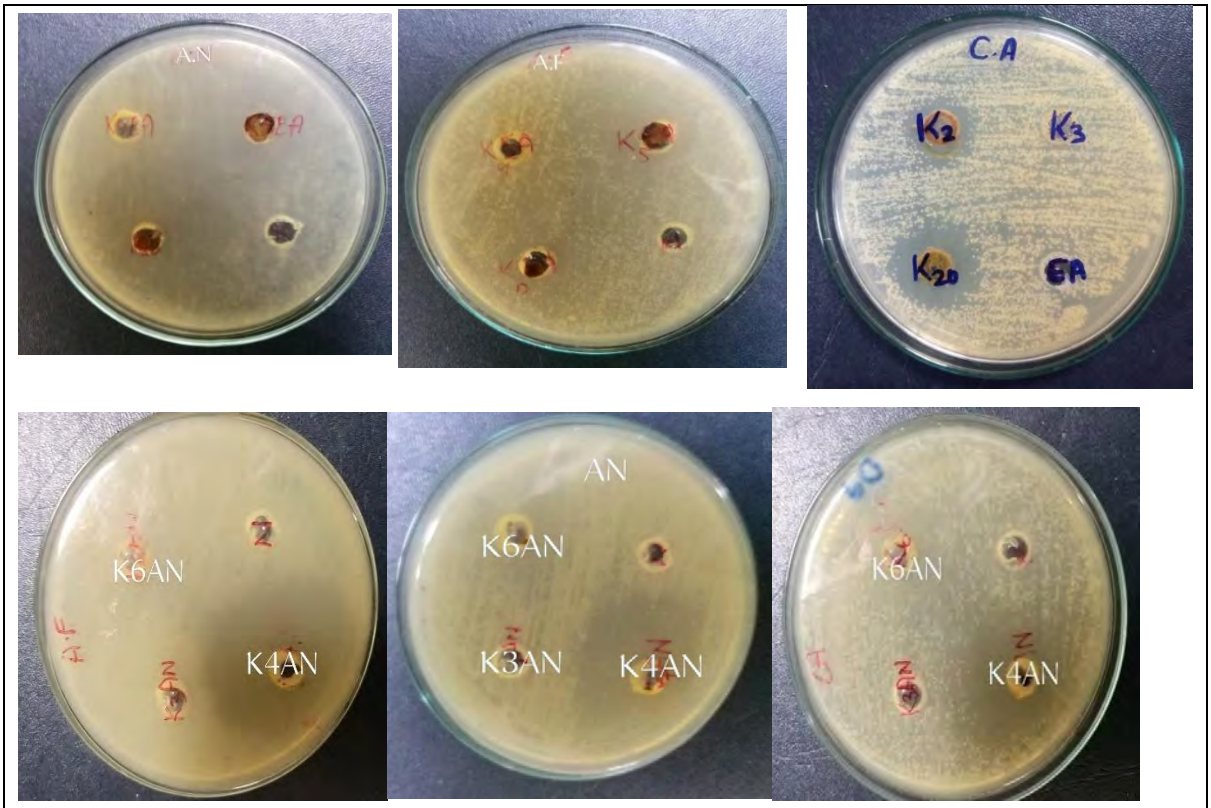

Figure 4.13. Antifungal activity of crude macrofungal extracts against A. niger, A. flavus and C. Albicans

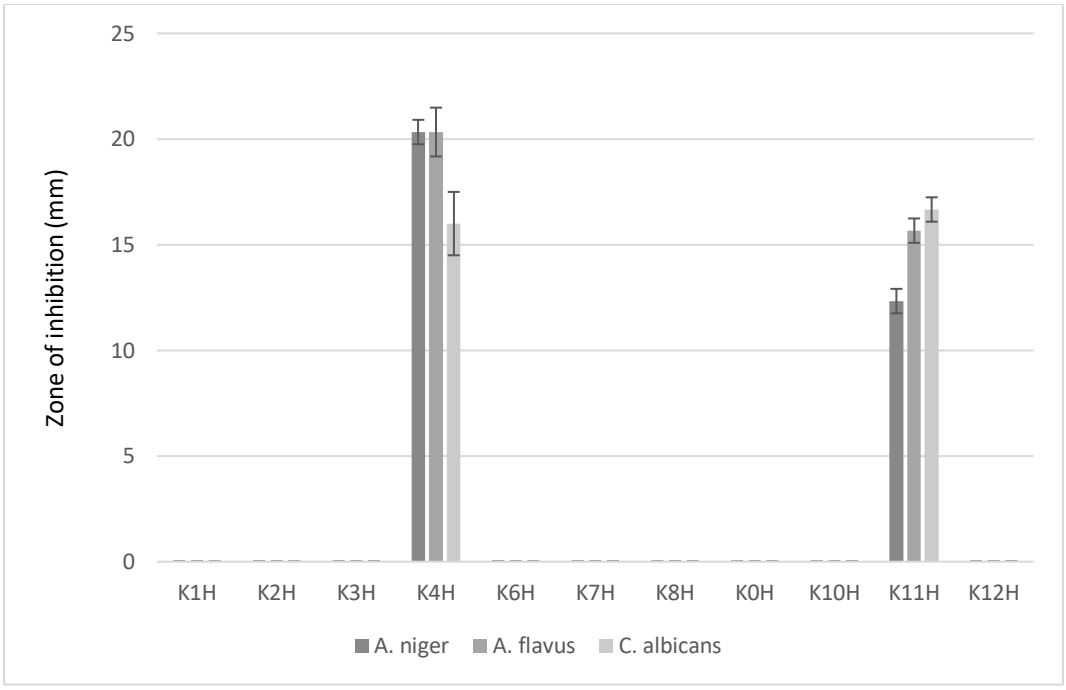

Figure 4.14. Inhibition zone (mm) of fungal strains against the hexane fractions of all 11 mushroom extracts.

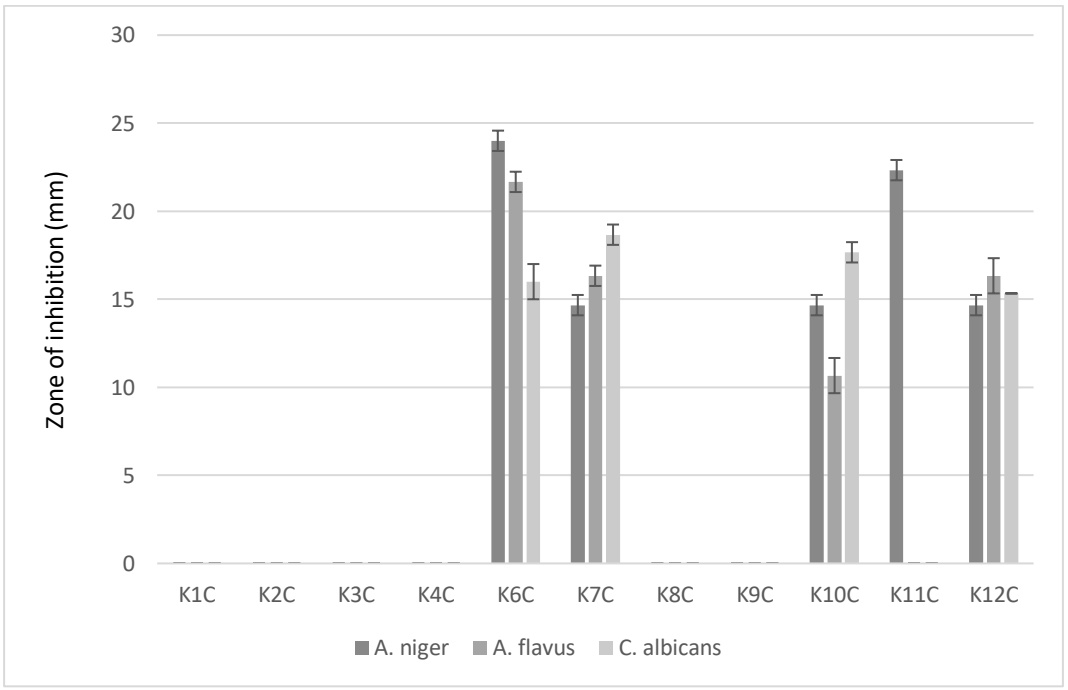

Figure 4.15. Inhibition zone (mm) of fungal strains against the chloroform fractions of all 11 mushroom extracts.

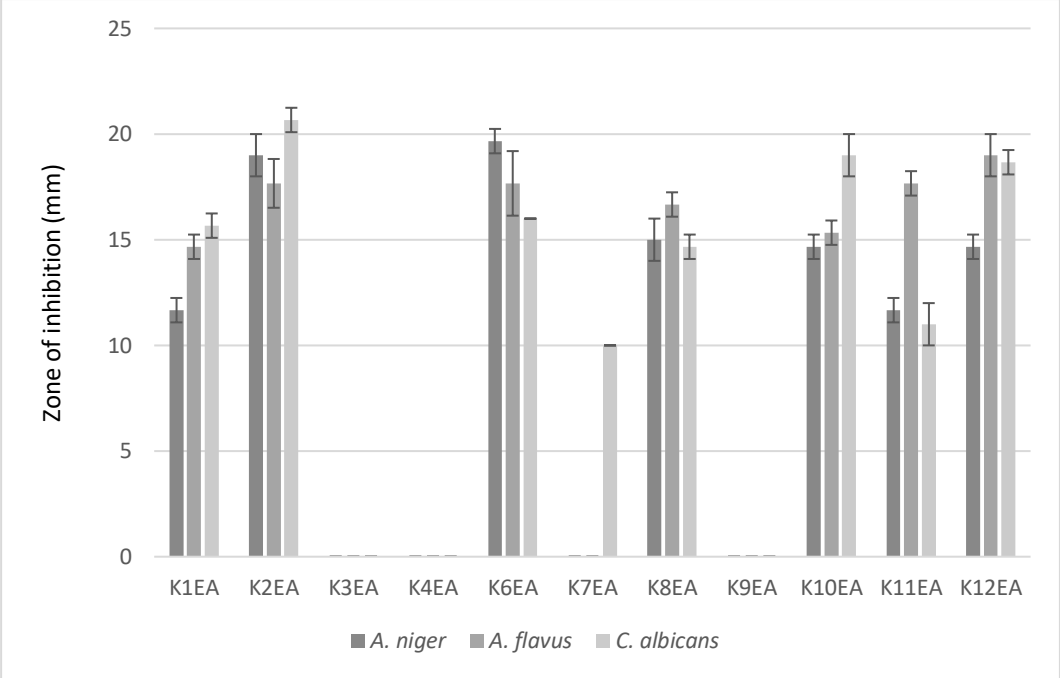

Figure 4.16. Inhibition zone (mm) of fungal strains against the ethyl acetate fractions of all 11 mushroom extracts.

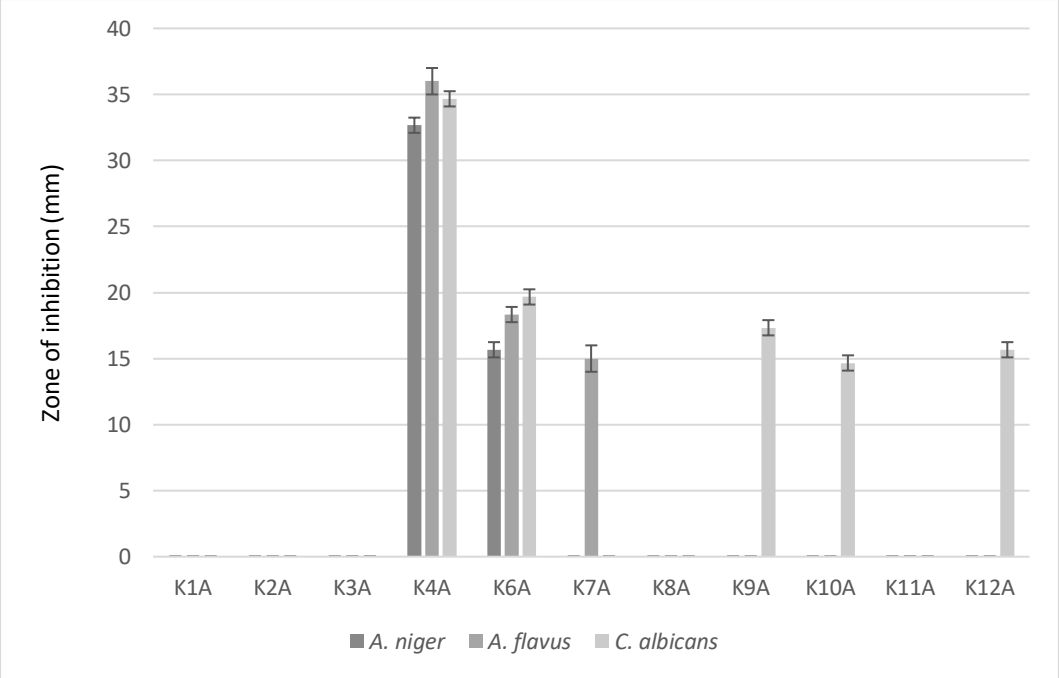

Figure 4.17. Inhibition zone (mm) of fungal strains against acetone fractions of all 11 mushroom extracts.

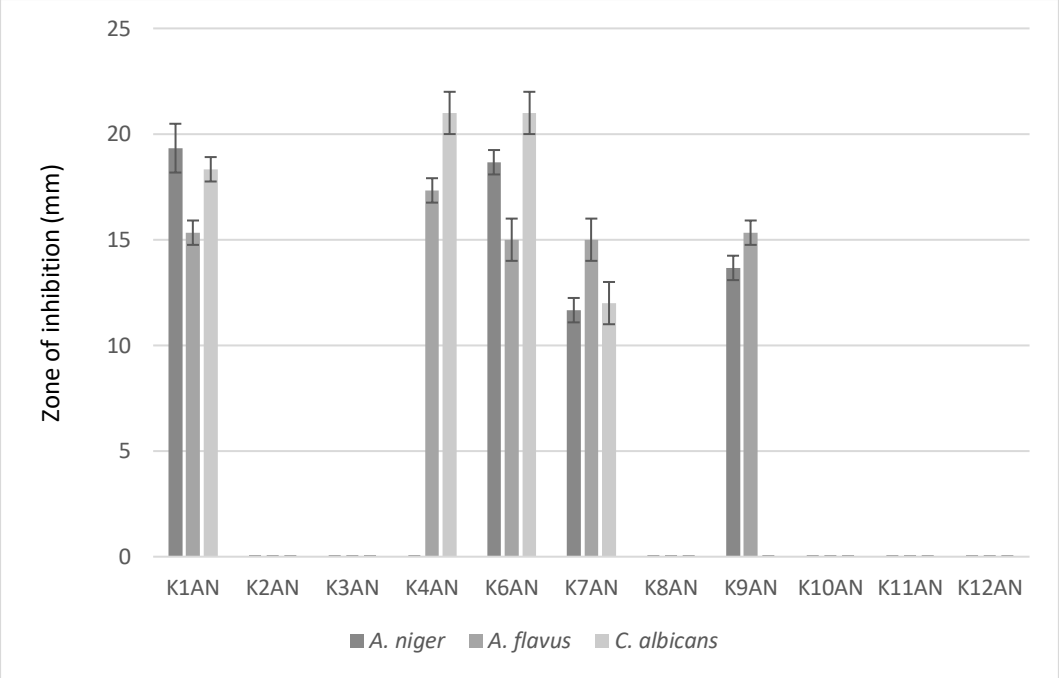

Figure 4.18. Inhibition zone (mm) of fungal strains against acetonitrile fractions of all 11 mushroom extracts.

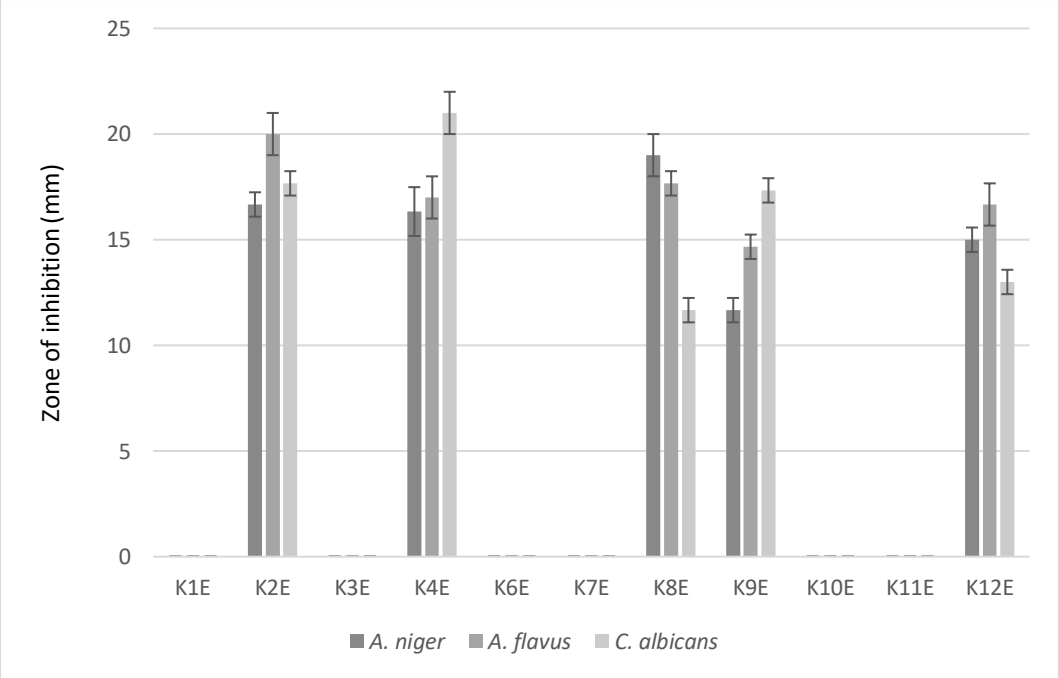

Figure 4.19. Inhibition zone (mm) of fungal strains against ethanol fractions of all 11 mushroom extracts.

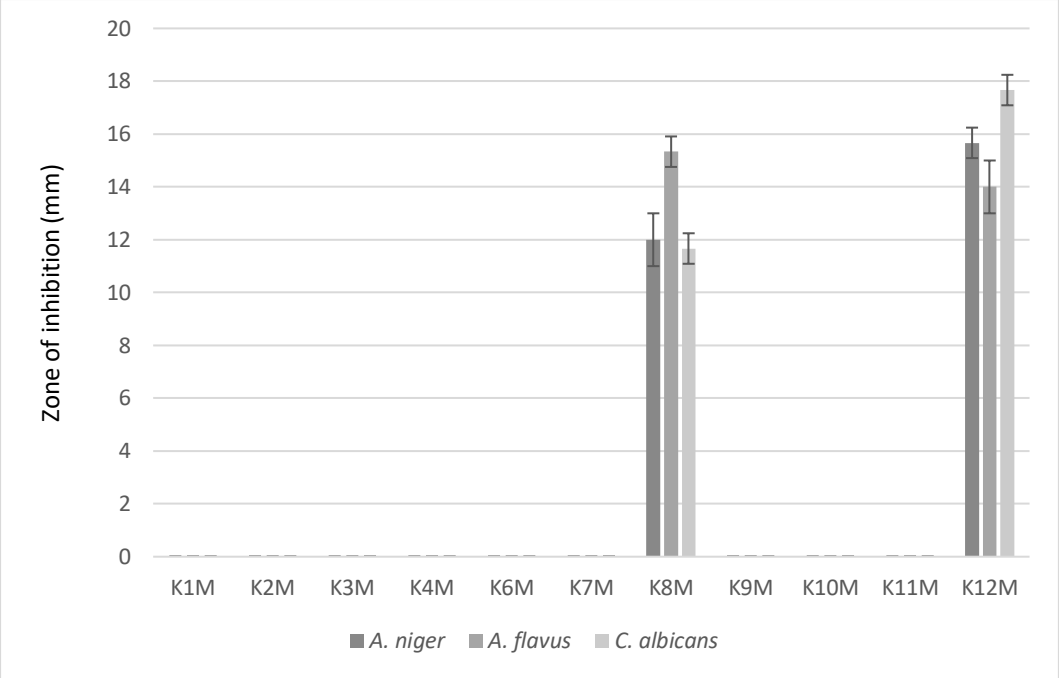

Figure 4.20. Inhibition zone (mm) of fungal strains against methanol fractions of all 11 mushroom extracts.

