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Conclusion should elucidate how the results communicate to the theory presented as the basis of the study and provide a concise explanation of the allegation of the findings.

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The Role of AI in Revolutionizing Agriculture for Food Security

OPEN ACCESS

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One of the major problems of present time is producing adequate amount of food, enough for survival of rapidly growing population. Several solutions have been applied to restore the food security. Production of resilient crops by increasing product yield, even under biotic and abiotic stress factors, is one of the biotechnological revolutions made in the field of agriculture. Molecular alteration, modifications of different crops to make them resistant to various kind of environmental fluctuations such as extreme temperatures, viral and fungal infections, and drought stress has restored the food production to a greater level. Moreover, different tissue culturing methods has optimized the resource usage due to which crop yield has been improved immensely in the recent years. But intensified change in climate in the past few years has affected the supply of food greatly. Integration of AI and machine learning in the agricultural biotechnology has shown new and fast ways to deal with these concerns. Soil monitoring before using it for the production of a crop is important as soil health play a vital role in producing good yield. Before AI, conventional methods like biomass estimation and soil mapping were used to address the problems with the soil and crop productivity but now with AI-assisted remote sensing technologies having deep learning algorithms, water and nutrient stress can be handled in a better way. Along with soil productivity, monitoring of forecast and generating the environmental data with help of AI integrated drones has also played a role in the crop productivity.

Furthermore, generating molecular data about gene positions, gene expression in the real time with the help of AI algorithms has advanced the research tremendously. AI has proved to be very useful in bringing the molecular based data and its alignment to design the projects regarding the improvement of crops. It has proved to be smart and time saving tool.

Another advancement that has been made in this field is the collaboration of AI and plant tissue culture. Though micropropagation has potential but at the same it is a labor intensive and time taking task. Such as finding the intricate relationship of elements and optimizing the culture media for plant treatment takes lot of time. Bit with AI model this task has also been simplified. It optimizes the media in short time and monitor the treatment in real time. Due to its effectiveness in tissue culturing it has become an important tool for plant tissue scientists.

By solving the climate change issues, facilitating the generation of modified crops, examination of environmental fluctuations and improving the effectiveness of plant tissue culture, Artificial intelligence has immensely transformed the agricultural world completely. Incorporation of AI in the agricultural improvement methods has created a sustainable food system.

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Review Article

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Actinomycetes: Ultimate Potential Source of Bioactive Compounds Production

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ABSTRACT

Every day, increased microbial resistance demands the search for new natural sources that can produce natural and effective antimicrobial compounds. Actinomycetes are attractive microorganisms with an enormous and unlimited potential to produce economically and biotechnologically important metabolites. Approximately 75% of all bioactive compounds produced so far originate from this group of bacteria. Many of these compounds have been successfully isolated and converted into valuable medications and other naturally derived synthetic compounds with antimicrobial and chemotherapeutic properties. The antimicrobial agents produced by this valuable group of prokaryotes were effectively used to rival parasites and other microbes for assets. They include many genera, each with the potential to produce various novel products. For example, one of the leading genera is Streptomyces, which contributes 70% of total antibiotics such as macrolide, aminoglycoside, Rifamycin, Ivermectin, chloramphenicol, and a large number of other medicinally valuable antimicrobial agents. It also includes anticancer agents as well. Similar to Streptomyces, Micromonospora is another major source of antibiotics producing Tetrocarcins, Fortimicins, Antlermicins, Sagamicins, Mutamicins, Verdamicins, Sisomicins, Calicheamicin, and gentamicin. Other rare actinomycetes are potential producers of novel and broad-spectrum antibiotics, including Salinosporamide A, Marinomycin A, Arenimycin, Vancomycin, Abyssomicins, and Proximicins. Due to the expanding studies, data on the production of various metabolites by this unique and outstanding phylum is expanding daily. This review has made an effort to improve the pre-available knowledge on producing and characterizing novel antimicrobial compounds with therapeutic potential from terrestrial and marine actinomycetes.

INTRODUCTION

Over the past three decades, there has been a rise in infections caused by opportunistic microorganisms due to immunocompromising diseases such as organ transplants, tumors, Human immunodeficiency virus (HIV), and other related diseases. Additionally, increasing reports of resistance to existing antimicrobial agents have become a worldwide issue [1]. Antibiotic resistance in pathogenic bacteria is a global problem correlated with morbidity and mortality. Increased multidrug resistance in Gram-negative and -positive pathogenic bacteria has led to difficulty in treatment and even non-treatable infectious diseases with traditional antimicrobial agents. This has prompted the pursuit of novel antibiotic molecules to address this worldwide challenge [2]. Actinomycetes are Gram-positive filamentous bacteria. They are primarily aerobic and possess a high GC content of up to 78% in their DNA. Therefore, they are highly metabolically active microorganisms. They produce metabolic active compounds that are beneficial as nutritional material, antitumor agents, and antimicrobial and immunosuppressive agents [3]. Actinomycetes are a prominent source of natural bioactive compounds. Out of 22,500 metabolites that have been isolated from microbes, 45% belong to this phylum. Among actinomycetes, the genus Streptomyces produces 70% of biologically active metabolites [4]. Actinomycetes have been reported to produce antibiotics of almost every class. Still, few of them are well known, like epoxides, macrolides, β-lactams, peptides, amino-coumarines, aminoglycosides, ansamycines, amino-coumarines, lincosamides and tetracyclines [5]. Natural compounds are the preferred source of antimicrobials with diverse structural and

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chemical properties associated with drug target sites [6]. However, using microbes as a potential cause of biologically active compounds has gained interest in the scientific community for the last two decades. Among microbes, phylum actinobacteria (order-actinomycetales) is a unique and auspicious source of novel and biologically active metabolites with broad-spectrum antimicrobial, anti-tumor activities and also with many other uses [7] (Figure 1).

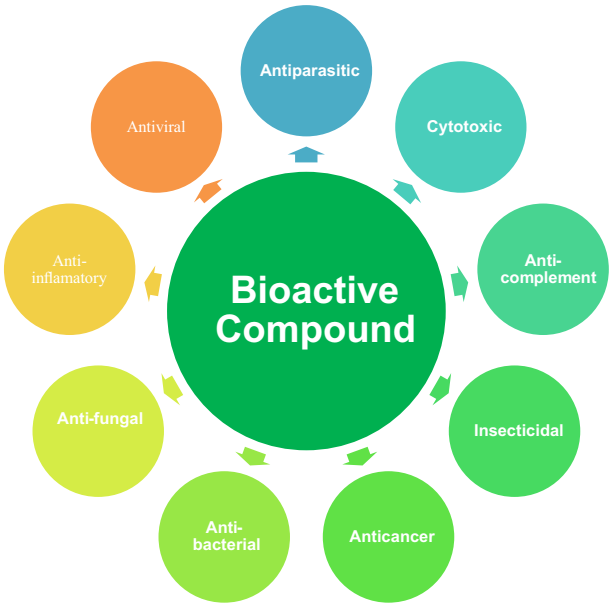

Figure 1: Usefulness of Bioactive Compounds Extracted from Actinomycetes

Approximately 75% of the total bioactive compounds are antitumor agents, immunosuppressive drugs, enzymes, and antibiotics produced by this phylum alone (Table 1).