4.4. Antioxidant Potential of Mushroom Extracts

The free radical scavenging assay results revealed that the percentage of scavenging depends on the concentration of extracts utilized (). By increasing the concentration of crude extracts, the antioxidant activity was enhanced. The peak activity of extracts was detected at a concentration of 1mg/mL, and activity decreases as the concentration of extract decreases. All fractions of *Russula cerolens, Amanita muscaria*, and *Auriculaia auricula-judae* extracts displayed unusually strong antioxidant activity, but *Cantharellus cinerus and Macrolepiota albuminosa* extracts displayed no activity at all. Acetone and methanol fractions of *Russula foetentula* showed respectively 78 ± 1.15%, 75 ± 1.5%, 72 ± 0.5%, 66 ± 0.5%, 60 ± 1% activity and 71 ± 1%, 67 ± 1.5%, 65 ± 1%, 62 ± 1%, and 59 ±0.5% activity with decreasing extract concentration. The antioxidant activity was demonstrated by *Russula cerolens* fractions in ethyl acetate, chloroform, and methanol. *Russula paludosa's* ethyl acetate and *Laccaria laccata's* acetonitrile have been shown to be powerful antioxidant sources. The hexane, chloroform, and acetone fractions of *Amanita muscaria*, as well as the chloroform fraction of *Chlorophyllum molybdites*, have high antioxidant potential. Ethanol and methanol fractions of

Auricularia auricula-judae demonstrated excellent antioxidant activity, with values ranging from 80 ± 0.5% to 75 ± 0.5%, 75 ± 0%, 58 ± 0.5%, and 37 ± 0.5% for ethanol and 73 ± 0.5%, 71 ± 1%, 67 ± 1%, 57 ± 1.15%, and 37 ± 0.5% for methanol.

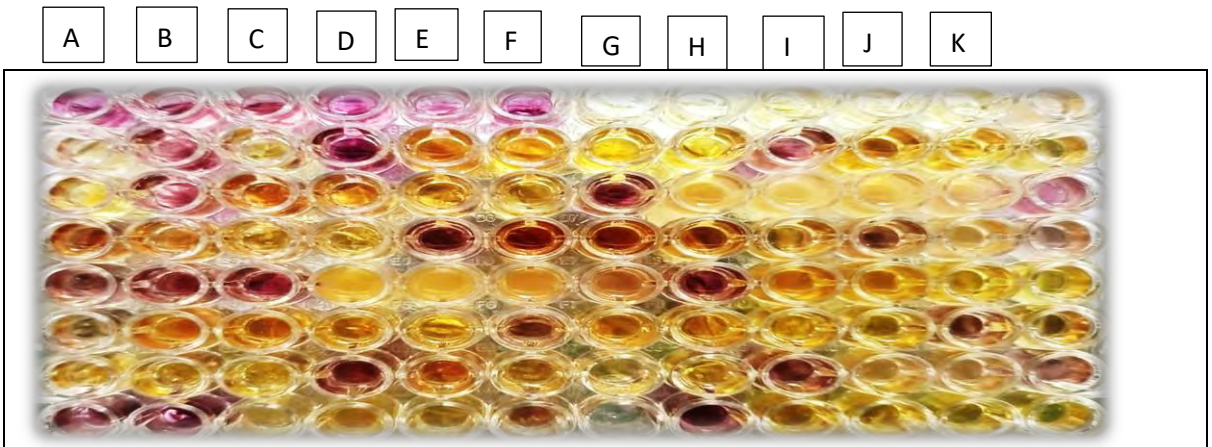

Figure 4.21. Antioxidant Activity of macrofungal crude extracts at different concentrations with A = Blank (DPPH); B, C, D, E, and F= Negative control (0.05, 0.25, 0.5, 0.75 and 1mg/mL); G, H, I, J, and K= Positive control (0.05, 0.25, 0.5, 0.75 and 1mg/mL).

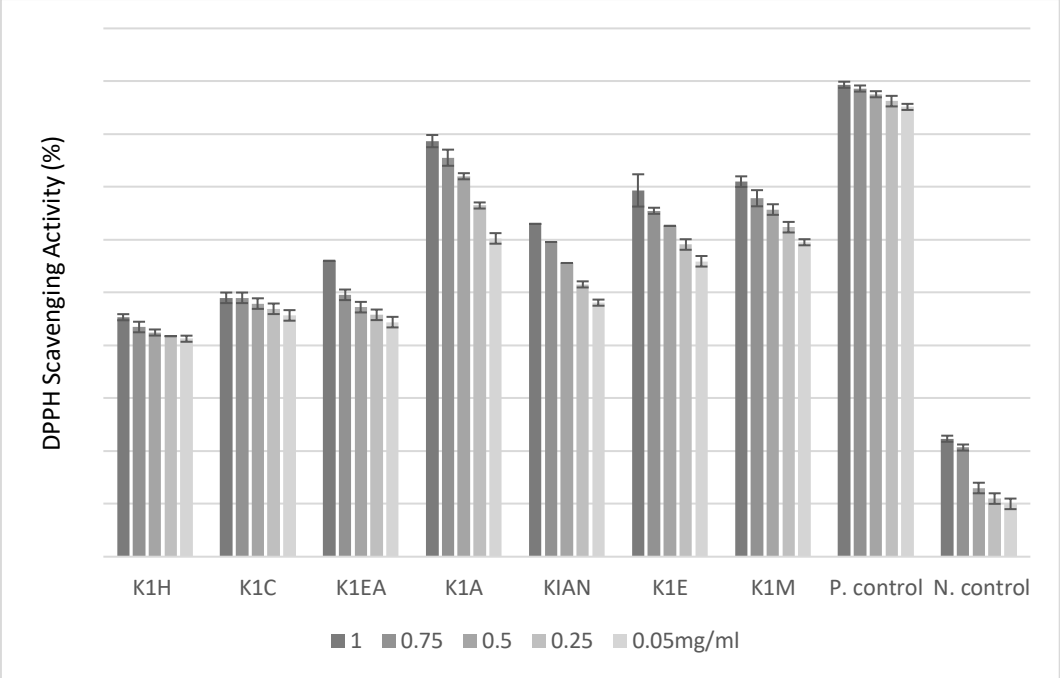

Figure 4.22. DPPH Scavenging Activity (%) of Russula foetentula crude extracts

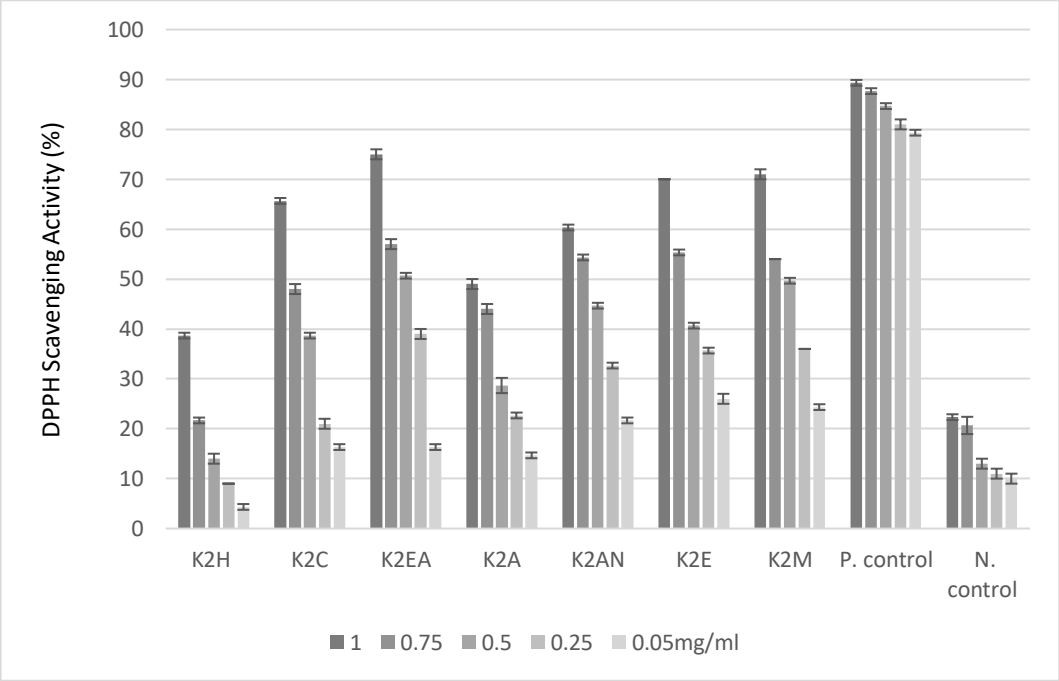

Figure 4.23. DPPH Scavenging Activity (%) of Russula cerolens crude extracts

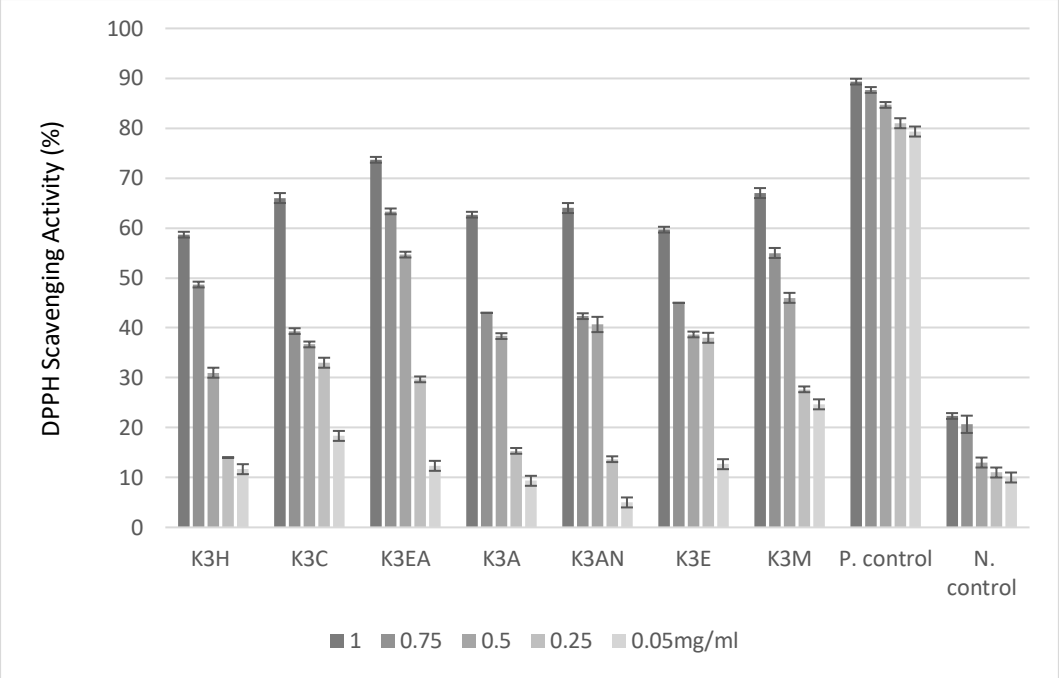

Figure 4.24. DPPH Scavenging Activity (%) of Russula muscaria crude extracts

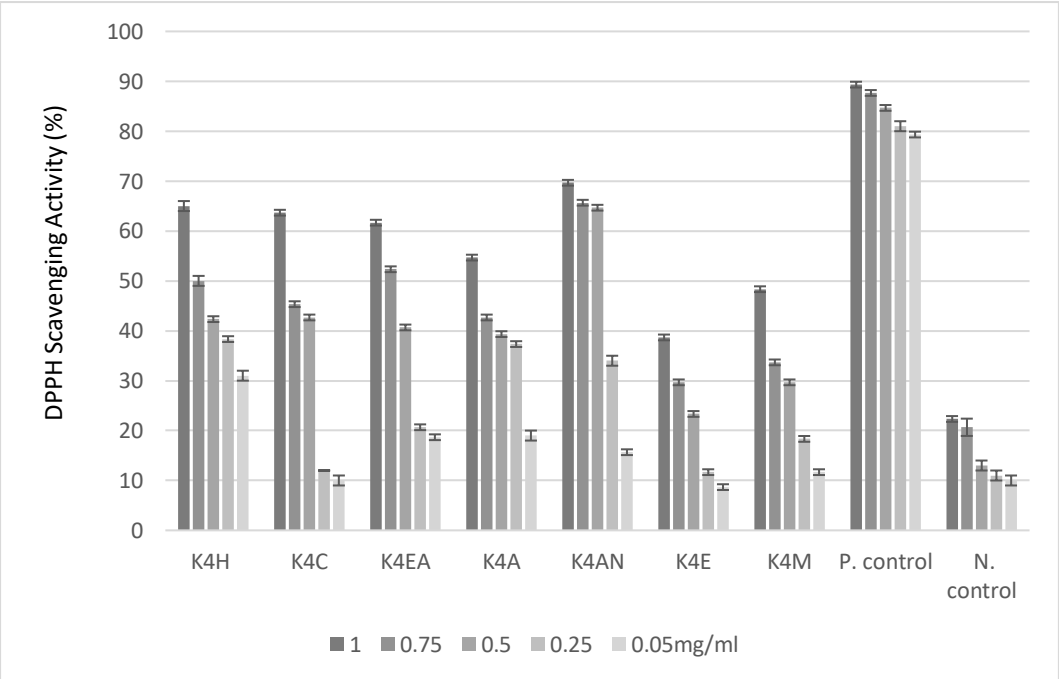

Figure 4.25. DPPH Scavenging Activity (%) of Laccaria laccata crude extracts

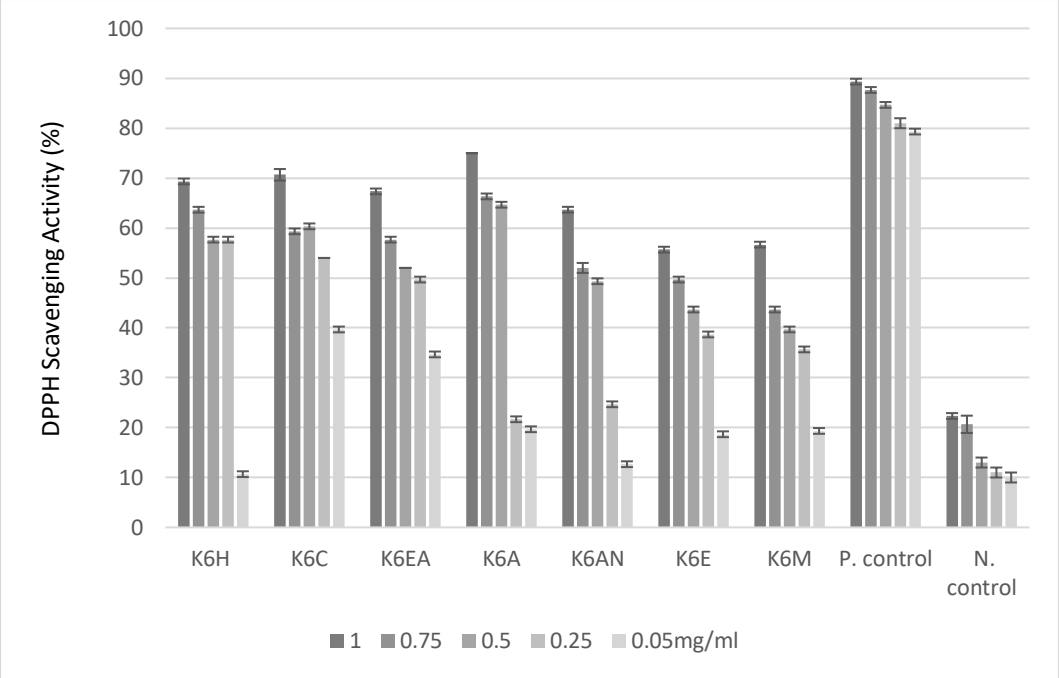

Figure 4.26. DPPH Scavenging Activity (%) of Amanita muscaria crude extracts

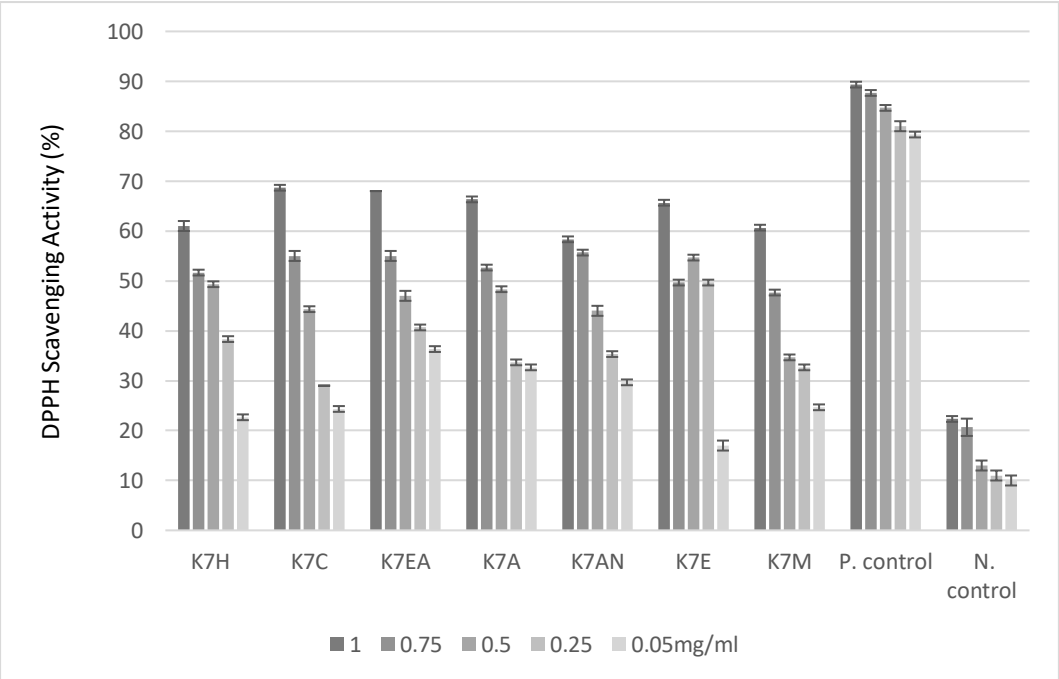

Figure 4.27. DPPH Scavenging Activity (%) of Cantharellus cinerus crude extracts

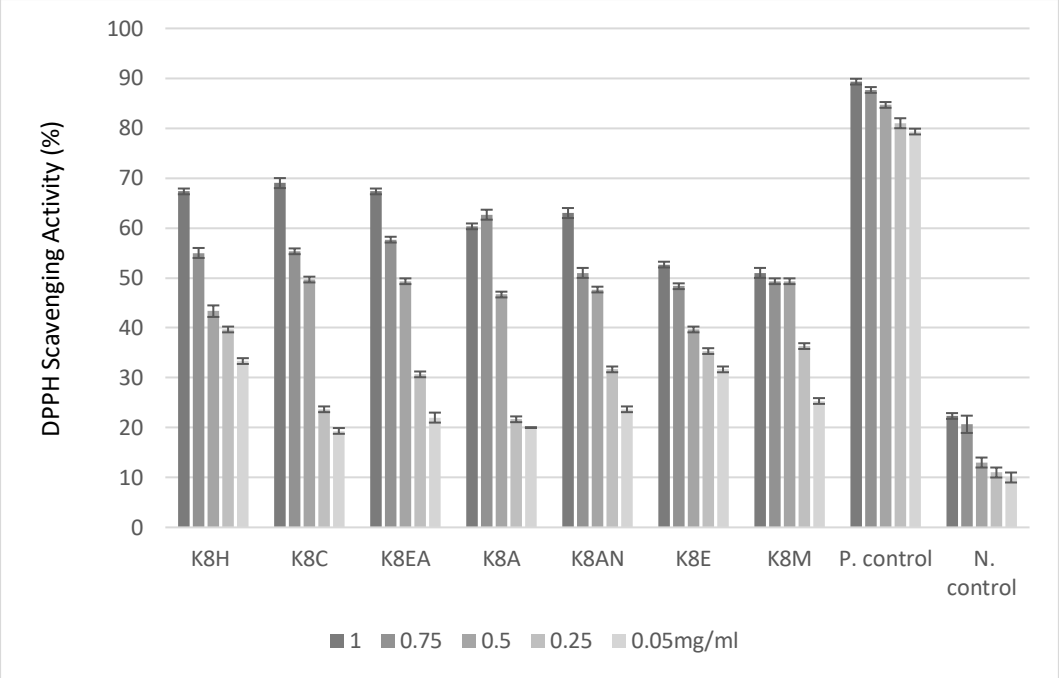

Figure 4.28. DPPH Scavenging Activity (%) of Chlorophyllum molybdites crude extracts