Table 1: New Novel Bioactive Compounds Extracted from Actinomycetes

Sr. No.	Compounds	Sources	Importance	References
1	Vancomycin	Amycolatopsis Orientalis	Used Against Methicillin-Resistant Staphylococcus Aureus	[8]
2	Tetracycline	Streptomyces Aureofaciens	Inhibits Aminoacyl-TRNA Binding	[9]
3	Chlora-mphenicol	Streptomyces Venezuelae	Interacts with 50S Subunit, Inhibiting the Activity of Peptidyl Transferase	[10]
4	Erythromycin	Saccaro-polyspora Erythraea	Binds with 50S Subunit, Block the Activity of Peptidyl Transferase	[11]
5	Rifampicin	Amycolatopsis Mediterranei	The Main Component of Anti-Tuberculosis Therapy	[12]
6	Novobiocin	Streptomyces Niveus	Inhibits Bacterial DNA Gyrase	[13]
7	Dia-zepinomicin	Micro-monospora Strains	Anticancer Agent Used In Phase II	[14]
8	Salino-sporamide A	Salinispora Tropica	Anticancer Agent	[15]
9	FK 506	Streptomyces Tsukubaensis	Antiviral Agent	[16]
10	Ivermectin	Streptomyces Avermitilis	Used to Treat Nematode Infections	[17]
11	Medecamycin	Streptomyces Mycarofaciens	Antibacterial	[18]
12	Rhamnose	Saccharo-polyspora Spinosa	Essential Components of Insect Control Agents Like Spinosad	[19]
13	Streptomycin	Streptomyces Sp.	Antibacterial	[20]
14	Lajollamycin	Streptomyces Nodosus	Antibacterial	[21]
15	Amphotericin B	S. Nodosus	Anti-Fungal	[22]
16	Avermictin	Streptomyces Sp.	Antiparasitic	[23]
17	Anthracyclines	Streptomyces Sp.	Anticancer	[24]
18	Chloram-phenicol	S. Venezuelae	Antibacterial	[25]
19	Amythiamicins	Amycolatopsis Sp.	Antibacterial	[26]
20	Meilingmycin	Streptomyces Nanchangensis	Antiparasitic	[27]
21	Nan-changmycin	S. Nanchangensis	Insecticidal	[28]
22	Eremomycin	A. Orientalis Sub Sp. Eremomycini	Antibacterial	[29]
23	Daptomycin (Com-mercialized As Cubicin)	Streptomyces Roseosporus	Antibacterial	[30]
24	Mithramycin	Streptomyces Argillaceus	Anticancer	[31]
25	Aclacinomycin A (Aclarubicin)	Streptomyces Galilaeus	Anti-Cancer	[32]
26	Tetracycline	S. Aureofaciens	Antibacterial	[33]

These bioactive compounds have different modes of action [34] (Figure 2).

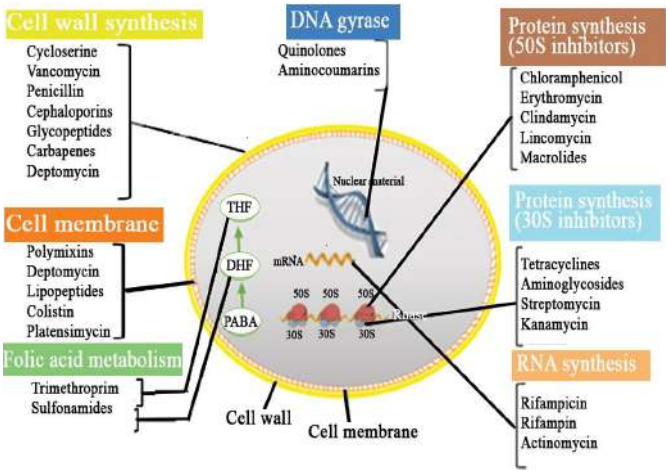

Figure 2: Mode of Action of Most Antibacterial Bioactive Compounds

Actinomycetes exhibit diverse habitats and are found in extreme environments like glaciers, hot springs, and high salt concentrations [35], but are commonly found in soil, marine water, and freshwater and also found as symbiotic

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organisms. Their association with different plants, animals and other organisms has been observed. However, they are categorized as marine or terrestrial based on the ecosystem in which they exist [36]. Terrestrial actinomycetes are commonly called soil actinomycetes. In soil, actinobacteria constitute a significant portion of microbes. According to a rough estimate, approximately 1 million actinomycetes may be obtained from one gram of soil [37]. They play an important role in soil biodegradation, humus formation, nutrient cycles, and inhibit the growth of many plant pathogens in the soil [38]. In contrast, soil actinomycetes have produced a wide variety of novel antibiotics. About 70% of total antibiotics are produced by soil actinomycetes [39]. Actinomycetes, isolated from marine ecosystems, have shown tolerance against NaCl up to 13%, which is very much as compared to actinomycetes isolated from terrestrial environments, which shows no tolerance to this concentration [40]. Moreover, actinomycetes, which were isolated from marine ecosystems, are metabolically more active than terrestrial ones. This is an exclusive adaptation of marine actinomycetes [41]. To understand this unique adaptation of marine actinomycetes, scientists started working on genomic sequencing of these bacteria. Genome sequencing of many novel marine actinomycetes has led to understanding this type of adaptation of marine actinomycetes. It also helped to discover their biosynthetic potential, and it discovered many new broad-spectrum antibiotics [42]. Marine actinomycetes are an attractive source of interesting research and bioactive compounds for most active researchers [43]. The actinomycetes isolated from marine environments have a broad spectrum of antimicrobial activities, including antifungal, antibiotic, toxic, neurotoxic, cytotoxic, antimitotic, antiviral, and antineoplastic. Recently, scientists started considering marine actinomycetes as an admirable probiotic source because marine actinomycetes also showed excellent antibiotic activity against various fatal pathogenic bacteria like Vibrio SP [44]. Study shows compounds extracted from marine actinomycetes with their importance and chemical groups. This is why scientists are now focusing more on marine actinomycetes to extract novel and broad-spectrum bioactive compounds (Table 2).

Table 2: Important Bioactive Compounds Extracted from Marine Actinomycetes

Sr. No.	Compounds	Chemical Form	Significance	Source	References
1	2-Allyloxyphenol	Allyloxyphenol	Antimicrobial Properties: Used in Food Preservation and Oral Disinfection	Derived From Streptomyces Sp.	[45]
2	Glaciapyrroles A, B, and C	Pyrrolosesquiterpenes	Effective Against Bacterial Strains	Isolated From Streptomyces Sp.	[46]
3	Lodopyridone	Alkaloid	Exhibits Antitumor Activity	Found In Saccharomonospora Sp.	[47]
4	Neomarinone	Sesquiterpene	Demonstrates Cytotoxic Effects	Strain CNH099	[48]
5	Saliniketal A, Saliniketal B	Polyketide	Potential in Anticancer Applications	Sourced From Salinispora Arenicola	[49]
6	Abyssomicin C	Polyketide	Known for Antibacterial Capabilities	Found In Verrucosispora	[50]
7	Daryamides	Polyketide	Effective in Anticancer and Antifungal Roles	Originates From Streptomyces Sp.	[51]
8	Actinofuranones A and B	Polyketide	Shows Cytotoxic Activity	Extracted From Streptomyces Sp.	[52]
9	Mechercharmycins	Peptide	Known for Antitumor Effects	Found In Thermoactinomyces Sp.	[53]
10	Saliniketal	Polyketide	Cancer Prevention Potential	From S. Arenicola	[54]
11	Arenimycin	Peptide	Dual Application as an Antimicrobial and Anticancer Agent	From S. Arenicola	[55]
12	Piperazimycins	Peptide	Useful in Cancer Therapy	Derived from Streptomyces Sp.	[53]
13	Dehydroxynocardamine and Desmethyleny-Inocardamine	Peptide	Inhibits Enzyme Sortase B	Sourced from Streptomyces Sp.	[56]
14	Tirandamycins	Dienoyl	Effective Antibacterial Agent	Produced by Streptomyces Sp.	[57]
15	Xanthone IB-00208	Polycyclic	Dual Role: Anticancer and Antibacterial	Isolated from Actinomadura	[58]
16	Piericidins C7 And C8	Piericidin	Known for Anticancer Properties	Found in Streptomyces Sp.	[59]
17	Resistomycin	Quinone	Exhibits Antimicrobial Activity	From Streptomyces Corchorusii AUBN (1) / 7	[60]
18	Proximicins	Aminofuran	Dual Role: Antibacterial and Anticancer	Found in Verrucosispora Sp.	[53]
19	Helquinoline	Quinone	Effective As An Antibacterial Agent	Found in Janibacter Limosus	[61]