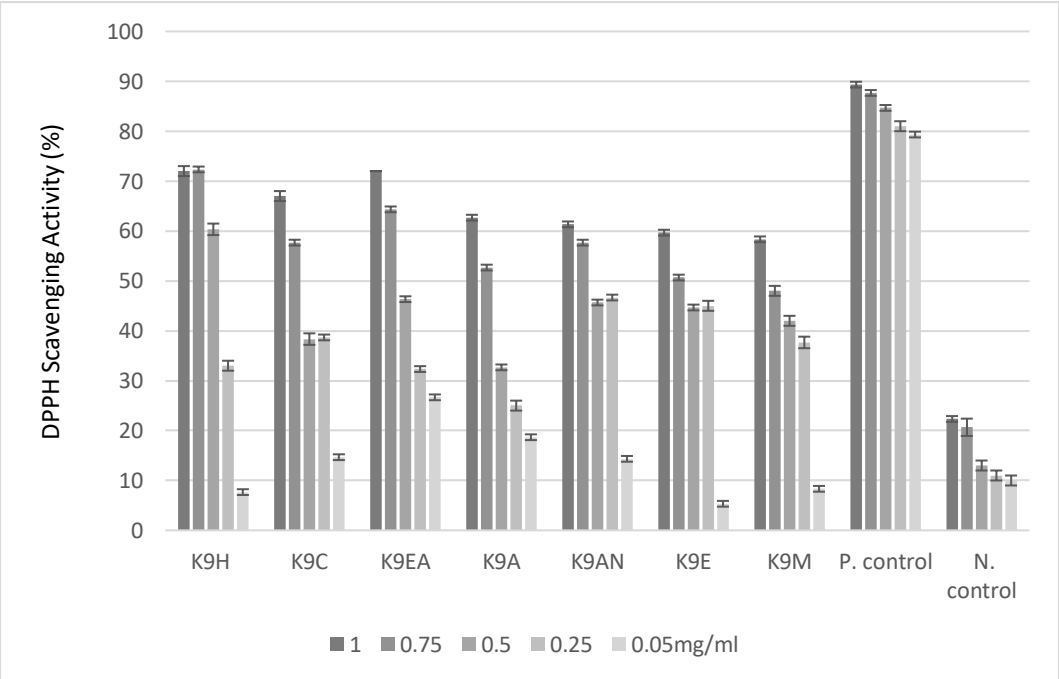

Figure 4.29. DPPH Scavenging Activity (%) of Russula emetica crude extracts

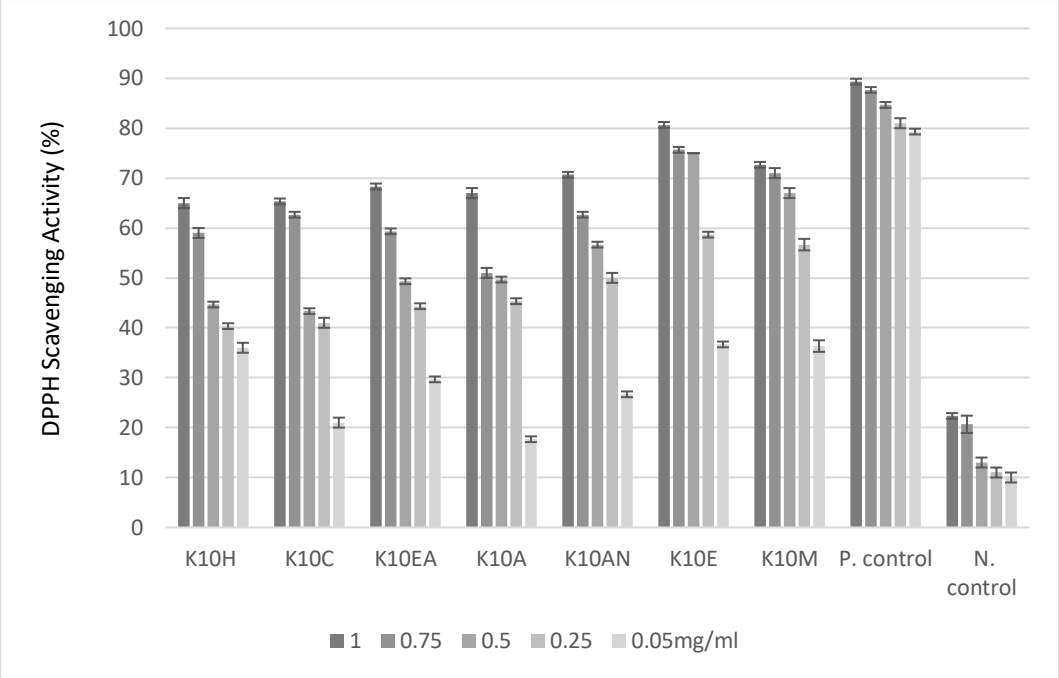

Figure 4.30. DPPH Scavenging Activity (%) of Auricularia auricula judae crude extracts

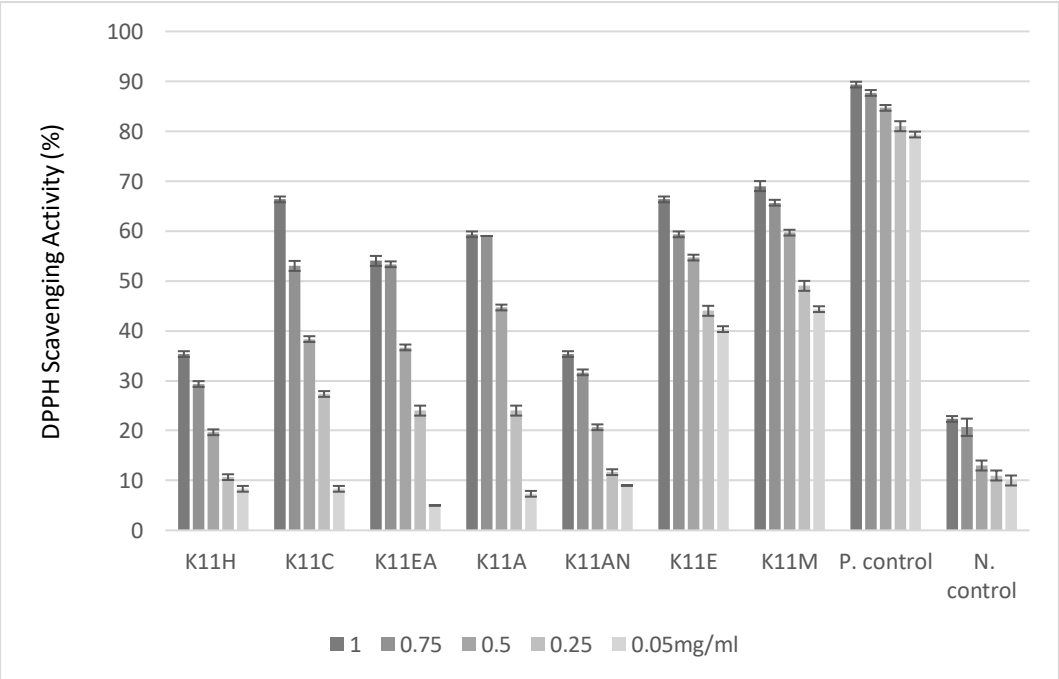

Figure 4.31. DPPH Scavenging Activity (%) of Lactarius deliciosus crude extracts

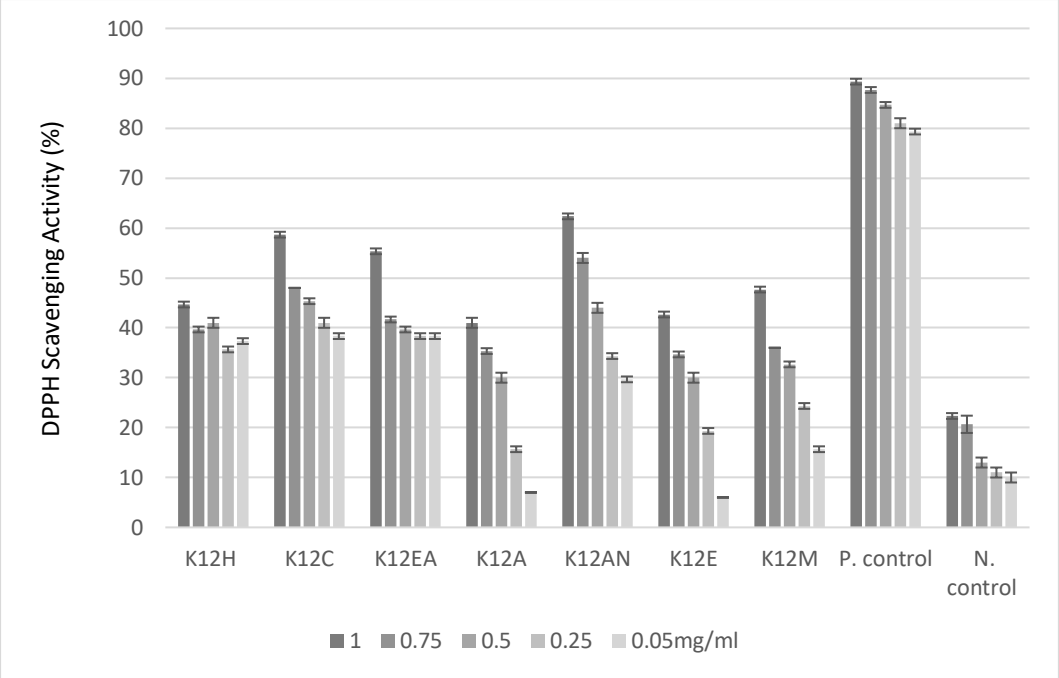

Figure 4.32. DPPH Scavenging Activity (%) of Macrolepiota albuminosa crude extracts

4.5. Cytotoxicity Activity of Mushroom Extracts

The cytotoxicity assay results were graphically shown by graphing the concentration of extracts vs the percentage of remaining brine shrimps, thereby noted as depending on concentration. It was discovered that when concentration decreased, the rate of survival increased. With a 0% survival rate, all fractions of *Russula foetentula, Russula carolens, Cantharellus muscaria*, and *Chlorophyllum cinerus* exhibited considerable cytotoxic potential. After 24 hours, all fractions of these four extracts at 1, 0.75, 0.5, 0.25, and 0.05mg/mL were shown to be 100% hazardous for shrimps. Overall, all fractions of *Russula paludosa* shown reduced cytotoxicity, with the exception of hexane and methanol fractions, where shrimp survival was 0% and 2% at 1 mg/ml concentrations, respectively. All fractions of *Auricularia auricula judae* showed less activity, with the exception of hexane, which showed 0% survival from start to finish. Except for the chloroform and methanol fractions, all five *Laccaria laccata* fractions demonstrated 100% motility at doses of 1, 0.75, 0.5, 0.25, and 0.05mg/mL. *Russula emetica* proved to be effective enough in all fractions and displayed 0% survival with the exception of the ethyl acetate fraction, whose activity was intermediate and exhibited 50% survival. In terms of cytotoxicity, *Auricularia auricula judae* was not very strong, but *Lactarius deliciosus* and *Macrolepiota albuminosa* were, and no survival of brine shrimps was found from high to low concentrations

in most cases. At 5 different doses of extract, the ethanol, acetate, and methanol fractions of *Amanita muscaria* were quite good and allowed for 100% mortality. In terms of cytotoxicity, all fractions of *Russula foetentula, Russula cerolens, Cantharellus cinerus, Chlorophyllum molybdites, Lactarius deliciosus*, and *Macrolepiota albuminosa* were extremely potent, while *Russula paludosa* and *Auricularia auricula judae* were less potent.

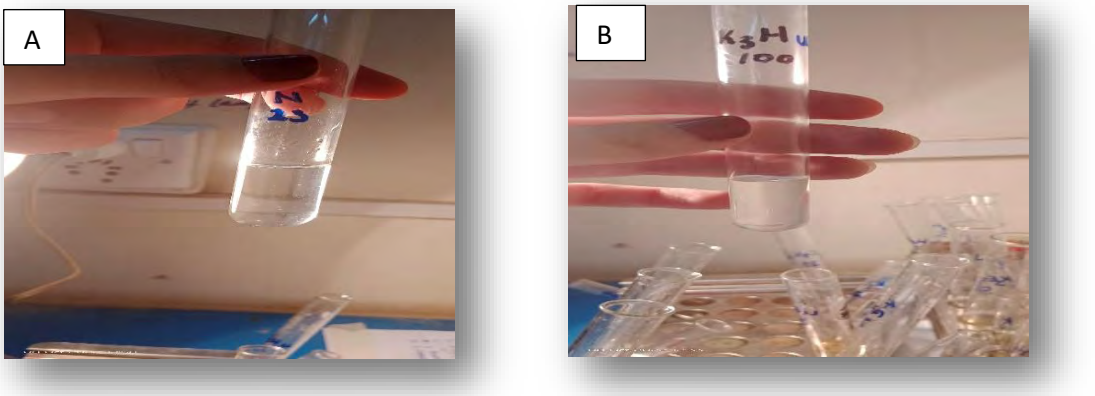

Figure 4.33. Cytotoxic activity of macrofungal crude extract with A = Negative control (DMSO) showing many shrimps after 24 hours and B = hexane fraction of Russula paludosa showing no sign of brine shrimps after 24 hours.

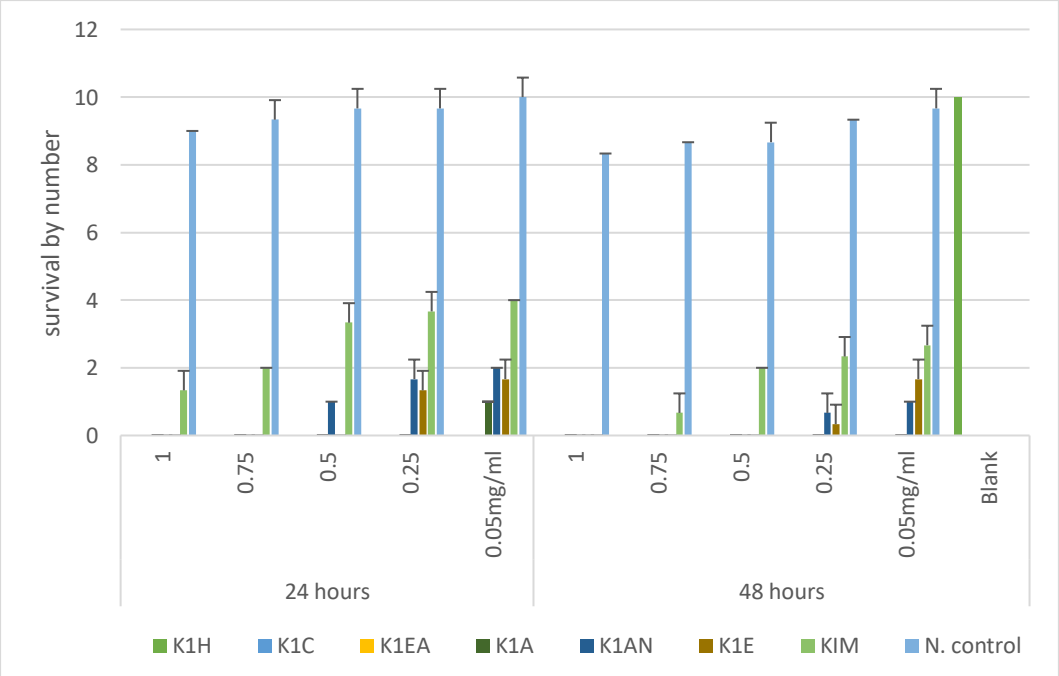

Figure 4.34. Cytotoxicity Assay of different extracts from Russula foetentula.

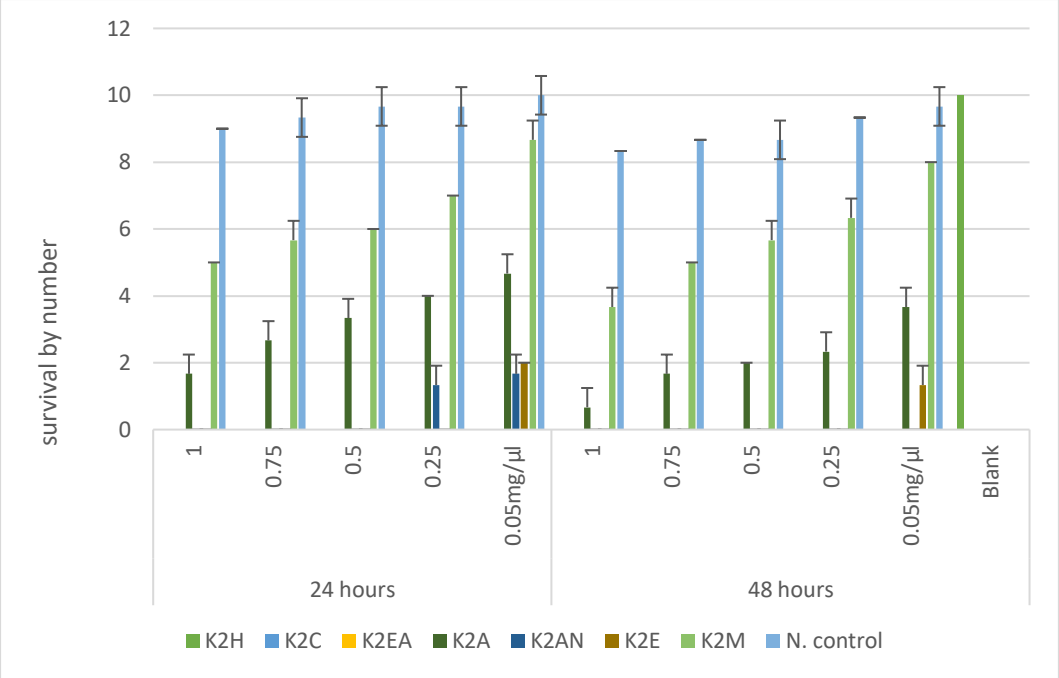

Figure 4.35. Cytotoxicity Assay of different extracts from Russula cerolens.

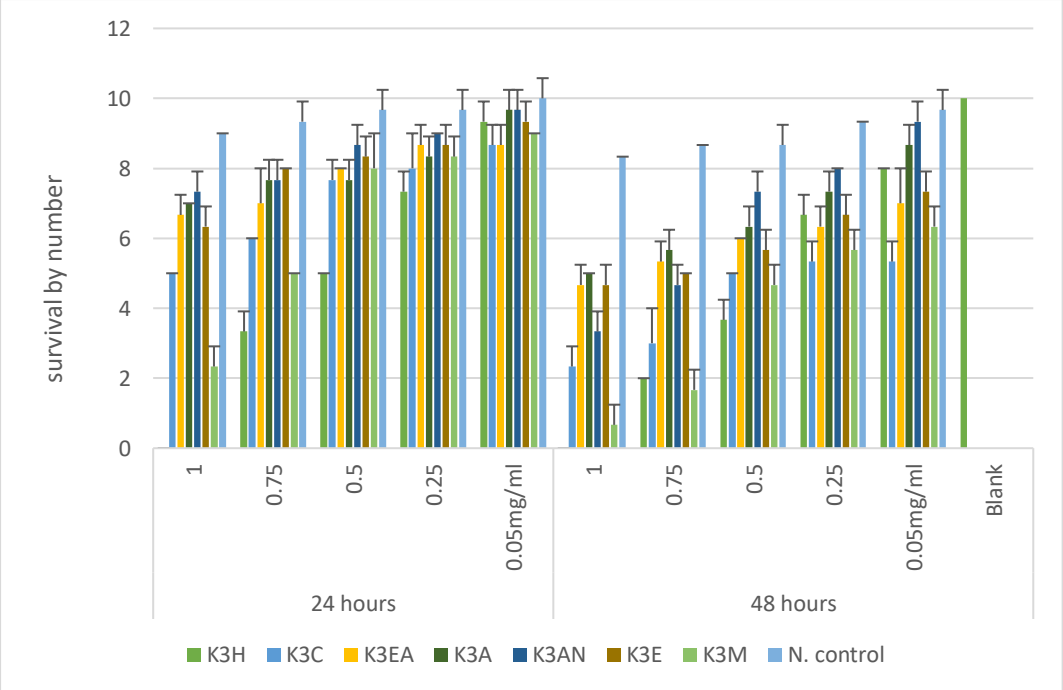

Figure 4.36. Cytotoxicity Assay of different extracts from Russula paludosa.

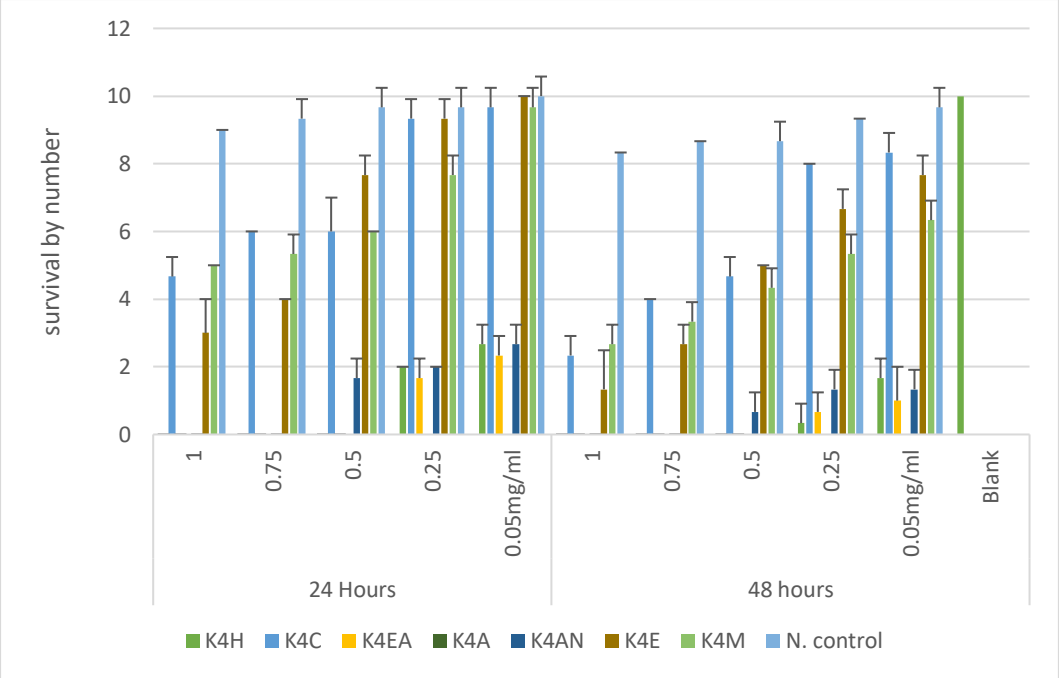

Figure 4.37. Cytotoxicity Assay of different extracts from Laccaria laccata.

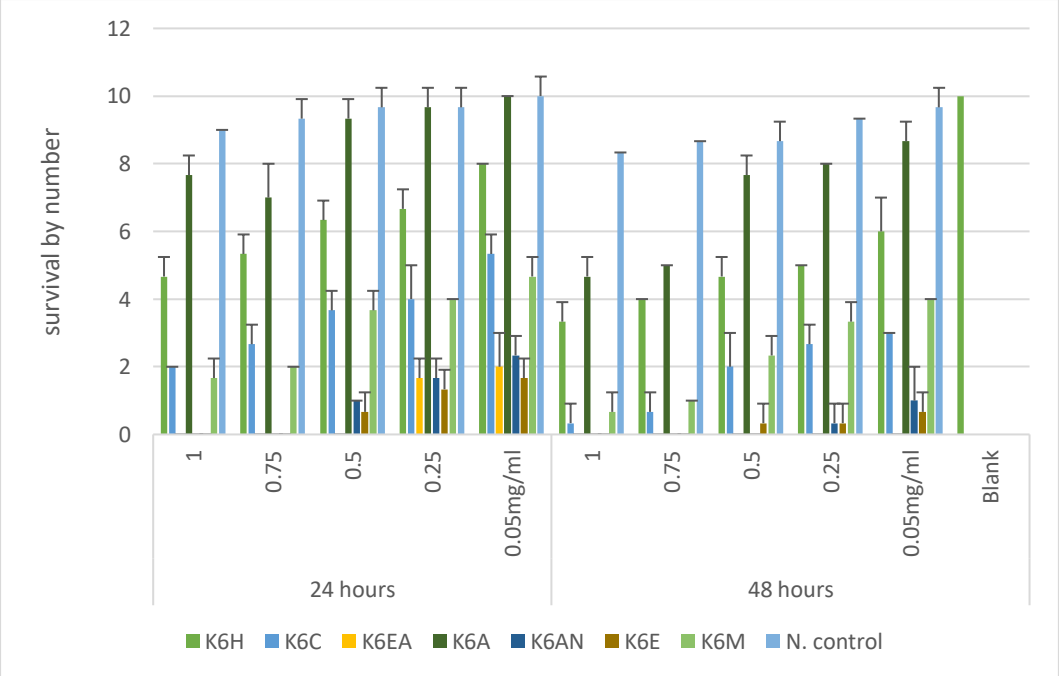

Figure 4.38. Cytotoxicity Assay of different extracts from Amanita muscaria.

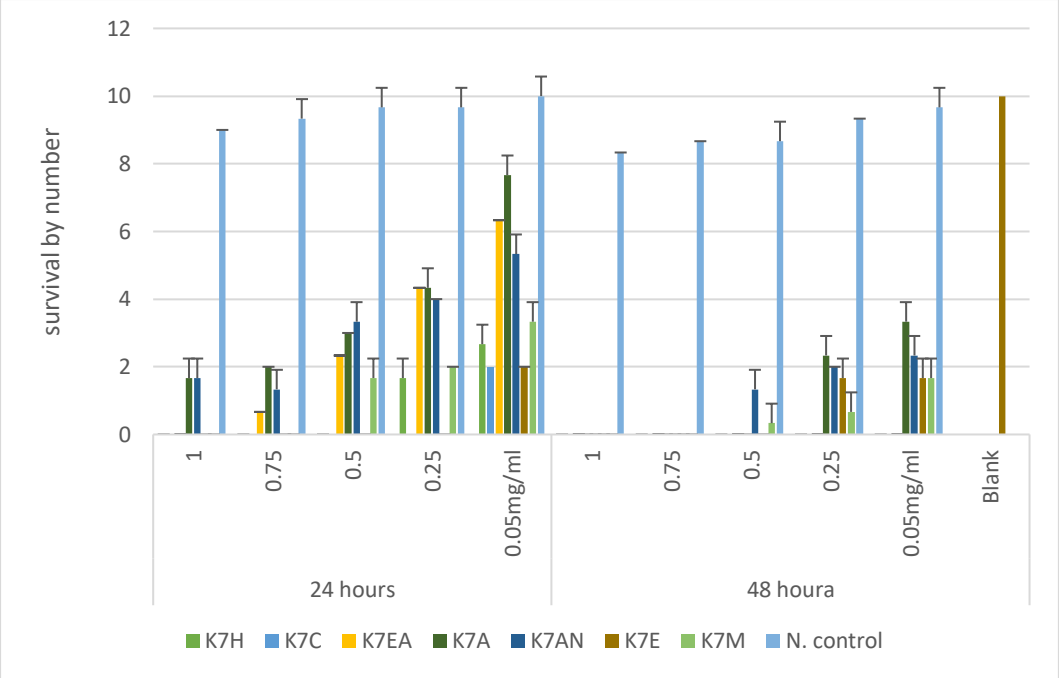

Figure 4.39. Cytotoxicity Assay of different extracts from Cantharellus cinerus.

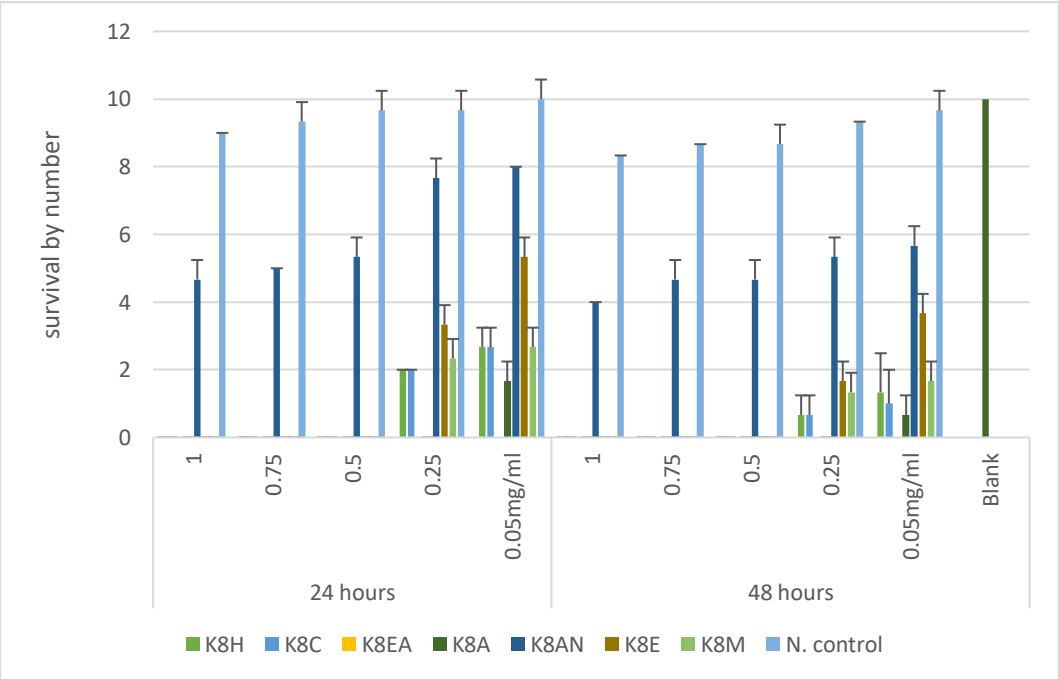

Figure 4.40. Cytotoxicity Assay of different extracts from Chlorophyllum molybdites.

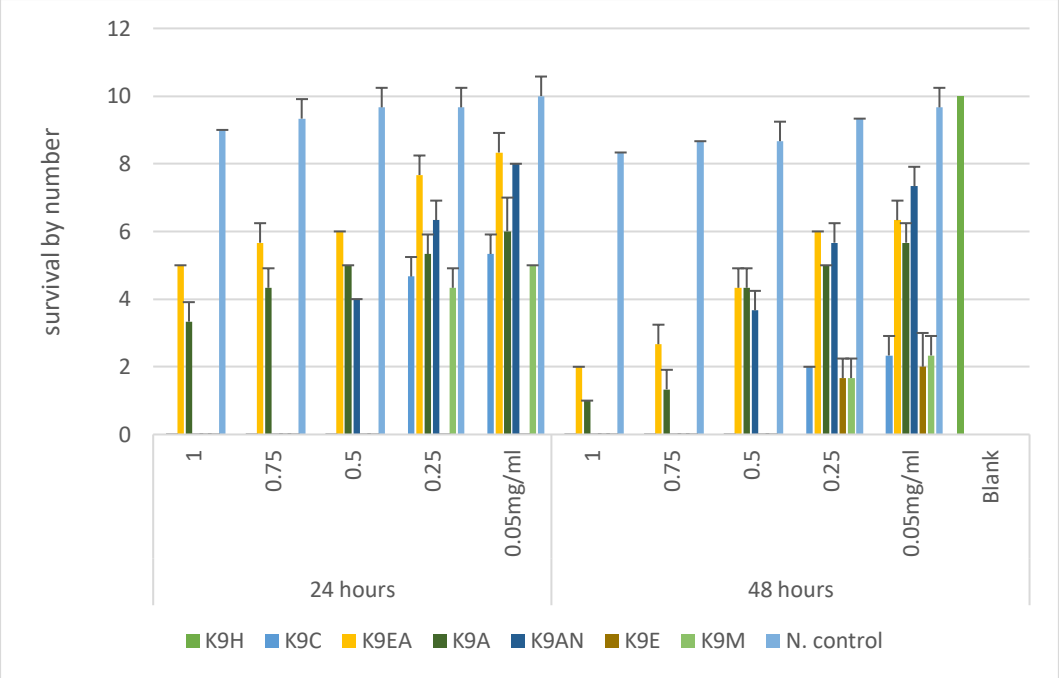

Figure 4.41. Cytotoxicity Assay of different extracts from Russula emetica.

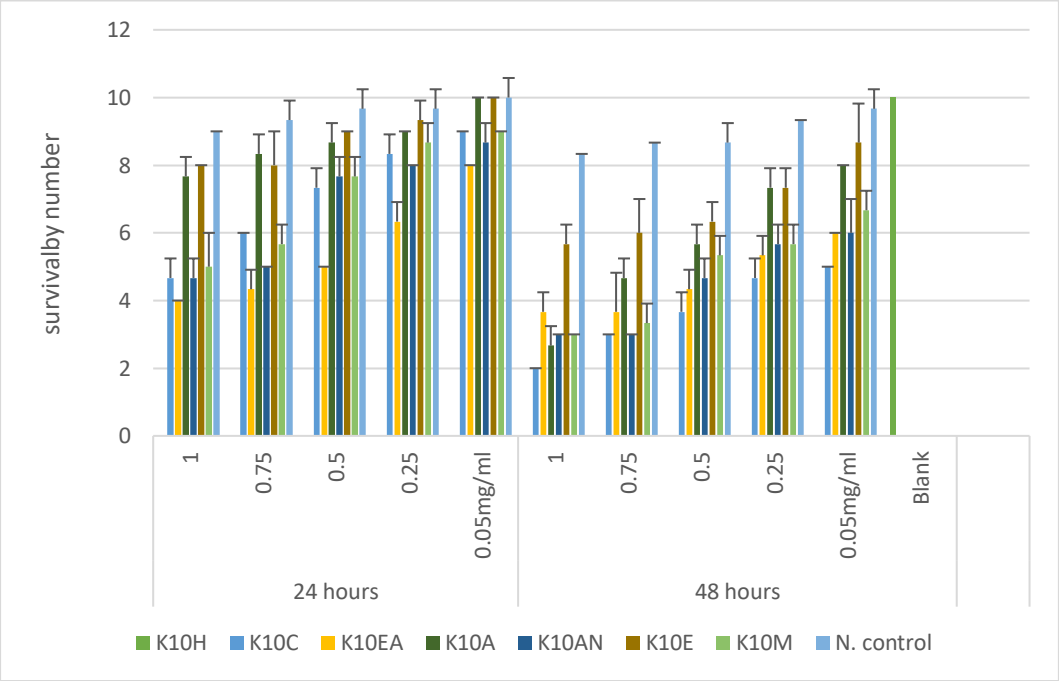

Figure 4.42. Cytotoxicity Assay of different extracts from Auricularia auricula-judae.

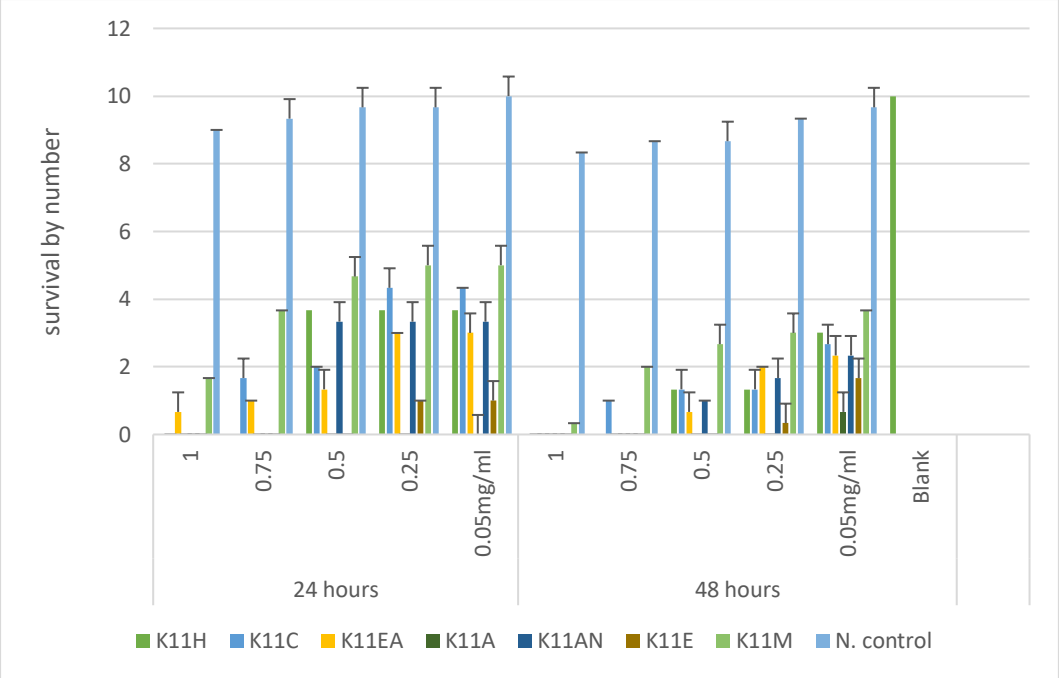

Figure 4.43. Cytotoxicity Assay of different extracts from Auricularia auricula-judae.

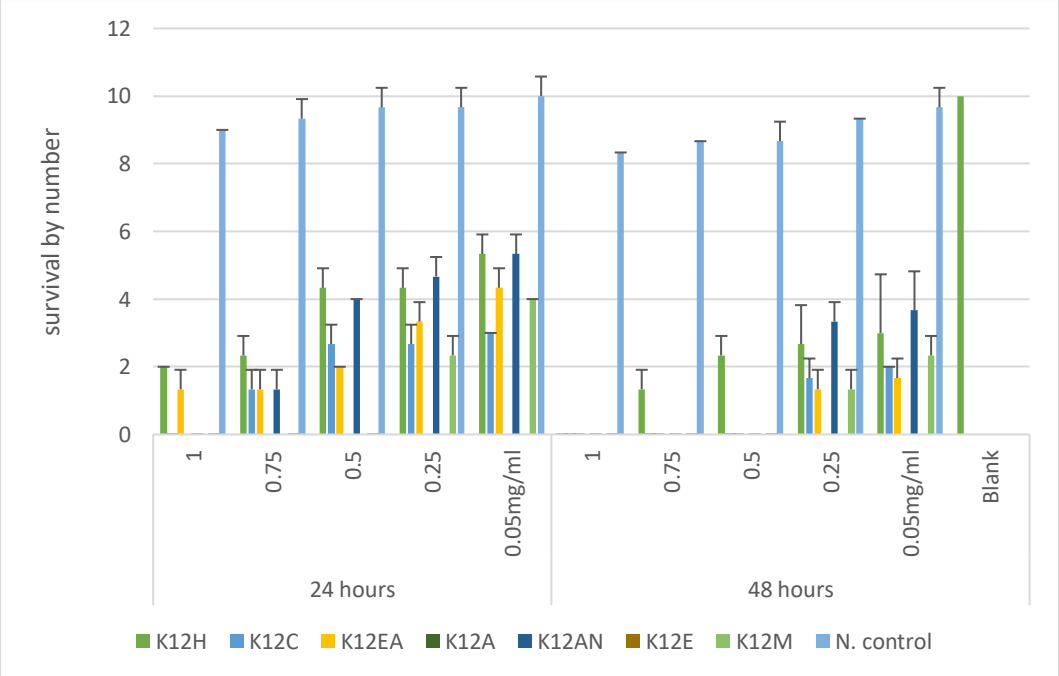

Figure 4.44. Cytotoxicity Assay of different extracts from Macrolepiota albuminosa.