Tunicamycin is a bioactive compound isolated from Streptomyces lavendulae DUT 11, a marine actinomycete that showed admirable anti-gastric cancer and breast cancer activity. Both types represent the most significant contributors to cancer-related deaths in the USA and are the most prevalent cause of cancer-related fatalities

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globally [62]. Tunicamycin accumulates unfolded protein in the lumen of the endoplasmic reticulum, thus reducing ER stress. It increases the level of nuclear translocation and CHOP protein expression, suppresses proliferation, reduces invasion, and leads to cell death [63, 64] (Figure 3).

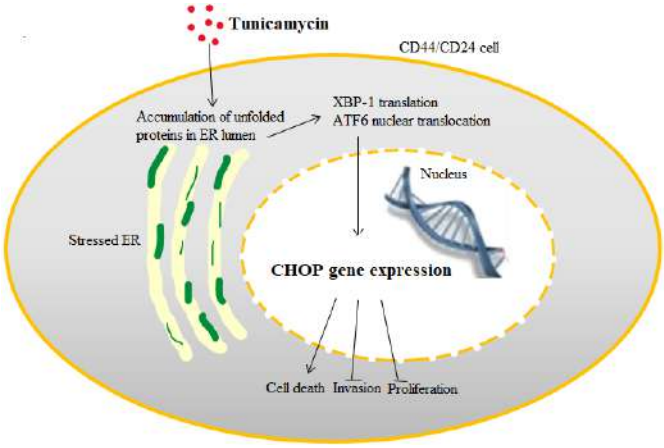

Figure 3: Mechanism of Action of Tunicamycin [65]

Caboxamycin was extracted from Streptomyces sp. NTK937, an obligate marine bacterium. Caboxamycin is a bioactive compound that belongs to the chemical group benzoxazole [66]. This bioactive compound shows potent activity against many pathogenic bacteria, including Staphylococcus lentus, Xanthomas campetris, Staphylococcus epidermis, etc. It also shows activity against the yeast Candida glabrata and the biofilm of Staphylococcus xybsus [67]. Abyssomicin C was identified from marine Verrucosispora sp. in 2004. It dramatically inhibited the synthesis of para-aminobenzoic acid (PABA). PABA is a key factor required for the biosynthesis of folic acid in prokaryotic cells [68]. Abyssomicin C demonstrates significant antibacterial efficacy against methicillin-resistant S. aureus (MRSA) and mycobacteria, which cause tuberculosis by targeting PABA synthesis. Abyssomicin C led to the discovery of next-generation antifolates and first-generation PABA inhibitors [69]. Mechercharmycin A and B were extracted from Thermoactinomyces sp. YM3-251, which is marine-derived. Mechercharmycin A has shown potent antitumor activity and cytotoxic action against human leukaemia and lung cancer, while mechercharmycin B is a linear congener. Crystallographic studies have shown that it is a cyclic peptide that contains four oxazoles and thiazole groups [70]. Pseudopterogorgia, isolated from marine Pseudopterogorgia elisabethae, was found to show intense, potent activity against Mycobacterium tuberculosis. M. tuberculosis is the well-known causative agent of TB. This disease always remains a global threat to the human population and leads to 2 million deaths and more than 9 million infections annually all over the world. Many studies have already reported that Pseudopterogorgia showed potent activity against vancomycin-resistant Enterococcus faecium (VRE) and methicillin-resistant S. aureus (MRSA) bacteria [71]. There are many genera in the phylum of actinobacteria producing novel bioactive compounds, which include Gulosibacter, Actinomadura, Actinomyces, Atopobium, Amycolatopsis, Actinobaculum, Micromonospora, Actinokineospora, Streptomyces, Kitasatospora, Salinispora, Actinomadura [72]. Study shows various genera and the quantity of new bioactive substances extracted from them (Table 3).

Table 3: Different Genera with Several Novel Bioactive Compounds [36]

Actinomycetales Genera	No. of Novel Antibiotics	Actinomycetales Genera	No. of Novel Antibiotics
Streptomyces	8,000	Actinomodura	345
Streptoverticillium	258	Saccharothrix	68
Kitasatosporia	37	Microbiospora	54
Thermoactinomyces	14	Actinosynnema	51
Microellobosporia	11	Micromonospora	740
Microtetraspora/ Nonomuria	26/21	Chainia	30
Actinoplanes	248	Thermomonospora	19
Dactylosporangium	58	Micropolyspora/Faenia	13/3
Saccharopolyspora	131	Nocardiopsis	41
Amycalotopsis/Nocardia	120/357	Nocordia	357
Kibdillosporangium	34	Mycobacterium	57
Psoudonocardia	27	Arthrobacter	25
Amycolata	12	Brevibacterium	17
Streptosporangium	79	Proactinomyces	14
Streptoalloteichus	79	Rhodococcus	13
Spirilospora	11	Actinosporangium	30
Planobispora	10	Microellobosporia	11

Streptomyces is the largest genus of phylum actinomycetes and is known for its novel biological compound production. Among various broad-spectrum antimicrobial compounds, over 70% of bioactive compounds have been isolated from the genus Streptomyces [73]. Many of these compounds have been reported to be important vitamins, alkaloids, and antibiotics. Streptomyces is the only prolific producer of novel biologically active compounds. These compounds include antimicrobial, anti-infective, anticancer agents, antiparasitic, antitumor, antifungals, antivirals, anti-hypertensives, and other critical medicinal compounds [74]. Similarly, Streptomyces nouseri produces nystatin, S. venezuelae produces chloramphenicol, Streptomyces fradiae produces neomycin, Streptomyces peucetius produces Amrubicin, and S. griseus produces streptomycin [75]. Tetracycline is a family of antibiotics that was first discovered in 1940. The application demonstrated efficacy against a range of microorganisms, including both Gram-positive and Gram-negative bacteria, as well as mycoplasmas, chlamydiae, rickettsia, and