4.6. Hemolytic Activity of Mushroom Extracts

Human erythrocytes were examined for hemolytic activity, with positive control SDS regarded 100% hemolytic and negative control DMSO considered non-hemolytic (). *Lactarius deliciosus* and *Macrolepiota albuminosa* extracts had the least hemolytic activity. *Russula emetica, Auricularia auricula judae, Russula foetentula,* and *Russula paludosa* all had low to moderate hemolytic activity. Overall, extracts of *Russula cerolens, Laccaria laccata, Cantharellus cinerus,* and *Chlorophyllum molybdites* shown high hemolytic activity. Methanol fractions of each extract had lower activity, while acetonitrile and ethyl acetate fractions had higher activity overall. The chloroform fraction of *Russula cerolens* demonstrated the strongest hemolytic activity, with % hemolysis values of 98 ± 1, 90 ± 1.1, 88 ± 1, 88 ± 0.5, and 74 ± 0.5% at 5 different doses of 1.0, 0.8, 0.6, 0.4, and 0.2mg/ml.

Laccaria laccata's acetone and acetonitrile fractions showed excessive hemolysis, but methanolic fractions caused the least hemolysis. The hexane fraction of *Amanita muscaria* caused excessive hemolysis, whereas the chloroform, ethyl acetate, acetone, acetonitrile, ethanol, and methanol fractions did not. *Chlorophyllum molybdites'* acetone fraction and *Cantharellus cinerus* methanol fraction have been shown to be less hemolytic. *Russula emetica* demonstrated moderate hemolytic activity, while *Lactarius deliciosus* and *Macrolepiota albuminosa* demonstrated reduced activity.

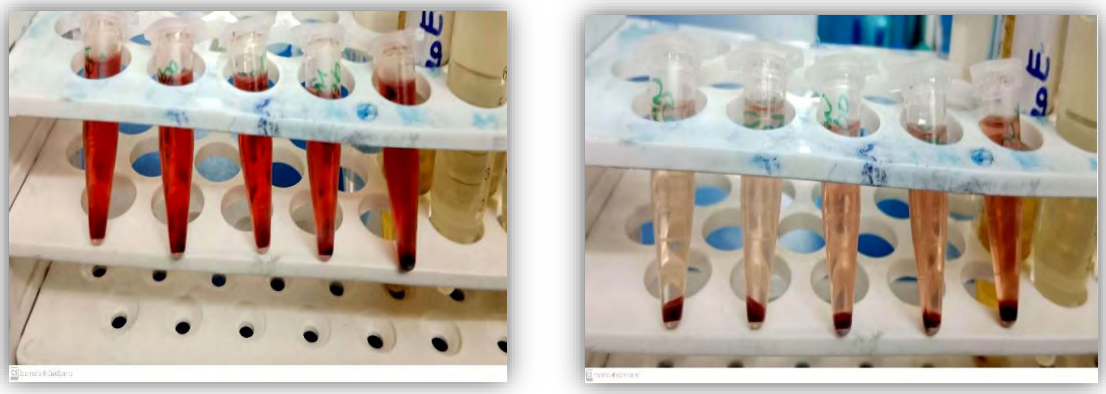

Figure 4.45. positive and negative controls of hemolytic activity.

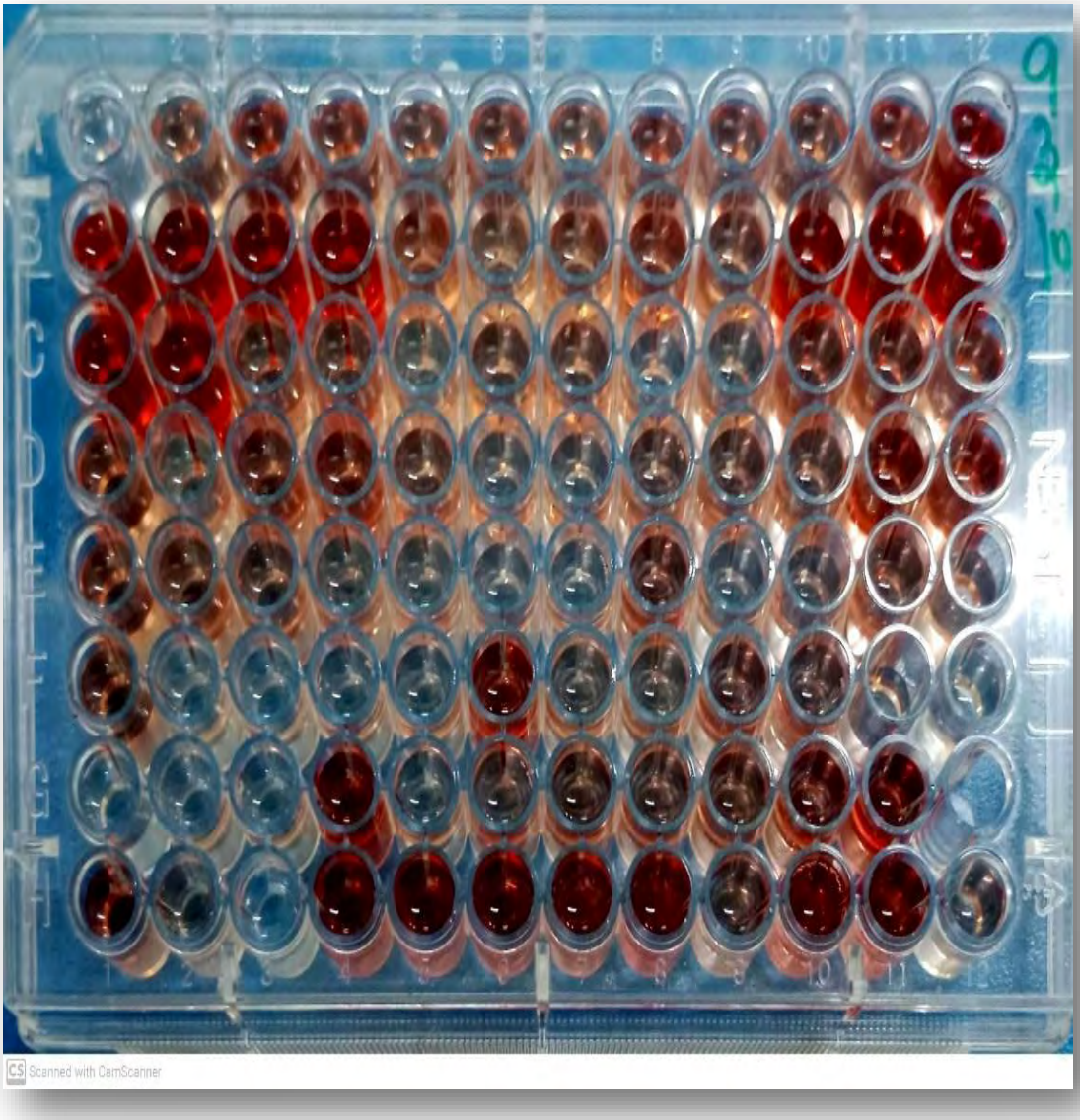

Figure 4.46. Hemolytic activity of crude extracts of mushrooms at different concentrations where dark red color indicates hemolysis while light color indicates no hemolysis.

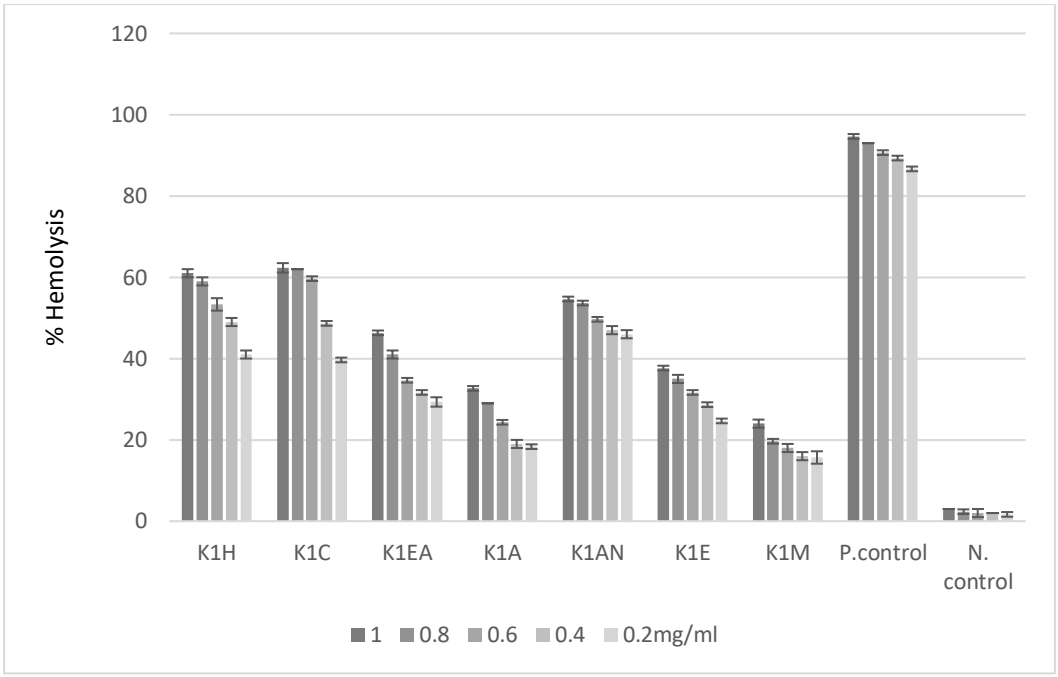

Figure 4.47. The dose-dependent hemolytic activity of crude extracts of Russula foetentula

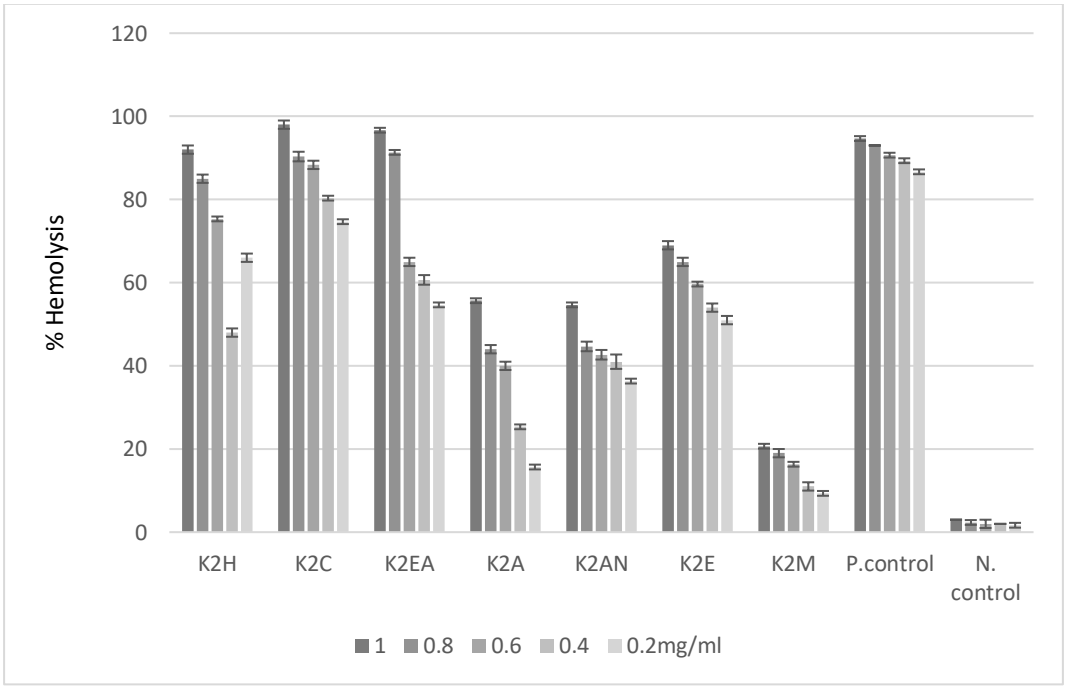

Figure 4.48. The dose-dependent hemolytic activity of crude extracts of Russula cerolens.

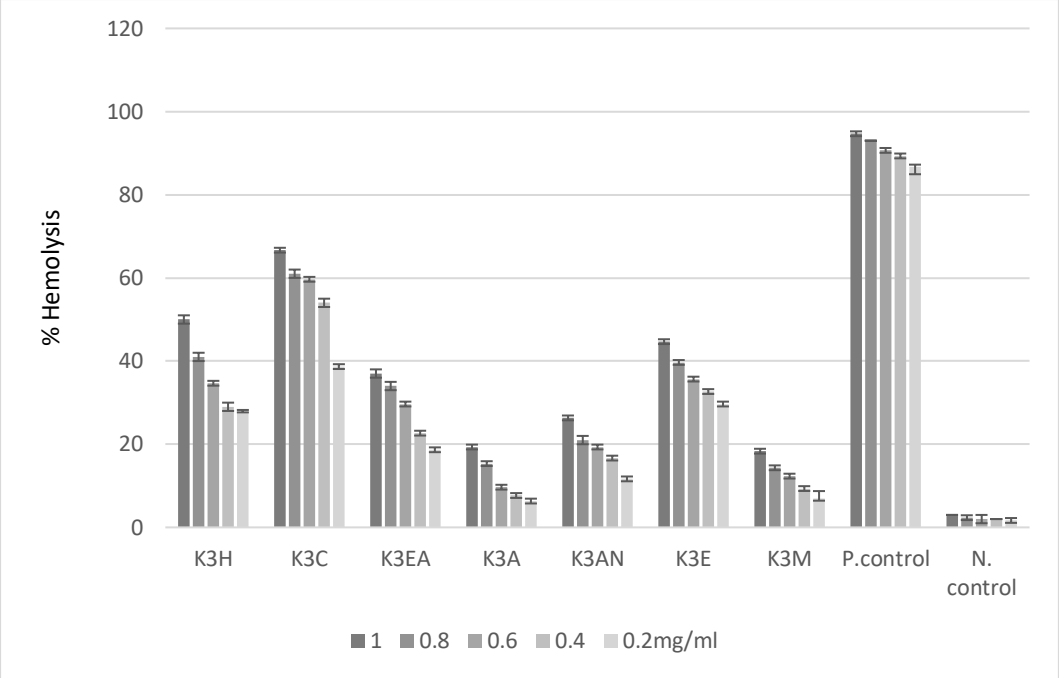

FigurE 4.49. The dose-dependent hemolytic activity of crude extracts of Russula paludosa.

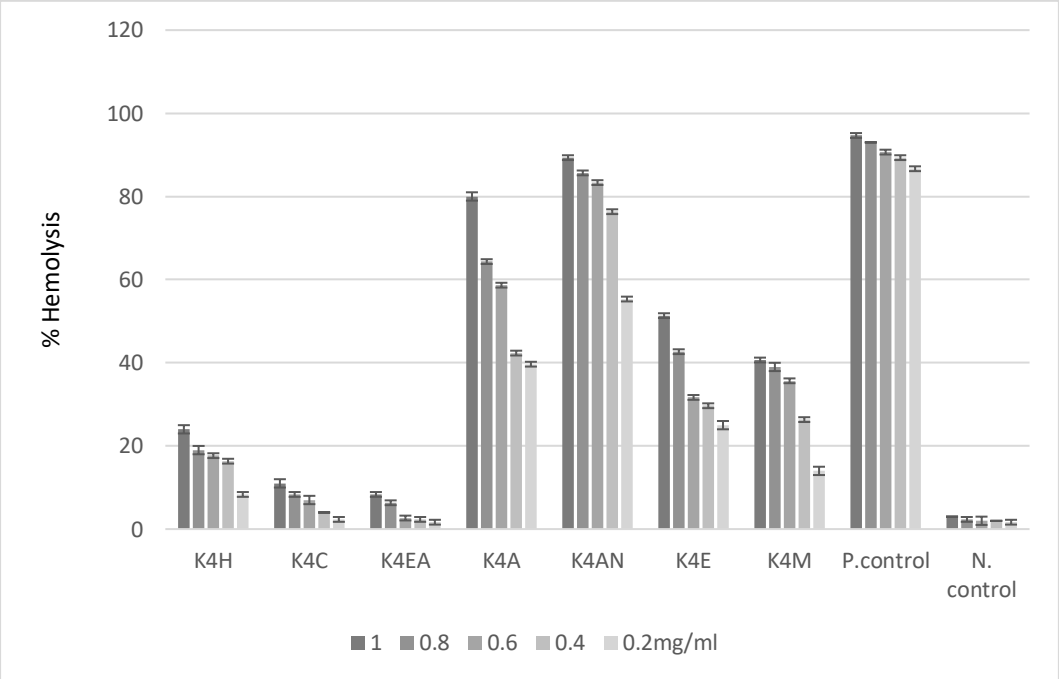

Figure 4.50. The dose-dependent hemolytic activity of crude extracts of Laccaria laccata.

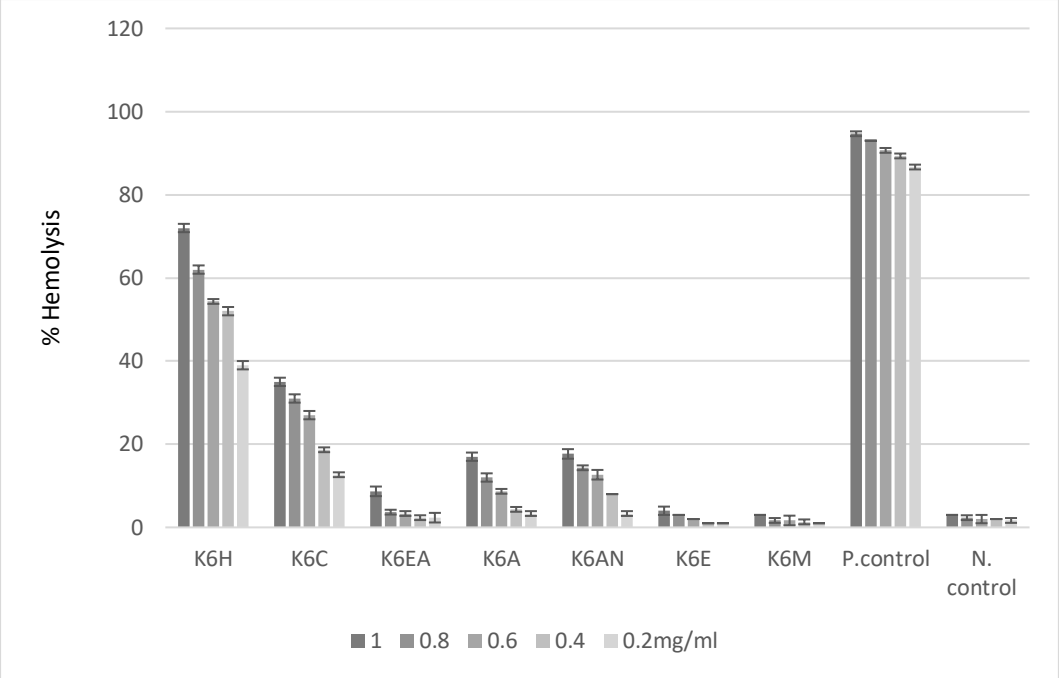

Figure 4.51. The dose-dependent hemolytic activity of crude extracts of Amanita muscaria.