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protozoan parasites [76]. Due to its broad-spectrum antimicrobial nature, tetracycline inhibits the protein synthesis mediated by binding with 30S bacterial ribosome. There are many reports that most pathogens have developed resistance to tetracycline [77]. To solve this problem, second-generation (such as minocycline) and third-generation (such as glycylcyclines) antibiotics have been developed [33]. A minocycline derivative, the tigecycline, was isolated from S. aureofaciens. Tigecycline is a novel antimicrobial agent against multidrug-resistant (MDR) pathogens [78]. These include strains resistant to methicillin, such as S. aureus, and those resistant to vancomycin, like enterococci. Tigecycline is structurally related to tetracycline and acts by inhibiting the protein translation in microbes [79]. Erythromycin is another broad-spectrum antibiotic from the genus Streptomyces, which exhibits activity against Gram-negative and Gram-positive bacteria [80]. It consists of a macrocyclic lactam ring to which compounds of sugar and amino are attached. Erythromycin is an alternative to Penicillin and Cephalosporins to treat infections, particularly those caused by β-hemolytic streptococci and pneumococci [81, 82]. Following Streptomyces, Micromonospora is regarded as the second most significant potential source of bioactive chemicals with therapeutic relevance. Out of these compounds, Maximum is aminoglycoside antibiotics. The aminoglycoside group includes mutamicin, neomycin B, gentamicin, fortimicin, antibiotic G-418, antibiotics JI-20, tetrocarcins, calicheamicins, sisomicin, verdamicin, antlermicins, and sagamicin [83, 84]. These medications have been utilized against Gram-positive and Gram-negative bacteria and are bactericidal as opposed to bacteriostatic action. These are also used against eukaryotic organisms, e.g., Protozoa. Another common type of antibiotic is the macrolide, which consists of a 16-, 15-, or 14-membered lactone ring linked to de-oxy sugars such as cladinose and desosamine. The antibacterial spectrum of macrolides is similar to penicillin. It is broad-spectrum; thus, it can be recommended to patients with penicillin allergy to illnesses such as soft-tissue infections and respiratory tract [85, 86]. Gentamicin (GM), which Micromonospora produces, was discovered in 1963 and first introduced into parenteral usage in 1971. Gentamicin is a broad-spectrum aminoglycoside antibiotic. This medication is frequently used to treat pelvic inflammatory disease, intra-abdominal infections, complicated infections, urinary tract infections, sepsis, endocarditis, affecting the skin, bones, and soft tissues, along with other severe infections induced by Gram-negative bacteria [87]. Tetrocarcinsis is a family of novel antibiotics that were isolated from Micromonospora chalcea. The findings demonstrated remarkable efficacy against Gram-positive bacteria and significant antitumor effects through various mechanisms of action. Recent studies have revealed that Tetrocarcins have a unique polycyclic aglycone (tetronolide) that functions in a trans-decalin system and also has tetronate moiety spiro-linked with a cyclohexane ring, which acts by inducing apoptosis in various cancerous cells [88]. Fortimicin is another broad-spectrum antibiotic from the class aminoglycoside. It was extracted from Micromonospora olivasterosporain in 1977. The mechanism works by binding to the 16S rRNA subunit of the 30S bacterial ribosome, thereby inhibiting prokaryotic protein synthesis and preventing the dissociation of 70S ribosomes. This antibiotic is effective against a broad spectrum of Gram-negative and Gram-positive infections [89, 90]. Calicheamicins is known as a novel family of antitumor agents. These compounds exhibit cytotoxic properties and were extracted from Micromonospora echinospora. They show a potency that is at least 1000-fold more significant than that of conventional cytotoxic chemotherapeutics. Calicheamicins bind with DNA in the minor groove, causing double-strand DNA to break, thus leading to cell death, and are more specific in their action than other antitumor agents [31]. Over time, the discovery rate of new novel antibiotics from Streptomyces has decreased; about 70% of antibiotics have been isolated from this genus alone, as discussed earlier. So, it is time to search for novel antibiotics from non-Streptomyces actinomycetes. Uncommon actinomycetes are the strains of the phylum actinomycetes isolated much less frequently than Streptomyces when employing conventional methods [75]. Recently rare actinomycetes are considered excellent potential sources of novel biologically active metabolites. The rare actinomycetes present significant challenges in isolation and cultivation and may possess unique potential for producing novel biologically active metabolites. Until 2005, the exploration for new antimicrobial compounds from rare actinomycetes resulted in the identification of over 2250 novel antimicrobial compounds [91]. Some genera of rare actinomycetes are Marinispora, Actinomadura, Actinoalloteichus, Actinoplanes, Amycolatopsis, Actinokineospora, Acrocarpospora, Actinosynnema, Catenuloplanes, Cryptosporangium, Dactylosporangium, Kibdelosporangium, Kineosporia, Kutzneria, Micro-bispora, Micro-tetraspora, Nocardia, Nonomuraea, Planomonospora, Planobispora, Pseudonocardia, Saccharomonospora, Saccharopolyspora, Saccharothrix, Salinispora, Streptosporangium, Spirilliplanes, Termomonospora, Termobifda, and Virgosporangium [3].

CONCLUSIONS

It was concluded that actinomycetes are a great source of antibiotics. About 45% of the biologically active compounds belong to actinomycetes. Actinomycetes are

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found everywhere in the biosphere. But they are categorized as terrestrial and marine actinomycetes. This is a large bacterial phylum which has more than 30 genera. Some of the actinobacterial genera are prominent like Streptomyces and other genera are known as rare actinomycetes. Almost all actinobacterial genera are known for producing biologically active compounds but Streptomyces are producing about 70% of total compounds that are discovered and isolated from actinomycetes.

Authors Contribution

Conceptualization: NM
Methodology: HK
Formal analysis: HK, NM
Writing review and editing: AT, HJ, RM, IL

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Review Article

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Impact of Titanium Dioxide Nanoparticles on Agricultural Crops Performance: A Review of Efficacy and Mechanisms

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ABSTRACT

The rapidly increasing global population has escalated the demand for food production, intensifying the pressure on agricultural systems to meet this rising need. Traditional farming methods often fall short of addressing this challenge due to limitations in crop yield and resistance to environmental stress. In response, nanotechnology has emerged as a promising solution, particularly through the application of titanium dioxide nanoparticles (TiO_2 NPs). TiO_2 NPs, due to their unique physicochemical properties, have gained attention for their potential to enhance agricultural productivity. Their mechanism primarily involves the modulation of light absorption, improving photosynthesis, and offering antimicrobial properties that protect crops from pathogens. Additionally, these nanoparticles can promote nutrient uptake and enhance plant growth, ultimately leading to higher crop yields. The utilization of TiO_2 NPs in agriculture offers a sustainable and efficient approach to boosting food production, making it a valuable tool in addressing global food security concerns. However, further research is essential to assess their long-term safety and scalability for widespread agricultural applications.

INTRODUCTION

Richard Feynman first put forward the idea of nanotechnology in 1959, and it has since developed into a major area of research and invention with potential uses in environment, healthcare, and agriculture. Considering their distinct physical and chemical characteristics, which make them valuable in a variety of industries and research sectors, Nanoparticles (NPs), particularly titanium dioxide nanoparticles (TiO_2 NPs), have drawn attention [1, 2]. TiO_2 NPs have been highlighted among metal oxides due to their advantages in environmental remediation, photocatalysis [3]. More recently, TiO_2 NPs have gained attention in agriculture, especially for enhancing plant growth and crop yield in stressful environments like soil with high salinity. Millions of hectares of agricultural land experience drop in the production as a result of soil salinity's negative effects on plant growth, which include oxidative damage and ionic balance disruption [4]. There are few traditional ways to counteract salinity, such as the application of chemical agents and development of genetically resistant crops. TiO2 NPs present a possible substitute since they promote the plant growth and enhance the nutrient absorption [5]. The capacity of TiO_2 NPs to control Reactive Oxygen Species (ROS), which are molecules that have two roles in stress reactions, is one of their main advantages. TiO_2 NPs shield plants from oxidative damage by regulating ROS levels [5]. Under stressful situations, TiO_2 NPs improve the plant's ability to uptake nutrients [6]. Additionally, TiO_2 NPs improve photosynthetic efficiency, which increases the amount of energy and biomass produced by stressed plants [7]. However, greater doses of TiO_2 NPs can be

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detrimental, resulting in cytotoxicity and genotoxicity, however, low amounts are advantageous. A careful consideration and administration of dosage is required to maximize the advantages and minimize the hazardous effects [8]. This study highlighted significance of safe integration of TiO2 NPs into large-scale farming while examining the effectiveness, processes, and possible uses of these particles in agriculture, specifically for treating soil salinity.