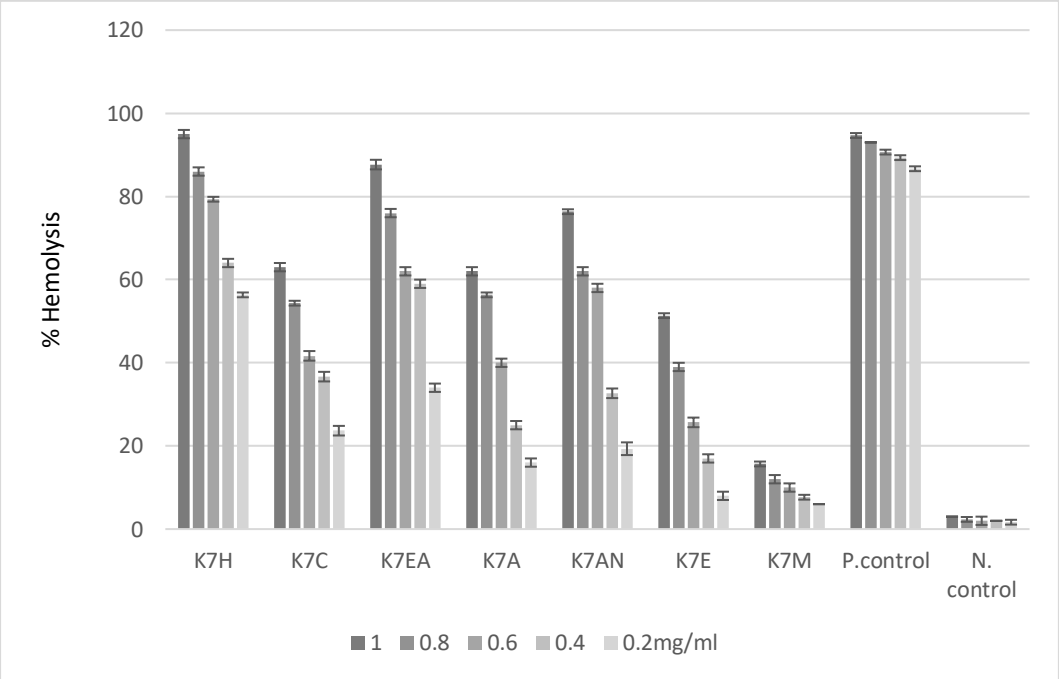

Figure 4.52. The dose-dependent hemolytic activity of crude extracts of Cantharellus cinerus.

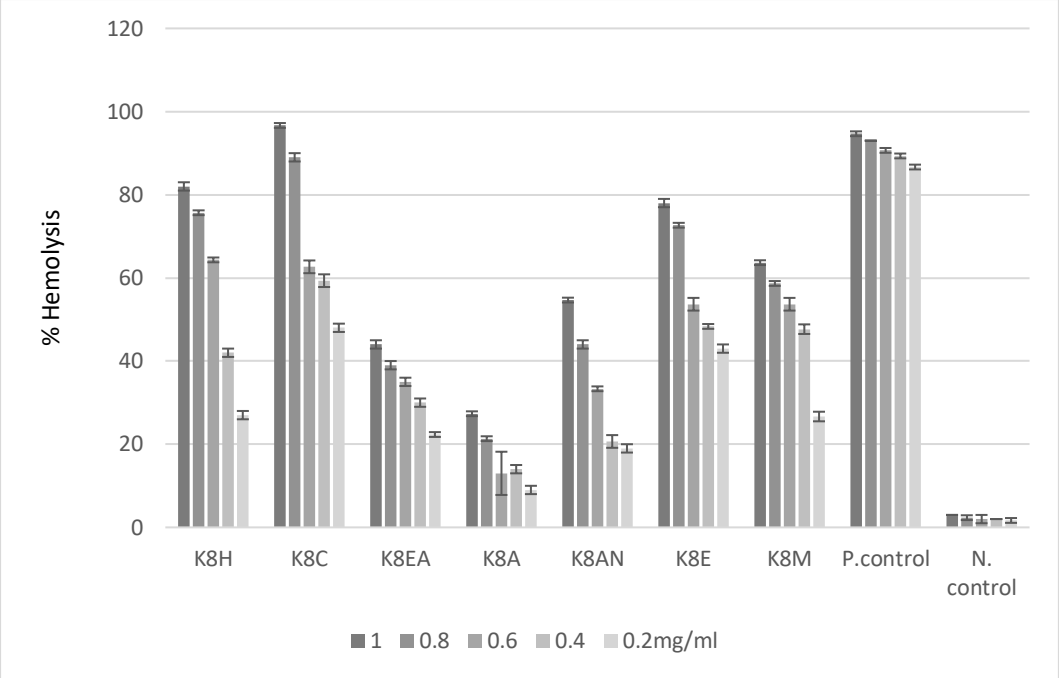

Figure 4.53. The dose-dependent hemolytic activity of crude extracts of Chlorophyllum molybdites.

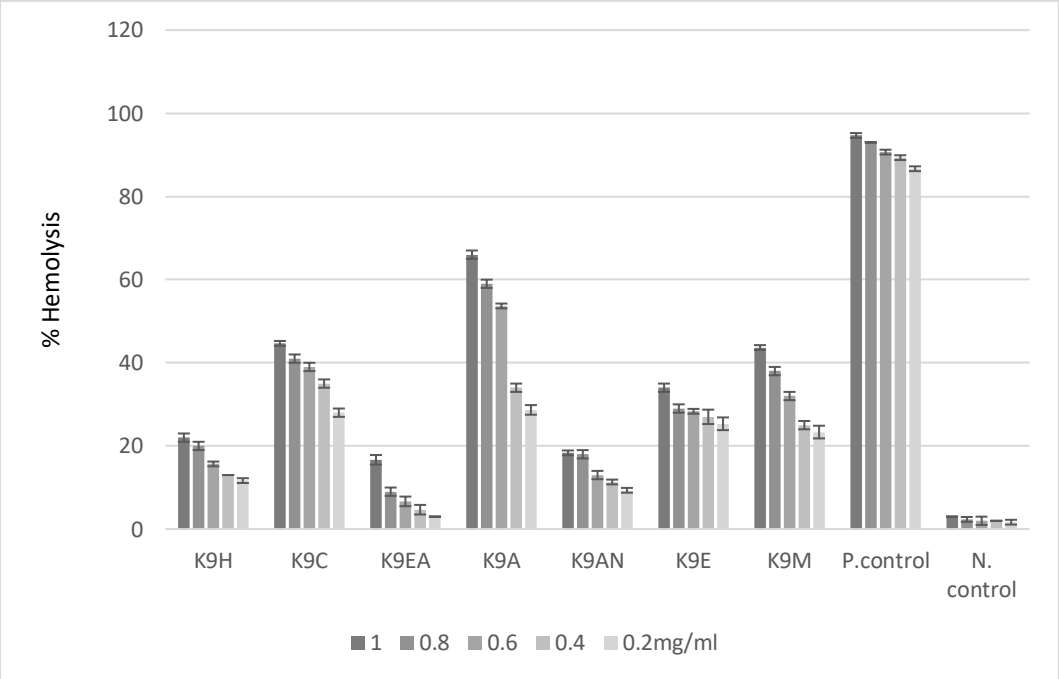

Figure 4.54. The dose-dependent hemolytic activity of crude extracts of Russula emetica.

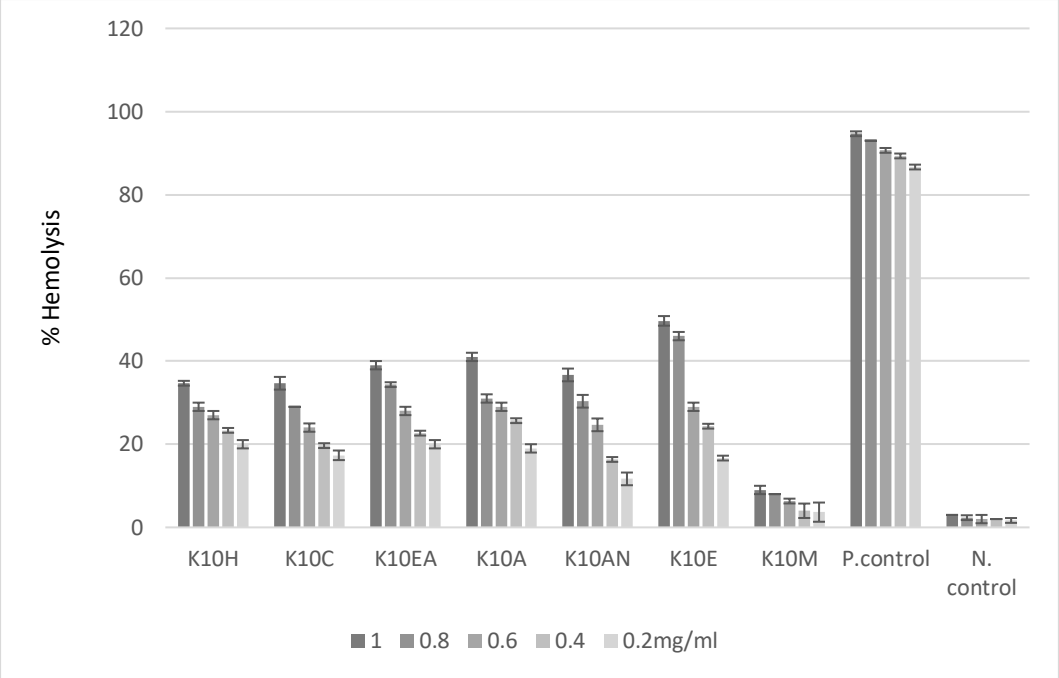

Figure 4.55. The dose-dependent hemolytic activity of crude extracts of Auricularia auricula-judae.

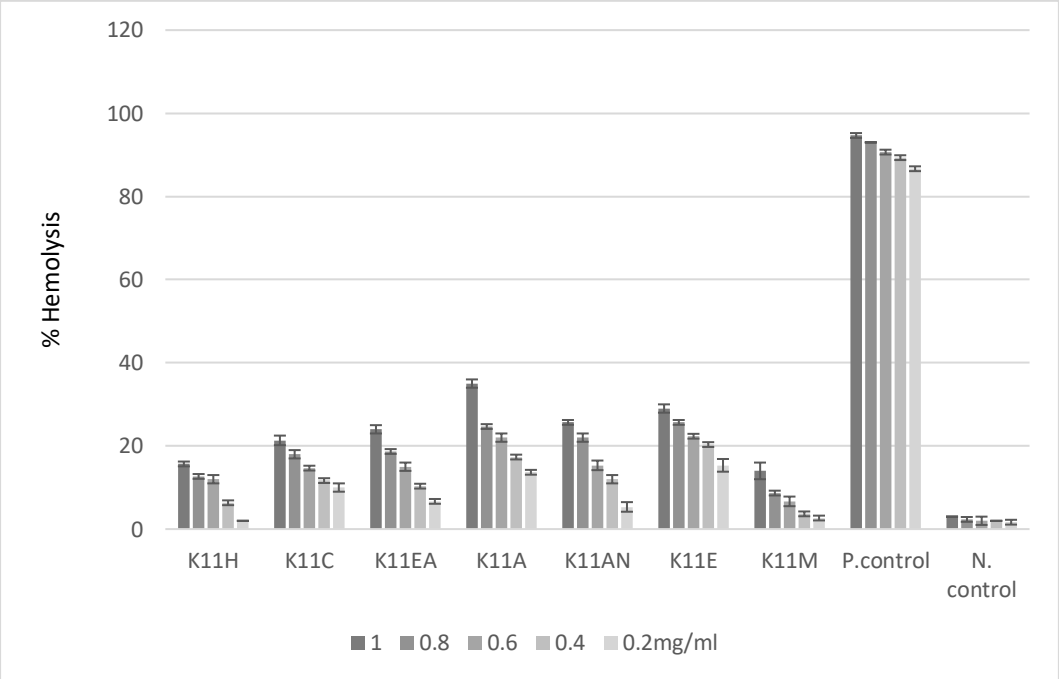

Figure 4.56. The dose-dependent hemolytic activity of crude extracts of Lactarius deliciosus.

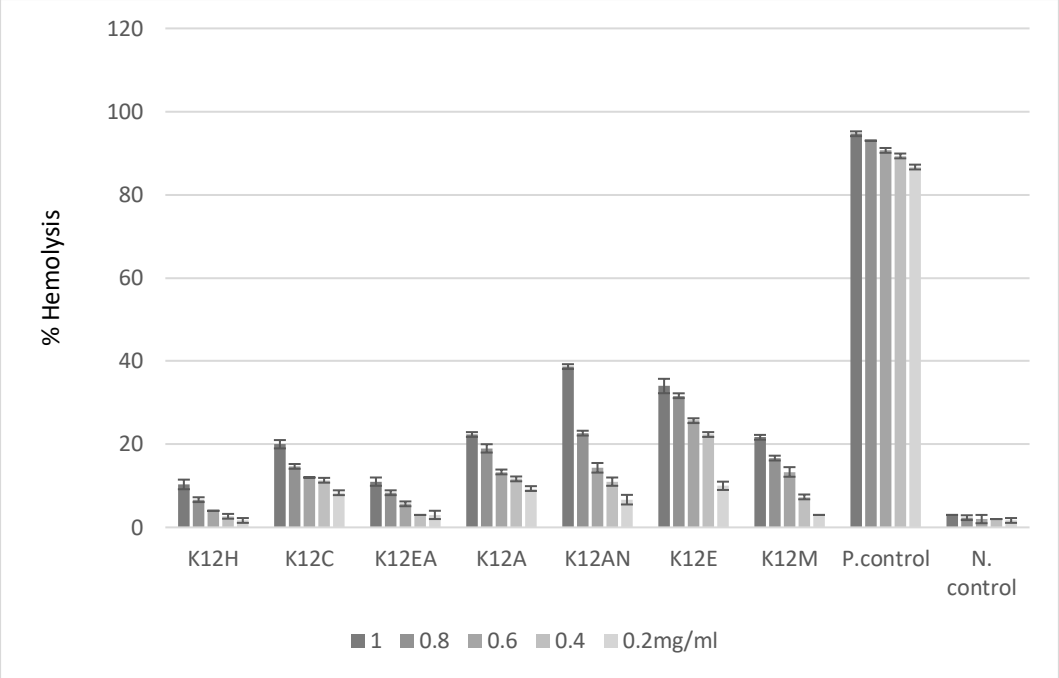

Figure 4.57. The dose-dependent hemolytic activity of crude extracts of Macrolepiota albuminosa.

4.7. Mycochemical Analysis

4.7.1. Saponins, tannins and steroid test

The medical benefit of any source is determined by its constituents, either separately or in combinations. Some of the significant mycochemicals with varied biological actions are flavonoids, phenolics, saponins, tannins, steroids, and terpenes. The discovery of mycochemicals may foresee a plant's pharmacological efficacy. Mycochemicals are now determined using a variety of contemporary techniques, while traditional qualitative assays are still used for early phytochemical screening of plants. In this context, a saponin test, also known as a foam test, is carried out, in which the production of foam following shaking reveals the presence of chemicals. The majority of the extracts dissolved in polar solvents such as ethanol and methanol tested positive for saponins. Saponin tests were positive for ethanol and methanol fractions of *Laccaria laccata, Amanita muscaria, Lactarius deliciosus*, and *Macrolepiota albuminosa*. While some extracts dissolved in polar solvents, such as *Russula cerolens, Amanita phalloids,* and *Cantharellus cinerus*, performed poorly in the foam test. Only the non-polar component of *Russula foetentula* tested positive for saponin. Tannins testing is also done

to determine the presence of tannins in the sample. Tannins have polar functional groups that allow them to dissolve in polar liquids. Because no black colour is visible after performing the FeCl3 test, all fractions *of Russula cerolens*, *Cantharelus cinerus, Chlorophyllum molybdites*, and *Lactarius deliciosus* were considered poor in terms of tannins content. In ethanol and methanol fractions of all other extracts, their presence was detected. Tannins were found in *Russula foetentula* hexane and chloroform fractions. Tannins were not found in the sample's acetone or acetonitrile fractions. The presence of steroids was detected in both polar and non-polar extracts using a steroid test. All fractions of *Russula foetentula*, polar fractions of *Russula paludosa*, and non-polar fractions of *Chlorophyllum molybdites* tested positive for steroids, as evidenced by the production of dark brown colour. *Russula cerolens, Cantharellus cinerus,* and *Lactarius deliciosus* did not show any signs of steroid content. The table below gives the data of saponins, tannins and steroid test result of all mushroom extracts.

Extracts	**Saponins**	**Tannins**	**Steroids**
K1H	+	-	+
K1C	+	-	+
K1EA	-	-	+
K1A	-	-	+
K1AN	+	-	+
K1E	-	+	+
K1M	+	+	+
K2H	-	-	+
K2C	-	-	-
K2EA	-	-	-
K2A	+	-	-
K2AN	+	-	-
K2E	-	-	-
K2M	-	+	-
K3H	-	+	+

K3C	-	-	-
K3EA	-	-	+
K3A	+	-	-
K3AN	+	+	-
K3E	-	+	+
K3M	+	+	+
K4H	-	-	+
K4C	-	-	+
K4EA	+	+	+
K4A	-	-	-
K4AN	-	-	-
K4E	+	+	+
K4M	+	+	-
K6H	-	-	-
K6C	-	-	+
K6EA	-	-	-
K6A	-	-	-
K6AN	+	-	-
K6E	+	+	+
K6M	+	+	-
K7H	+	-	-
K7C	-	-	-
K7EA	-	-	-
K7A	-	-	-
K7AN	+	-	-
K7E	-	-	-
K7M	-	-	-
K8H	-	-	+
K8C	-	-	+

K8EA	-	-	-
K8A	-	-	-
K8AN	-	-	+
K8E	+	+	-
K8M	-	-	-
K9H	-	-	-
K9C	-	-	-
K9EA	-	-	-
K9A	-	-	+
K9AN	+	-	-
K9E	+	+	-
K9M	+	+	-
K10H	-	-	-
K10C	-	-	+
K10EA	-	-	-
K10A	+	-	-
K10AN	+	-	-
K10E	+	+	-
K10M	-	+	-
K11H	+	-	-
K11C	-	-	-
K11EA	-	-	-
K11A	+	-	-
K11AN	+	-	-
K11E	+	-	-
K11M	+	-	-
K12H	-	-	-
K12C	-	-	-
K12EA	+	-	-
K12A	+	-	-

K12AN	+	-	-
K12E	+	+	+
K12M	+	+	-

Table 4.2. Results of saponin, tannins and steroids tests

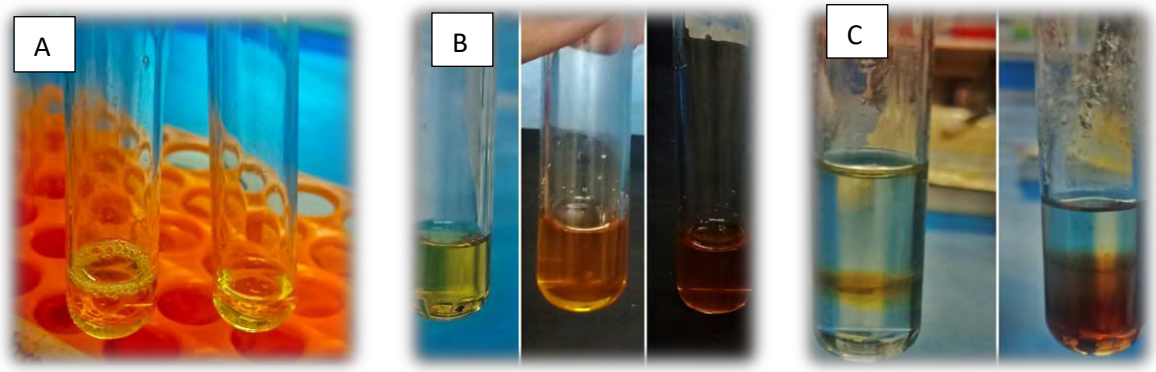

Figure 4.58. In A Saponins test indicates positive and negative test result. In B Tannins test result of extract which indicates thepresence of less to high tannins from left to right. In C Steroid test which indicates the absence to presence of steroids from left to right.

4.7.2. Phenolic and flavonoid content test

Aluminium chloride calorimetric assay was used to determine the total flavonoid content and total phenolic content was determined by using Fc reagent. The absorbance values of all samples were compared with the standard curve or control, which is quercetin in case of flavonoids and gallic acid in case of phenols. The total phenolic content of acetonitrile fraction of *Russula foetentula* is 100mg GAE/g and hexane and chloroform fractions of *Russula paludosa* is 120mg GAE/g and 130mg GAE/g respectively which is quite high. In the case of *Russula cerolens*, phenolic content was comparatively low. In *Laccaria laccata*, only hexane fraction was potent and in *Amanita muscaria* chloroform and acetone fractions were potent to give values in the range of 120mg GAE/g to 150mg GAE/g. *Cantharellus cinerus* and *Chlorophyllum molybdites* gave the values in the ranges of 70mg QE/g to 100mg GAE/g. All non-polar fractions of *Russula emetica* proved to be rich in phenolic content as value of chloroform fraction is 120mg GAE/g. Similarly, hexane fraction of *Macrolepiota albuminosa*

gave the value of 120mg GAE/g. The total flavonoid content of acetonitrile and ethanol fractions of *Russula foetentula* was quite high and its values were 125 and 130mg QE/g. Hexane and methanol fraction of *Russula cerolens* exhibited presence of flavonoids with the values 170 and 173mg QE/g. Similarly chloroform fractions of *Laccaria laccata* and *Amanita muscaria* exhibited too much presence of phenols with the values of 192mg QE/g and 163mg QE/g.

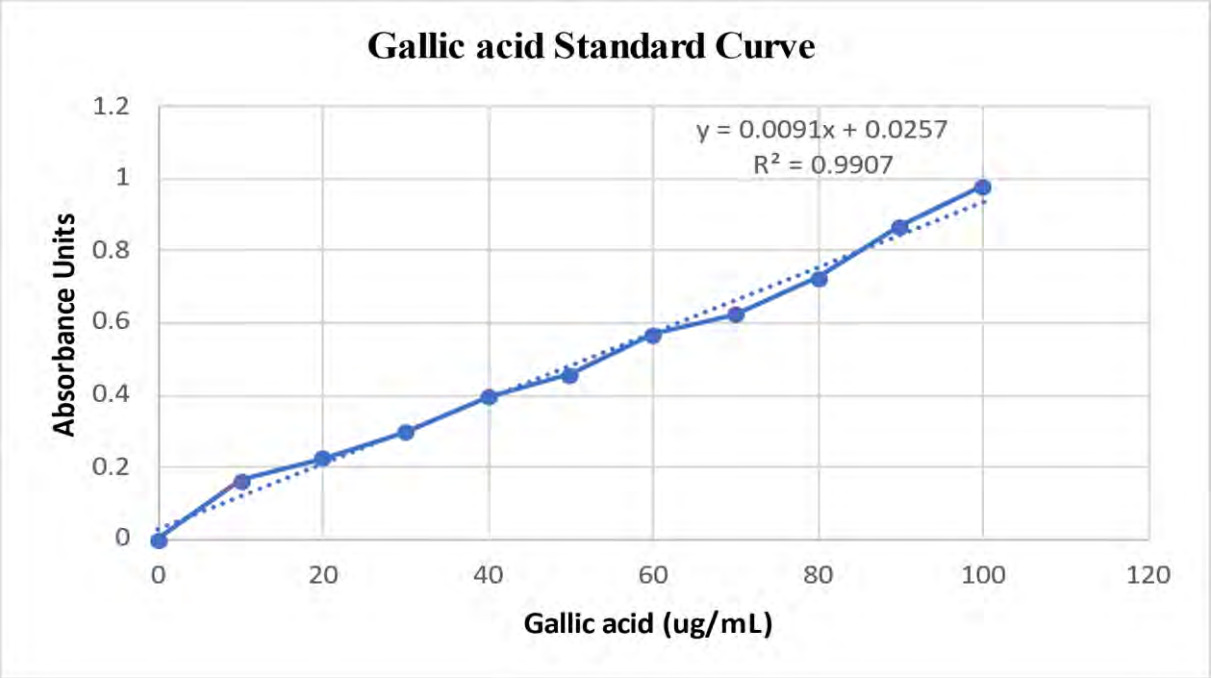

Figure 4.59. Standard curve of Gallic acid

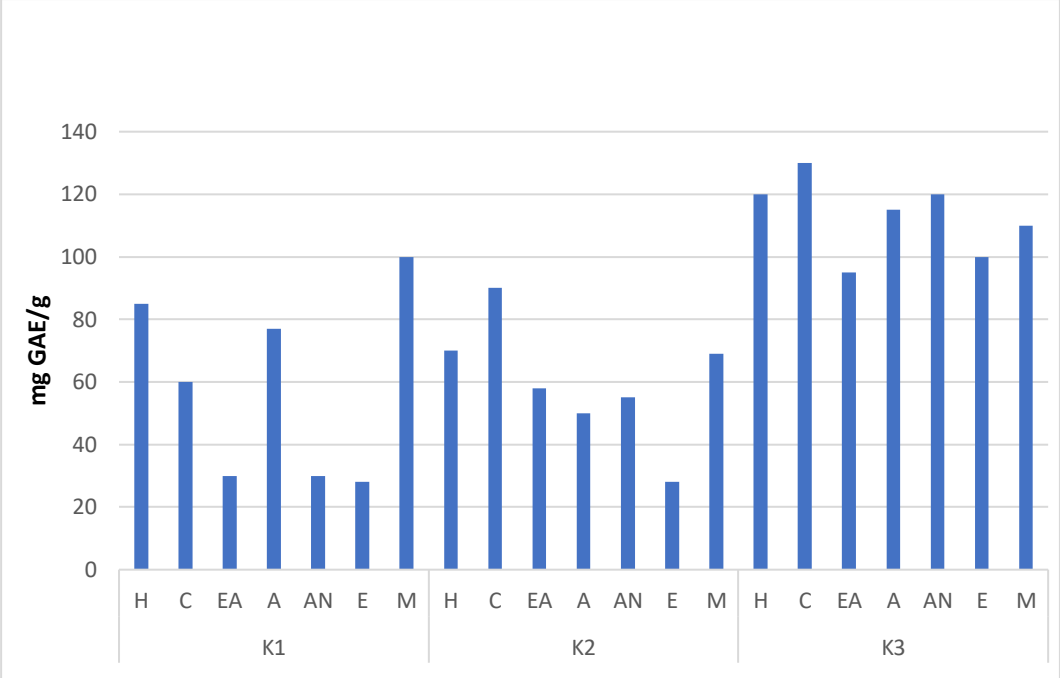

Figure 4.60. Indicating phenolic content of all extracts of Russula foetentula, Russula cerolens and Russula paludosa.

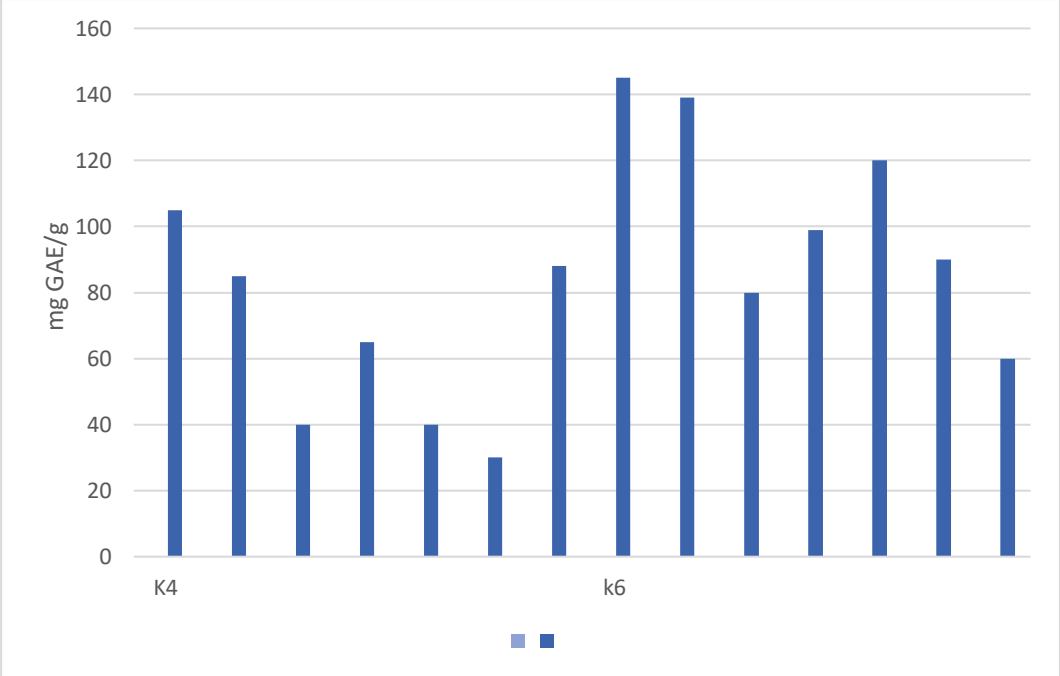

Figure 4.61. Indicating phenolic content of all extracts of Laccaria laccata and Amanita muscaria.

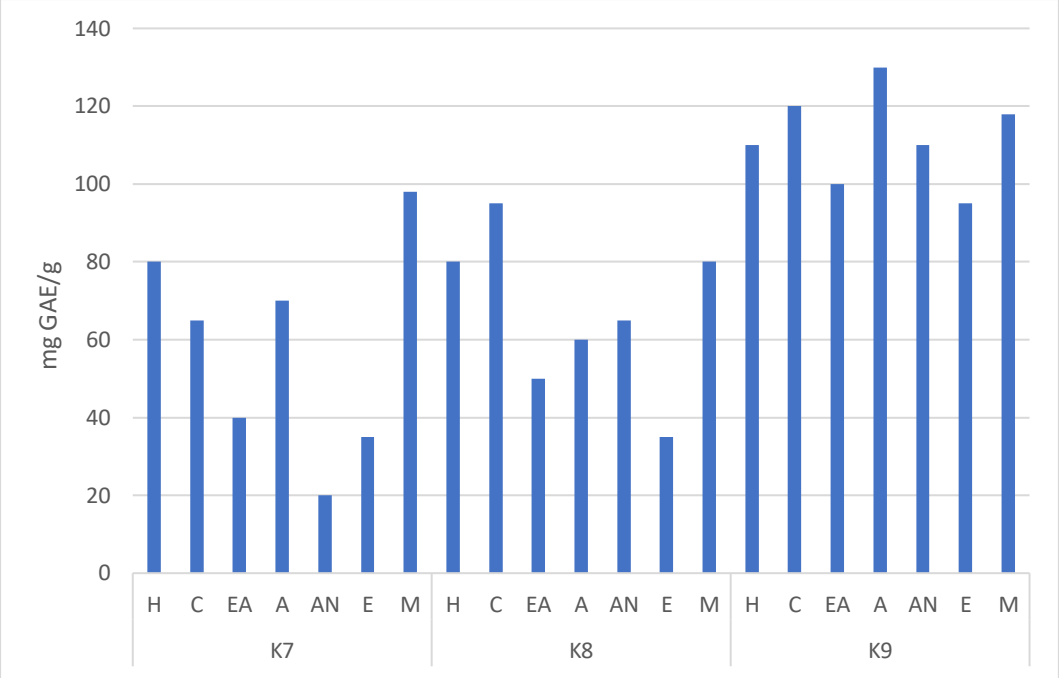

Figure 4.62. Indicating phenolic content of all extracts of Cantharellus cinerus, Chlorophyllum molybdites, and Russula emetica.

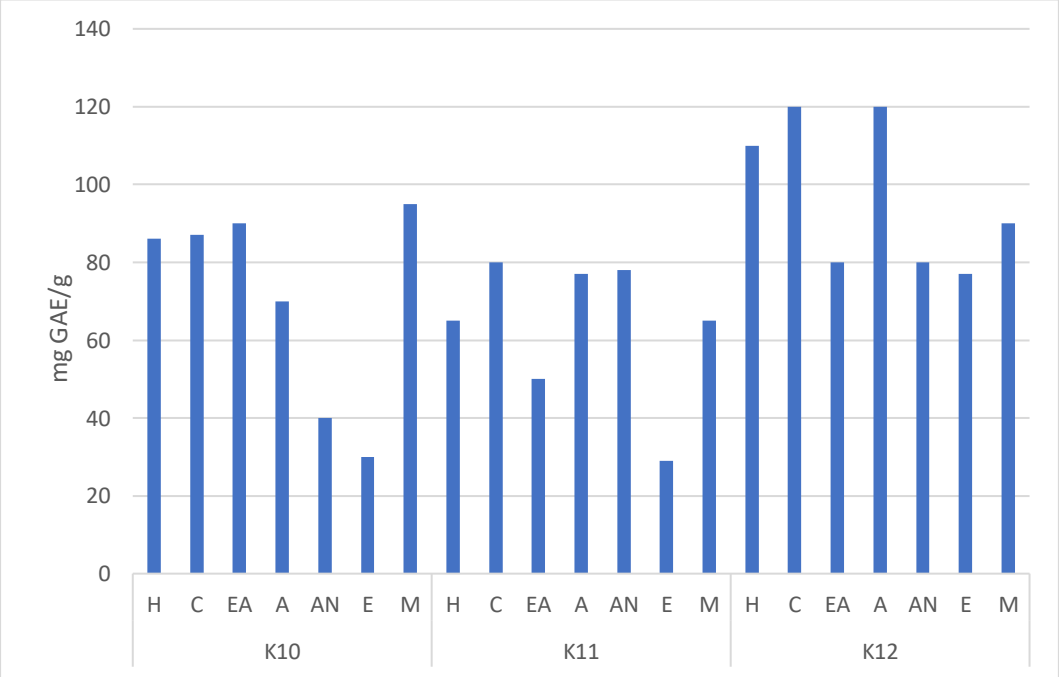

Figure 4.63. Indicating phenolic content of all extracts of Auricularia auricula-judae, Lactarius deliciosus and Macrolepiota albuminosa.

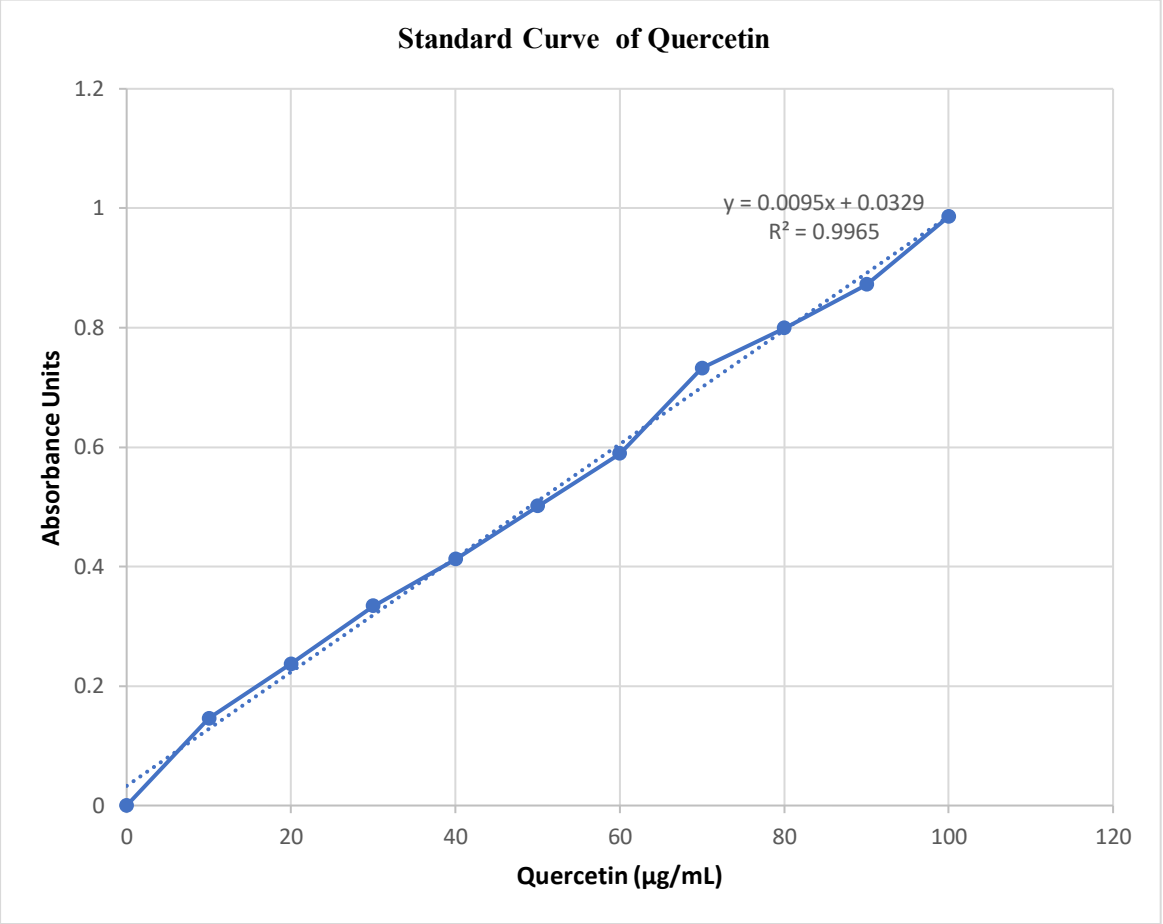

Figure 4.64. Standard curve of quercetin

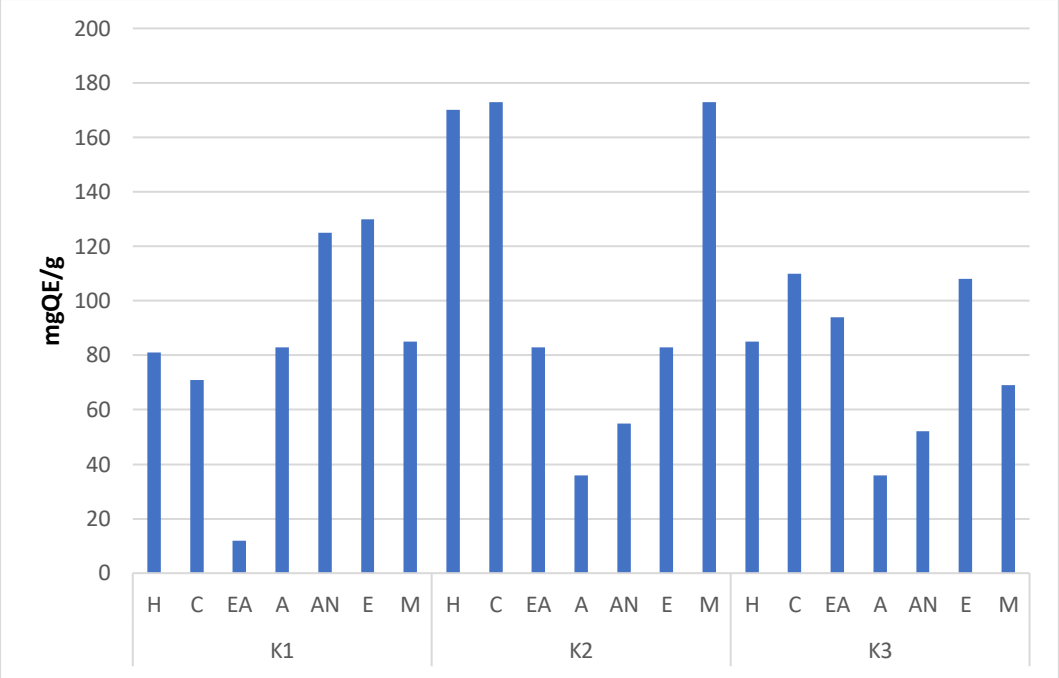

Figure 4.65. Indicating flavonoid content of all extracts of Russula foetentula, Russula cerolens and Russula paludosa.