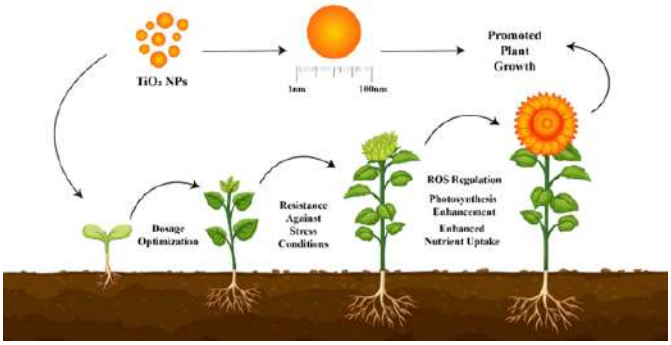

Figure 1: Mechanisms and Benefits of TiO_2 Nanoparticles in Agricultural Crops [9]

Mechanism of Action of TiO_2 Nanoparticles in Plants

1. Photosynthesis

Titanium dioxide nanoparticles have been reported enhance photosynthesis. In a study on spinach (*Spinacia oleracea*). Acting as photocatalysts, TiO_2 NPs improve light penetration and energy transfer to photosynthetic pigments, enhancing biomass, moisture content, and root and shoot lengths. The chlorophyll-a and chlorophyll-b levels were found to be increased by 14% and 33%, respectively, in TiO_2 NP-treated plants compared to controls [9]. TiO_2 NPs also enhance metabolic pathways and overall plant health, without causing oxidative stress at low concentrations [10]. These findings highlight the potential of TiO_2 NPs, under optimal dosages, to enhance photosynthesis and plant productivity.

2. Nutrient Uptake

TiO_2 NPs improve nutrient absorption in plants. When applied at optimal levels (up to 400 mg/kg), they enhance the uptake of Potassium (K), Iron (Fe), Manganese (Mn), and Phosphorus (P) by increasing root exudation and nutrient bioavailability. However, higher doses (above 600 mg/kg) can lead to toxicity and reduced nutrient absorption. TiO_2 NPs also catalyze antioxidant enzyme activity, lowering oxidative stress and enhancing nutrient metabolism. Wheat plants, in particular, exhibit improved growth parameters and nutrient concentrations when treated with optimal TiO_2 NP dos ages [11]. In a study, P uptake in wheat plants increased significantly when treated with TiO_2 NPs in soil over 60 days. Specifically, a 1.0-fold increase in P uptake was observed with a treatment concentration of 60 mg/kg TiO_2 NPs compared to the control. All tested TiO_2 NP concentrations demonstrated higher P uptake than the control, indicating their effectiveness in enhancing nutrient absorption [12].

3. Stress Tolerance

TiO_2 NPs enhance plant's resistance against abiotic stress, such as salinity, by enhancing the antioxidant defense system. At low doses (e.g., 0.01%), they increase the activity of antioxidant enzymes like Peroxidase (POD), Catalase (CAT), and Superoxide Dismutase (SOD), reducing oxidative damage caused by Reactive Oxygen Species (ROS) like Hydrogen Peroxide (H_2O_2). TiO_2 NPs also promote the production of osmo-protectants, such as proline and soluble sugars, which maintain osmotic balance and mitigate stress-induced growth inhibition [7]. In a study, the foliar application of 50 mg/kg TiO_2 NPs significantly increased the leaf Relative Water Content (RWC) by 6% and 12% under deficit irrigation conditions compared to control and full irrigation, respectively. However, applying 100 mg/kg TiO_2 NPs under severe drought conditions reduced leaf RWC below control levels, suggesting a threshold concentration beyond which adverse effects may occur. These findings indicate that optimal concentrations of TiO_2 NPs can improve drought stress tolerance [13].

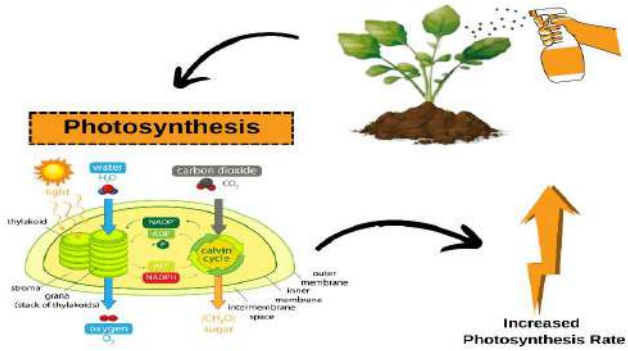

Figure 2: Foliar Application of TiO_2 can Increase the Rate of Photosynthesis [14]

Effect of TiO_2 Nanoparticles on Various Crops

1. Cereal Crops

TiO_2 NPs affect the development and yield of barley in both positive and negative ways. In general, they stimulate plant growth by increasing the plant height and leaf area. A study conducted on barley in soil with various concentrations of NPs showed that amongst all the concentrations, a concentration of 1000mg/kg of TiO_2 NPs had significant positive impact on the growth parameters, including taller plants and greater number of tillers compared to untreated barley plants. Additionally, the plants possessed an increased photosynthetic rate, gaseous exchange, stomatal conductance and transpiration during early developmental stages. Moreover, TiO_2 NPs mitigated the negative effects of cerium dioxide nanoparticles which caused a stunt growth. Generally, TiO_2 NPs have the potential to influence growth and yield of barley when

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administered at an optimal concentration [14]. The application of TiO_2 NPs has been regarded to influence plants' physiological functions by providing the protection to chloroplasts as well as enhancing the growth rate. TiO_2 NPs supply stabilization to the chloroplast's membrane, prevents aging and improve chlorophyll content during reproductive phase. A research conducted on spinach demonstrated that TiO_2 NPs lead to increased chlorophyll content as compared to TiO_2 application in bulk. The small size of the particle led to greater cellular absorption. Furthermore, better light absorption, faster energy transfers and protection of chloroplast led to improved photosynthetic rate. In addition, an improvement in anthocyanin content has been observed in various studies when compared to control groups [15]. Another study explored the effects of TiO_2 NPs on Cadmium (Cd) toxicity and migration in the soil-rice system. Results demonstrated that TiO_2 NPs positively influenced the physiological parameters of *Oryza sativa* such as increasing plant height, biomass, and chlorophyll content while reducing Malondialdehyde (MDA) levels and antioxidant enzyme activities. Such changes suggested that TiO_2 NPs mitigated the stress levels induced by Cd. Although TiO_2 NPs showed positive effects on growth but they demonstrated no effect in reducing the Cd content in rice grains below the maximum legal limits. Therefore, the usage of TiO_2 NPs is not feasible in reducing the toxicity generated by Cd content. Thorough research investigating the interaction between nanoparticles and environment, particularly rhizosphere, is necessary [16].