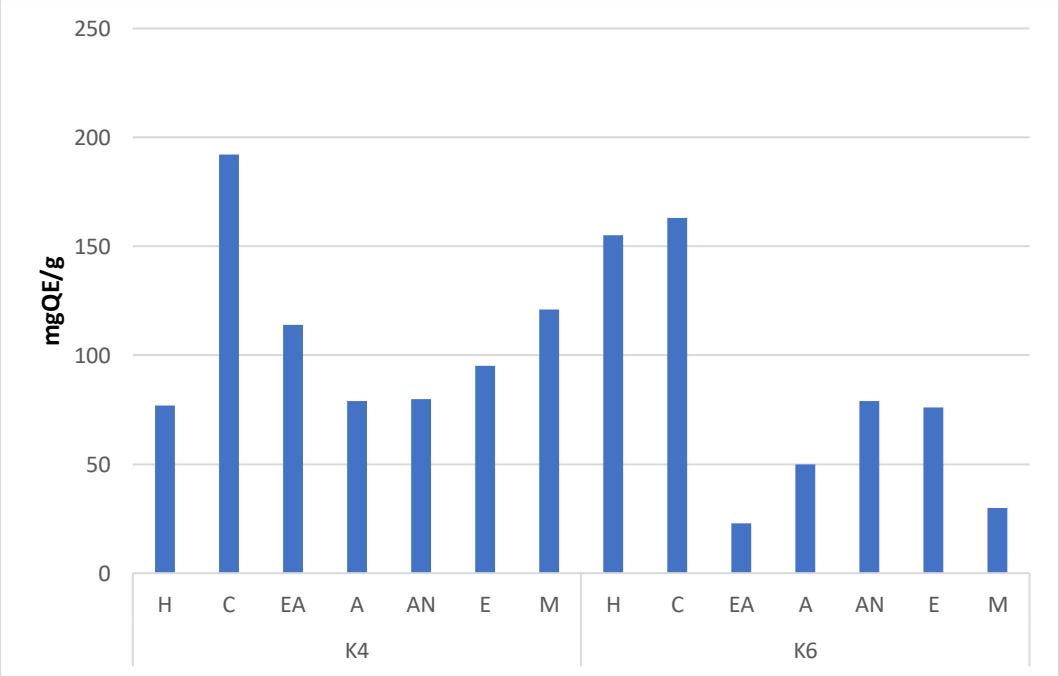

Figure 4.66. Indicating flavonoid content of all extracts of Laccaria laccata and Amanita muscaria.

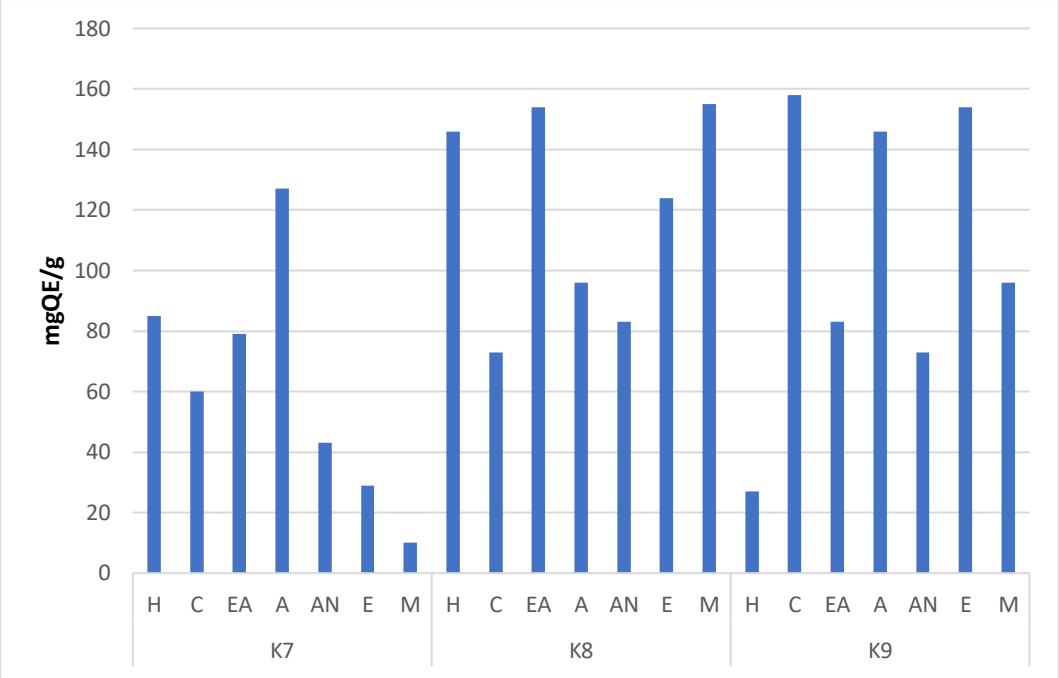

Figure 4.67. Indicating flavonoid content of all extracts of Cantharellus cinerus, Chlorophyllum molybdites, and Russula emetica.

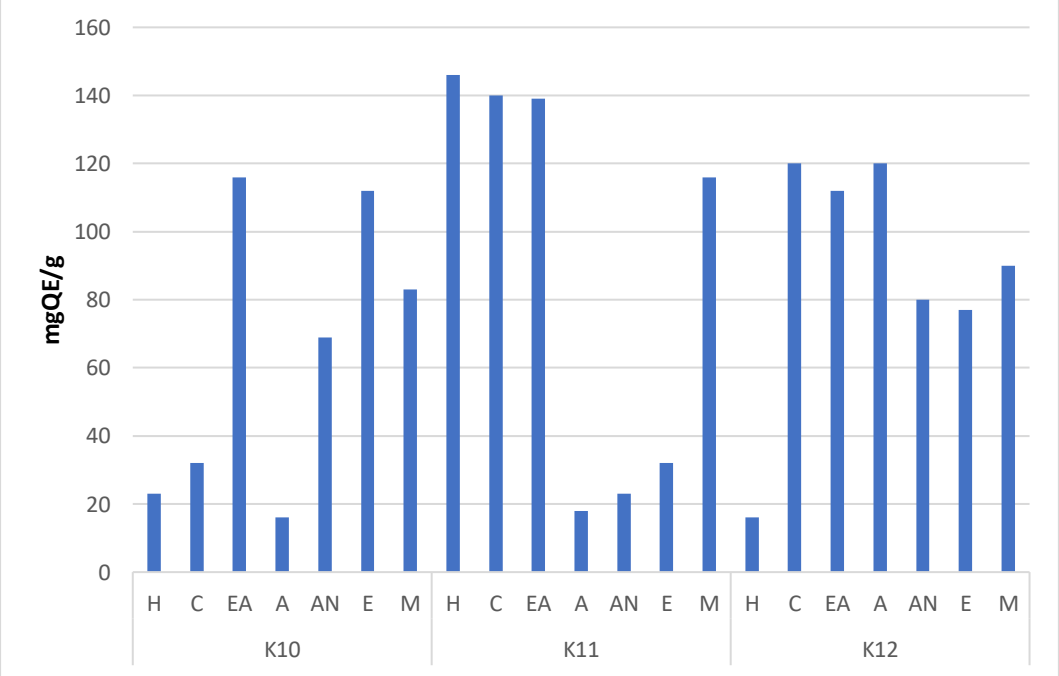

Figure 4.68. Indicating flavonoid content of all extracts of Auricularia auricula-judae, Lactarius deliciosus and Macrolepiota albuminosa

5. DISCUSSION

Antimicrobial resistance is giving rise to superbugs, making simple infections harder to cure and surgery dangerous. Risk evaluations undertaken by WHO have revealed that the South Asian Region is most likely the most vulnerable portion of the planet. AMR not only affects the physical and mental wellness of people in the region, but it also has implications for public health and well-being in general. Pakistan is the third-highest consumer of antibiotics among nations with middle to low incomes. During 2000 and 2015, antibiotics utilization increased by 65%, reaching an average daily consumption of roughly 20 DDDs per 1000 people (Klein et al., 2018). In the research of 71 countries, this put Pakistan in the mid-table ranking thirty-fourth with regard to of antibiotic usage. Antimicrobial resistance has emerged as an international health issue that could result in 10 million deaths per year by 2050. To address the issue of antimicrobial resistance, it is critical to look for alternative sources of bioactive compounds with novel characteristics. Several research have been undertaken in recent years to investigate the medicinal properties of macrofungi.

The current study entails a preliminary screening of eleven wild mushrooms collected from various regions of Azad Jammu and Kashmir, Pakistan. The acetonitrile fractions of *Russula foetentula* and *Laccaria laccata* and the ethyl acetate fractions of *Auricularia auricula judae* and *Macrolepiota albuminosa* demonstrated the best activity against all bacterial strains, including *Bacillus subtilis, P. aeruginosa, E. coli, S. aureus* and *Klebsiella pneumoniae*. Several bioactive chemicals have been discovered in *Russula* species in earlier investigations. Hot water extracts of *Russula veca* showed antibacterial activity against *Proteus mirabilis, Salmonella typhi,* and *Escherichia coli*. (Nwachukwu & Uzoeto, 2010). The best activity against *K. pneumoniae* was consistently produced by hexane fractions of *Cantharellus cinerus, Chlorophyllum molybdites, Laccaria laccata,* and *Russula emetica*. The reason for this could be that all of these mushroom extracts particularly target the pathways unique to *K. pneunonia*, rendering it more sensitive to these four extracts than other MDR strains. One thing is noteworthy that most of the extracts didn't show activity for *E. coli* but the ethanol and methanol fractions of *Chlorophyllum molybdites, Russula emetica and*

Macrolepiota albuminosa. According to Poirel et al., 2018 *E. coli* is already sensitive to practically all clinically important antimicrobial treatments, this bacterial species has a high capability for resistance gene accumulation, primarily by horizontal transmission of genes. There is also a possibility that the specific antibacterial compounds in the mushroom extract that are effective against *E. coli* may have polar characteristics. Phenolic molecules, flavonoids, and other polar secondary metabolic products are examples of these substances. In comparison to nonpolar solvents, ethanol and methanol would be better in extracting such chemicals.

In the current study, *E. coli* remained resistant to the majority of extracts, although *K. pneumonia* and *Psudomonas aeruginosa* proved to be vulnerable to the majority of macrofungal crude extracts. Previous research has shown that Gram-positive bacteria are more sensitive to raw extracts than Gram-negative bacteria. (Gebreyohannes et al., 2019) concluded that extracts from various mushrooms were more vulnerable to Gram-positive bacteria than Gram-negative bacteria. Previous research has also shown that the disparity in sensitivity of gram-positive and gram-negative bacteria to bioactive compounds is primarily due to structural variations. Lipopolysaccharides in Gram-negative bacteria's outer membrane render the cell wall impermeable to lipophilic substances (Ciznár & Krcméry, 1967).

However, with the exception of E. coli, crude mushroom extracts were similarly effective against gram negative bacteria in this investigation. *Laccaria laccata, Cantharellus cinerus,* and *Russula emetica* hexane fractions all showed high effectiveness against *K. pneumoniae.* Methanol fractions of *Chlorophyllum molybdites* showed specific efficacy against *E. coli, Pseudomonas aeruginosa,* and *K. pneumoniae.* These mushrooms must have a stronger affinity for the distinctive structures found in Gram-negative bacteria, making them more susceptible to antimicrobial actions. Efflux pumps, which actively remove hazardous chemicals from the cell, are common in Gram-negative bacteria. These pumps make it more difficult for antimicrobial chemicals to concentrate within the bacterial cell, yet these mushroom extracts inhibited these efflux pumps effectively. Based on present data, it was concluded that a fraction

of acetonitrile, ethyl alcohol and methanol obtained from *Chlorophyllum molybdites* exhibited antibacterial potential against the tested organisms.

Ethyl acetate fractions of *Russula foetentula, Russula cerolens, Amanita phalloides, Chlorophyllum molybdites, Auricularia auricula-judae* and *Lactarius deliciosus* gave the best antifungal activities. According to the mycochemical study conducted by Zhao et al., 2019, a lot of phytochemicals were purified from ethyl acetate fraction from *Russula aruea*. So, these chemicals must have been the cause of best antifungal activity of these mushroom extracts. Hexane fractions of *Laccaria laccata and Lactarius deliciosus* gave the activity against 3 test fungal strain. A study was conducted by Vazirian et al., 2014, in which hexane and chloroform fractions of *Ganoderma lucidum* gave a secondary metabolite ganodermadiol which had antifungal activities. Acetone fractions of Amanita species and Russula species showed great antifungal activity. According to SEVİNDİK et al., 2019, Amanita genus, which includes both edible and toxic species, has a wide range of biological activity.

All fractions of *Russula cerolens, Amanita phalloides*, and *Auriculaia auricula-judae* extracts displayed unusually strong antioxidant activity, but *Cantharellus cinerus and Macrolepiota albuminosa* extracts displayed no activity at all. Ethanol and methanol fractions of *Auricularia auricula-judae* demonstrated excellent antioxidant activity. The hexane, chloroform, and acetone fractions of *Amanita phalloides*, as well as the chloroform fraction of *Chlorophyllum molybdites*, have high antioxidant potential. According to Barros et al., 2007, phenolic molecules were found in greater quantities than other bioactive chemicals in these mushrooms. The abundance of strong phenolic compounds is responsible for all of the species' significant antioxidant capabilities. The occurrence of these active medicinal chemicals in edible mushrooms is due to habitat or substrates that are rich in functional molecules. These compounds are classified as anthocyanidins, beta-glucans, triterpenes, or cordycepin. The substances detected in these extracts demonstrate that growing mushrooms on substances rich in functional compounds accounts for at least a portion of the functional compounds (Vamanu & Nita, 2013). Methanol fraction of *Lactarius deliciosus* gave exceptionally high antioxidant activity and likewise, zhou et al., conducted a study in which 25 types of

volatile aromatic compounds found; acids were the most common compounds in terms of content, while aldehydes were the most abundant in terms of diversity and both ethanol and aqueous extracts of *L. deliciosus* demonstrated high antioxidant activity. Ethanol and methanol extracts of *Auricularia auricula judae* also showed extravagantly high antioxidant potential, and the same activity was conducted by Yu et al., 2013, on the same mushroom where the DPPH radical scavenging capabilities of ethanol and acetone extracts were very high.

As far as cytotoxic potential is concerned, all fractions of *Russula foetentula, Russula carolens, Cantharellus phalloides*, and *Chlorophyllum cinerus* exhibited considerable cytotoxic potential. After 24 hours, all fractions of these four extracts at 1, 0.75, 0.5, 0.25, and 0.05mg/mL were shown to be 100% hazardous for shrimps. Overall, all fractions of *Russula paludosa* showed reduced cytotoxicity, with the exception of hexane and methanol fractions, where shrimp survival was 0% and 2% at 1 mg/ml concentrations, respectively. *Auricularia auricula judae* was not very strong, but *Lactarius deliciosus* and *Macrolepiota albuminosa* were, and no survival of brine shrimps was found from high to low concentrations in most cases. Chelela et al., 2014 conducted a study in which Ethanol extracts were shown to be more damaging to cells than chloroform and other. This shows that extracts of ethanol could be a viable solvent for the isolation of anticancer agents. The fatality of brine shrimp demonstrates the bioactivity of the extract, which in most circumstances corresponds with cytotoxic and anti-tumor activities reasonably well. In their study, inedible *A phalloides* and *L. denigricans* mushrooms were examined. It was discovered that some mushroom species exhibit greater cytotoxicity than others. Because of its high cytotoxicity, certain species propose why certain kinds of mushrooms are not edible in most places of the world.

The majority of the mushroom extracts had low to moderate hemolytic activity. The activity values of *Russula foetentula's* ethanol and methanol content ranged from 20 to 25%. The acetone and acetonitrile fractions of *Laccaria laccata* produced significant hemolysis, whereas the methanolic fractions caused the least. The hemolytic activity of *Russula cerolens* fractions in hexane, chloroform, and ethyl acetate was excessive.

Laccaria laccata ethanol extract demonstrated extremely poor hemolytic activity, whereas hexane and chloroform extracts had very significant hemolytic activity. In this manner, a trend is noticed in which all non-polar fractions of mushrooms generated more hemolysis whereas polar fractions caused the least. Similarly, *Russula emetica* hexane and chloroform fractions demonstrated unusually high hemolytic activity. Nonpolar solvents may preferentially absorb some hazardous compounds or lipophilic substances that solvents that are polar do not extract as effectively. Some mushrooms are said to possess lipophilic poisons that might cause hemolysis. Nonpolar solvents, such as hexane or chloroform, are more effective at recovering lipophilic chemicals from mushrooms. As a result, utilising nonpolar solvents may result in the extraction of more of the aforementioned toxic substances which can lead to hemolysis. Pu et al., 2023 also conducted a study in which Russula species caused hemolysis. *Russula subnigricans* is now one of China's most dangerous mushroom species, with a fatality rate of over 50%. According to one patient's report, severe hemolysis occurred on the second day after intake and was accompanied by a drop in haemoglobin concentration.

In the course of mycochemical screening, the bulk of the extracts proved positive for saponins and tannins in polar solvents such as ethanol and methanol, while steroids were detected in both polar and non-polar solvents. Saponins and tannins are polar chemicals that allow them to dissolve in polar solvents such as ethanol and methanol. Steroids, on the other hand, have varying degrees of solubility based on the steroid molecule. Some steroids are more polar and absorb well in polar solvents, whereas others are less polar and dissolve more in solvents that are not polar. The phenolic and flavonoid content of Russula species is also high. Niazi & Ghafoor, 2021 conducted a study on *A. cinnamomescens* and *A. pakistanica* and tested them for the presence of mycochemicals and extracts had notable total phenolic and total flavonoid concentrations.

CONCLUSION

Drug-resistant bacteria are a growing health concern worldwide, and natural sources are being investigated as an antibiotic alternative. In this study, mushroom extract as a natural source was investigated for usage as an antibiotic alternative. Seventy-seven fractions were obtained after treating mushroom samples with seven solvents in sequential order from non-polar to polar. The crude extracts of various wild mushrooms demonstrated strong antibacterial activity against MDR strains such as *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis,* and *Klebsiella pneumoniae*. The best effectiveness against *B. subtilus, P. aeruginosa*, and *K. pneumoniae* was demonstrated by the ethyl acetate fraction of *Russula foetentula, Russula carolens, Amanita phalloides, Cantharellus cinerus,* and *Chlorophyllum molybdites*. The mushrooms also showed strong antifungal activity against *Aspergillusflavus, Candida albicans,* and *Aspergillus niger*. Hexane, acetone, acetonitrile, and ethanol fractions of *Laccaria laccata* were found to be effective against pathogenic fungal strains. All fractions of *Russula cerolens, Amanita phalloides*, and *Auriculaia auricula-judae* extracts displayed unusually strong antioxidant activity. In terms of cytotoxicity, *Auricularia auricula judae* was not very strong, but *Lactarius deliciosus* and *Macrolepiota albuminosa* showed 100% mortality of brine shrimps at a very low concentration of 1mg/mL. All fractions of *Russula foetentula, Russula carolens, Cantharellus phalloides*, and *Chlorophyllum cinerus* exhibited considerable cytotoxic potential. *Lactarius deliciosus* and *Macrolepiota albuminosa* extracts had the most minor hemolytic activity. While, *Russula emetica, Auricularia auricula judae, Russulafoetentula,* and *Russula paludosa* all had low to moderate hemolytic activity. Further, mycochemical analysis confirmed the presence of important chemicals that may contribute to their pharmacological potential.

In conclusion, ethyl acetate, acetonitrile, ethanol and methanol fraction gave considerably better results than the rest, and in terms of mushrooms, *Russula foetentula, Laccaria laccata, Auricularia auricula-judae* and *Macrolepiota albuminosa* gave the best results of all activities.

FUTURE PERSPECTIVES

Although mushrooms have more therapeutic significance, there is an urgent need to document and conserve these economically significant mushroom species. Additionally, more research is needed to fully comprehend the mechanism and metabolic processes of mushroom bioactive substances for their pharmacological actions. To provide greater shelf life and less waste of resources, research investigations on mushroom processing and preservation techniques must be conducted. Alterations in the mycochemical makeup of mushrooms, as well as modifications to the processes of the bioactivities, must be explored. Furthermore, the sequencing of genomes and the implementation of modern technologies like metabolomics, proteomics, and transcriptomics might bring useful knowledge and fresh ideas for medicinal mushroom research. More research into the medical benefits of medicinal mushrooms, as well as medication development, is required. Effective human-based clinical trials using excellent mushroom-derived substances for disease therapy, as well as developing affordable methods of producing these products under strict guidelines, are critical challenges. In order to accomplish appropriate cultivation of wild edible fungi, detailed investigations on the impact of cultivation conditions, substrate makeup, and harvest timing on the mycochemical content and nutritious value of these mushrooms must be conducted. Furthermore, more research investigations, including thorough clinical trials on humans, are needed to comprehend the biological processes and metabolic routes of mushrooms. More extensive research on undiscovered wild consumable varieties and production settings is needed to discover the full potential of mushrooms for human wellness and survival.

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