2. Leguminous Crops

Several physiological and biochemical responses have been recorded upon the foliar application of TiO_2 NPs at various growth stages in pinto beans. These responses contributed to the enhanced stress tolerance. Increased light absorption and improved photosynthetic efficiency has been attributed to the photo-catalytic characteristics and thermal conductivity of TiO_2 NPs. Additionally, TiO_2 NPs treatment reduced ROS accumulation and stimulated antioxidants activity in pinto beans which were exposed to stress. Such treatment led to increased biomarkers such as Malondialdehyde (MDA) and 8-OH-2-DG, demonstrating an improved ROS management in pinto beans. These findings indicate the TiO_2 NPs prove to be an effective agent in inducing tolerance against various stress [17].

3. Horticultural Crops

Foliar application of TiO_2 NPs on tomatoes during the rainy season under varying light conditions revealed mixed effects on yield and growth of plant. Improved photosynthetic rate and electron transfer rate with increased fruit yield was observed at a concentration of 100mg/kg of TiO_2 NPs. However, at a higher concentration such as 200mg/kg, there was no observable improvement in the fruit yield but a decline in photosynthetic rate. Moreover, the weight of fruit decreased with an increase in its hardness, regardless of the TiO_2 NPs concentration. These findings demonstrated that certain factors such as light intensity, nanoparticle concentration and type of crop being treated, influence the efficacy of TiO_2 NPs in both negative and positive manner. Therefore, such variables must be carefully analyzed before applying TiO_2 NPs in agricultural settings to gain the positive outcomes [18]. TiO_2 NPs were applied to coriander, an increase in the absorption of vital nutrients like nitrogen, potassium and phosphorous was observed. Moreover, growth parameters and physiological function were influenced positively due to an increase in total chlorophyll contents, carotenoids, sugar, indoles, amino acids and phenols. Overall, TiO_2 NPs improved the growth properties as well as yield in coriander crop. These studies suggested that TiO_2 NPs have the potential to replace the conventional fertilizers and act as cost-effective nano-fertilizer for raising the overall crop productivity [19].

Table 1: Effect of Nanoparticles on Growth, Photosynthesis, and Stress Tolerance in Different Crops

Crop	Effect on Growth	Photosynthesis Rate	Stress Tolerance	Reported Effective Concentration	References
Barley	Significant increase in plant height and tillers	Enhanced leaf photosynthetic rate and stomatal conductance	Mitigated the negative effects of Cerium Dioxide NPs	1000mg/kg	[20]
Spinach	Increased overall plant growth	Increased Chlorophyll content	Protection of Chloroplast membranes and prevention of aging	20mg/kg	[21]
Rice	Increased plant height and biomass	Improved Chlorophyll content and MDA levels	Alleviated Cadmium toxicity stress though Cd in grains remained high	50-100mg/kg	[22]
Pinto Beans	Enhanced water uptake and light absorption	Increased photosynthetic efficiency through Rubisco activation	Boosted Antioxidants activity and reduced ROS accumulation	16-80mg/kg	[23]
Tomato	Increased fruit yield	Higher photosynthetic rate and electron transfer rate at optimal concentration	Fruit hardness increased but higher concentration led to negative effects	100mg/kg	[24]

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Coriander	Improved plant growth	Enhanced Carotenoid and nutrient content	Increased essential nutrients (Nitrogen, Potassium, Phosphorous)	6mg/kg	[25]

4. Other Crops

TiO_2 NPs boosted the Rubisco carboxylation that led to higher photosynthetic carbon reactions, enhanced photosynthetic rate, growth parameters, carotenoid and chlorophyll content [26]. Plants which were treated with 24mM and 75mM of TiO_2 NPs had reduced lesions (85-93%) caused by Xanthomonas axonopodis [27]. Despite their benefits for pathogen resistance, TiO_2 NPs can negatively affect soil microbes, reducing denitrification enzyme activity and altering bacterial community structure after 90 days [28]. In Moldavian balm, TiO_2 NPs reduced oxidative damage, boosted essential oil content, and improved stress tolerance, though higher concentrations (200 mg/kg) caused toxicity through increased ROS levels [29]. Water deficiency stress significantly reduces leaf area in plants due to inhibited cell division and limited leaf enlargement. In sunflower, foliar application of Salicylic Acid (SA) and TiO_2 NPs helped sustain leaf area under water stress, with both treatments showing similar effects [30]. SA promotes mitotic activity in growth apices, aiding leaf maintenance under stress, while TiO_2 NPs protects leaves from salt stress and upregulates stress-related genes [30-32]. However, SA and TiO_2 NPs lessened these effects, with SA improving photo-assimilate translocation to seeds and maintaining cell turgidity [32, 33], while TiO_2 NPs protected chloroplasts and increased leaf phenolic content, mitigating oxidative stress [34]. Water stress decreased sunflower seed oil content and increased oil acid value and free fatty acid content, lowering biodiesel yield. Both SA and TiO_2 NPs improved oil quality by preventing the rise in free fatty acids, which is crucial for higher biodiesel yield [35]. TiO_2 NPs improved the production of biodiesel by stabilizing the TiO_2 nanocatalysts [36]. Severe water stress adversely impacts the biodiesel production and growth of sunflower. A combination of Salicyclic acid and TiO_2 NPs alleviated these impacts when applied at a concentration of 5mg/kg and 50mg/kg respectively. The combined application of these two enhanced the leaf area, biodiesel yield and oil quality. These treatments induced tolerance in the plant against the drought and other stress conditions as well as increased the total phenolic content [37]. Furthermore, a study specified an increase in the essential oil content in rosemary upon the application of TiO_2 NPs, till the concentration of 200mg/kg. This study introduced a novel approach for understanding the metabolic changes, uptake rate and translocation induced by nanoparticles in medicinal plants [38]. TiO_2 NPs improve the light absorption and thus the plant growth, stimulate Rubisco activity, increase the uptake of nitrate, and promote the transformation of inorganic substances to organic materials [39-41]. They positively influence photosystem II, thylakoid membranes, mitosis and plant hormones such as cytokinins and gibberellins [42, 43]. TiO_2 NPs extend the functionality of chloroplast by the increased light absorption, converting light energy into chemical energy [21]. Such photocatalytic characteristics make them significantly effective in decomposing organic contaminants and disinfecting viruses, bacteria and even cancerous cells [44]. TiO_2 NPs also facilitate in stabilizing CO_2 [45]. Such findings highlight the significance of TiO_2 NPs in improving plant performance and their potential environmental applications [46]. Figure 3 illustrated the positive impact of TiO_2 nanoparticles (NPs) on photosynthesis, highlighting how their application can enhance chlorophyll content and stimulate better photosynthetic efficiency in plants.

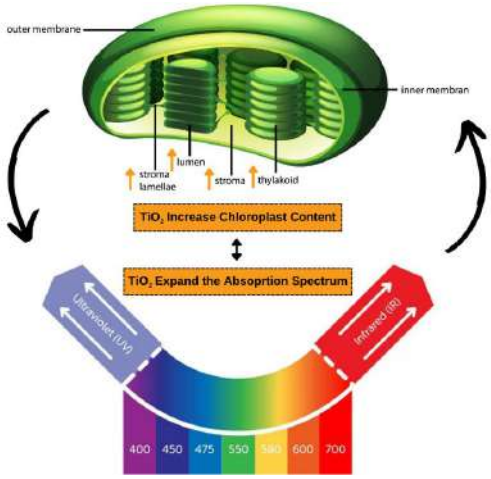

Figure 3: TiO_2 NPs' Positive Impact on Photosynthesis [47]

Efficacy of TiO_2 NPs under Varying Environmental Conditions

1. Soil Quality and Type

The performance of TiO_2 NPs in agricultural applications is significantly affected by soil characteristics such as pH, structure, organic matter content, etc. [47]. Studies have shown that TiO_2 NPs perform optimally in soils with a neutral to slightly alkaline pH value where NPs are more valuable stability and reduced agglomeration, thus enhancing their bioavailability to plants [48]. Soil structure also plays an important role, this is because soil rich in organic matter facilitates the diffusion of TiO_2 NPs, improving their interaction with plant roots and promote nutrient absorption [49]. In addition, the presence of essential nutrients such as nitrogen and phosphorus in together with TiO_2 NPs can enhance photosynthesis and increase stress tolerance in nutrient-deficient soil [38]. Table 2 summarized the performance of TiO_2 NPs in

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different types of soil. It emphasizes the effect of pH and organic content on nanoparticle performance.

Table 2: Performance of TiO_2 Nanoparticles across Different Soil Types

Types of Soil	pH Range	Organic Matter Content	TiO_2 NPs Performance	Reference
Sandy Loam	5.5 - 7.0	Low	Medium	[50]
Black Cotton Soil	7.0 - 8.5	Medium	High	[50]
Fine Sand	6.0 - 8.0	Medium	High	[51]
Chalky Soil	7.5 - 8.5	Low	Medium	[51]
Silty Loam	6.0 - 7.5	Medium	High	[52]
Coarse Sand	6.5 - 8.5	Low	Medium	[53]
Desert Soil	8.0 - 9.0	Very Low	Low	[53]
Alluvial Soil	6.5 - 8.0	High	High	[54]
Peaty Soil	5.0 - 7.0	Very High	Low	[54]

2. Irrigation and Water Management

Water availability is another important factor affecting the performance of TiO_2 NPs in agriculture. Research showed that adequate irrigation is essential for proper diffusion and mobility of TiO_2 NPs within the matrix of soil in order to reach the root zone where nutrients are absorbed [50]. In drought conditions, the performance of TiO_2 NPs tends to decrease due to limited mobility, restricting their interaction with plant roots. However, in a good irrigation system TiO_2 NPs were found to improve water use efficiency by reducing evaporation and improving column conductivity, especially under stress from heat and salinity [51]. Additionally, studies have shown that TiO_2 NPs can retain essential nutrients in the soil, reducing leaching during heavy irrigation and improve the availability of nutrients to crops [52]. This increased water and nutrient management results in improved growth and yield in many crops especially under conditions of abiotic stress [53].

3. Climatic Factors

The effectiveness of TiO_2 nanoparticles is also affected by climate, light intensity especially ultraviolet light. It is necessary to start the photocatalytic process that breaks down pollutants. Reduced efficiency of TiO_2 due to limited ROS generation under low light conditions [50]. High temperature increases the reaction rate of TiO_2, although too high temperature can cause nanoparticle agglomeration. As a result, the surface area available for the reaction is reduced [51]. In the same way, moderate humidity levels support the formation of hydroxyl particles. But excessive moisture reduces photocatalytic activity by forming a water film on the nanoparticle surface [51].

Potential Risks and Environmental Concerns

1. Ecotoxicity and Environmental Persistence

TiO_2 NPs are widely used in various industries, including agriculture, but concerns have emerged about their long-term impact on the environment. In soil ecosystems, studies have shown that concentrations as low as 1 mg/kg of TiO_2 NPs can disrupt microbial activity, leading to reduced nitrogen fixation by bacteria like Rhizobium that are crucial for plant growth [51]. Additionally, TiO_2 NPs have been shown to affect earthworm reproduction, with one study reporting a 27% reduction in the number of cocoons produced at concentrations of 100 mg/kg. When NPs enter water systems, they pose risks to aquatic life. For instance, research has shown that at 10 mg/kg, TiO_2 NPs can reduce the growth rate of algae by 30%, which can have cascading effects on the food web [54].

2. Human Health Considerations

There is growing evidence that TiO_2 NPs could pose risks to human health, particularly through bioaccumulation in the food chain. A study demonstrated that plants exposed to TiO_2 NPs at concentrations of 500 mg/kg exhibited significant uptake, potentially leading to human consumption. In laboratory studies on mammals, ingestion of TiO_2 NPs has been linked to inflammatory responses in the gastrointestinal tract, and at doses as low as 5 mg/kg body weight, they were found to cause oxidative stress in liver cells. While the exact mechanisms of toxicity in humans are still being researched, these findings underscore the potential risks of long-term exposure through contaminated food, particularly given that TiO_2 is classified as a Group 2B carcinogen by the International Agency for Research on Cancer (IARC) [55, 56].

3. Regulatory and Safety Guidelines

The regulation of TiO_2 NPs in agriculture and food systems is inconsistent across different regions. In the European Union, the use of TiO_2 NPs in food products has been banned since 2022, following the European Food Safety Authority's conclusion that their safety could not be established. In contrast, the United States has no specific regulations limiting TiO_2 use in food, though the U.S. Food and Drug Administration (FDA) allows it as a food additive, provided it does not exceed 1% by weight of the food's total composition. In agriculture, no global limits have been established for the use of TiO_2 NPs in soil or water, despite evidence suggesting that levels above 50 mg/kg can cause significant environmental damage. As the use of nanotechnology grows, there is an urgent need for more comprehensive, standardized guidelines on nanoparticle use to protect human and environmental health [57].

CONCLUSIONS

Titanium dioxide nanoparticles hold tremendous potential in revolutionizing agriculture by enhancing plant growth, improving stress tolerance, and increasing nutrient absorption, particularly in crops exposed to challenging conditions such as soil salinity. Their ability to improve photosynthetic efficiency, regulate ROS levels, and boost antioxidant defense mechanisms makes them a promising tool for modern farming. However, while the benefits of TiO_2 NPs are well-documented, their potential risks to the

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environment and human health cannot be overlooked. Future research must focus on optimizing their concentration, application methods, and safety protocols to harness their full potential while minimizing adverse effects. Comprehensive risk assessments and regulatory frameworks will be critical in ensuring that TiO_2 NPs can be safely integrated into large-scale agricultural practices, paving the way for sustainable and resilient food production systems in the face of global challenges like climate change and population growth.

Authors Contribution

Conceptualization: LZ, AT
Methodology: LZ, AT, EUN, AR, AA, DM, HE
Formal analysis: LZ, AT, EUN, AR, AA, DM, HE
Writing, review and editing: LZ, AT, EUN, AR, AA, DM, HE

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

All the authors declare no conflict of interest.

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Review Article

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Biotechnological Approaches to Discovery of Drugs for Veterinary Use

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ABSTRACT

This review examined the application of biotechnology to veterinary drug discovery, highlighting its efficiency and potential for creating novel therapies for a wide range of animal diseases. Compared to conventional methods, biotechnological models offer several advantages, such as reduced time and cost. These models also allowed for an abysmal empathetic of disease mechanisms, facilitating the development of highly targeted treatments. Gene therapy is a significant area of research, demonstrating considerable potential in addressing various veterinary conditions. Its successful application includes the management of ocular disorders in dogs, cardiovascular and renal issues in cats, osteoarthritis in horses, and metabolic disorders like diabetes in dogs. Advances in genome sequencing and proteomics have enabled researchers to understand animal proteomes better, leading to the documentation of potential drug targets and the development of more precise therapies. vetinformatics, which uses computational tools and big data analysis, is vital for accelerating research and development in veterinary science. The convergence of biotechnology and Artificial Intelligence (AI) presents considerable promise for the future of veterinary drug discovery. AI-powered algorithms can analyses large datasets, identify patterns, and predict drug efficacy, thus expediting the drug development process and creating more effective treatments. Continued investment in these areas is essential to realize the transformative potential of biotechnology for improving animal health and advancing veterinary science.

INTRODUCTION

The ability to employ live things or substances to enhance or rebuild an artefact, develop floras or faunas, or evolve microorganisms for specific uses is a broad definition of biotechnology [1]. An excellent example of a well-established use of biotechnology is traditional animal breeding, which involves the collecting and breeding of phenotypically desired individuals [2]. The most modern biotechnology, however, is derived from new discoveries like recombinant DNA, which is a genetic material found in all existing belongings, from microbes to elephants, and which limits and controls every function of living things [3]. Through genetic operations by means of microorganisms and vector hosts, DNA machinery and related methods, monoclonal antibody methods, embryo manipulation technology, and Polymerase Chain Reaction (PCR) have highlighted the feasibility of modifying biological systems for the benefit of humankind [4]. Despite the fact that biotechnology appears to have benefited human medicine the most, affluent countries have largely been the only ones to successfully implement veterinary biotechnology. In particular, there are very few examples of biotechnology being successfully applied to advance animal farming and

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health in underdeveloped countries. Therefore, the tenacity of this study is to evaluate readily obtainable biotechnologies that may be used in the diagnosis and treatment of diseases, identify those that have been or may be used in Africa specifically, and in other countries. The description of each portion is not given much weight, given the breadth of the subject matter. In contrast, an attempt is completed to highlight the skills that are thought to have present or future use in the veterinary medical profession. This review study ends with a brief summary of the challenges relating to the potential environmental risks of inherited engineering and other biotechnologies, which call for their moral assessment for a worldwide regulatory framework [5]. Gene Therapy in Veterinary Medicine: Gene therapy's use in veterinary medicine One of the outcomes of developments in molecular biology is gene therapy, a beneficial approach in which a functional gene is introduced into a cell to treat a metabolic defect or to add a new function [6]. In both human and veterinary medicine, gene therapy holds promise for treating cancer and other hereditary illnesses [7]. Combining chemotherapy and cytotoxicity with immunomodulatory therapy's anti-tumor immune responses inhibits the growth of tumors in a variety of cancer types, and Electroporation (EP) seems like a feasible way to carefully and effectively combine these treatments [8]. Therefore, electroporation is a legitimate method for introducing substances into host cells, including plasmid DNA (pDNA) and chemotherapeutics. Since EP is a safe and effective way to deliver a range of materials (such as ions, cytotoxic medicines, and nucleic acids) into target cells and tissues without endangering them, it is being employed more and more in the scientific and medical professions [9]. In EP, the agents are transported into the cytosol by short electric pulses that open temporary holes in the cell membrane. Numerous veterinary clinical trials have shown the protection and effectiveness of Electro Chemotherapy (ECT), chemotherapy administered using EP, since EP frequently does not result in any serious negative side effects [10]. Gene therapy has also demonstrated efficacy in large animal models of X-linked retinitis pigmentosa, potentially leading to its eventual translation into human treatments: Aden associated virus-functional coagulation factor VIII (AAV-FVIII) liver gene therapy was successful in two outbred, privately owned dogs with severe Hemophilia A (HA) involved; they prevented 90% of expected bleeding episodes and demonstrated persistent expression of 1-2 percent of normal FVIII levels [11]. Aden associated virus-functional coagulation factor VIII (AAV-FVIII) liver gene therapy demonstrated that coexpression of Glucokinase (GCK) and insulin can create a "glucose sensor" in skeletal sway, improving glucose absorption and reversing hyperglycemia in diabetic mice.

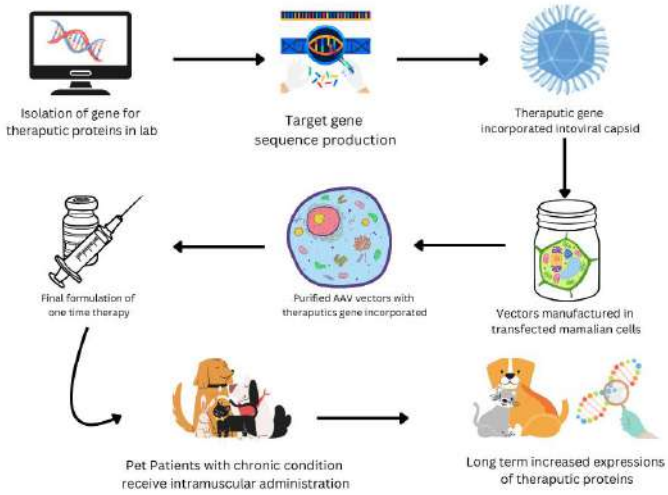

Figure 1: Development Process of Gene Therapy for Pets with Chronic Conditions

This diagram outlined the process of developing a gene therapy for pets with chronic conditions. It begins with isolating and producing a target therapeutic gene, which is incorporated into viral capsids to create viral vectors. These vectors are manufactured in mammalian cells, purified, and formulated into a one-time treatment. The therapy is administered via intramuscular injection, leading to long-term expression of the therapeutic protein to manage the condition effectively. Animal Ocular Conditions: The treatment of eye disorders is some of the primary applications of gene analysis in internal faunas. Many writers believe that keratitis of different etiologies will benefit from the application of gene therapy. Furthermore, it may help with glaucoma in dogs as well as disorders like neoplasia, corneal dystrophy, desmoids, chemical tingles, cuts, and corneal deterioration [12]. Adenovirus (AV), Lentivirus (LV), and the Adeno-Associated Viral Vector (AAV) containing the target genes are among the methods that have been suggested for the transmission of inherited material for the therapy of this condition [13]. Most hereditary retinal disorders are currently treatable. Researchers are particularly interested in gene therapy for degenerative diseases because some of them might also be natural models of these conditions [14]. A recessive modification in the RPE65 genetic factor causes congenital night blindness in Braid dogs, and Leber's congenital amaurosis type 2 is thought to be the human counterpart of this condition. A genetic factor builder based on AAV encompassing the beneficial gene was injected subrationally into 26 dogs in order to correct this condition. Of the 26 eyes that established gene therapy, 23 had positive results, and the treatment's effects persisted for at least five years. Throughout their lives, the canines showed no signs of negative effects from the genetic therapy. In RPE65-mutant dogs, another study showed that ocular

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administration of a therapeutic dose of AAV-2/2. RPE65 did not result in systemic or retinal toxicological effects [15]. Gene Therapy for Cardiovascular System Disorders: In the United States, almost 2 million domestic cats suffer from Chronic Renal Failure (CRF) and the related erythropoietin tin-sensitive anemia [16]. Using viral vectors to transfer the erythropoietin gene to cats is one of the possible gene therapy uses that is presently being investigated. The kidneys generate the hormone erythropoietin, which controls the bone marrow's erythrocyte production. Insufficient levels of hormone are frequently produced when a cat's kidneys are injured. As a result, anemia affects many cats with renal disease, which can lead to lethargy and decreased appetite [17]. Currently, human erythropoietin injections are used for treatment. Many cats' immune systems, however, view this erythropoietin as alien. We can prevent frequent injections of foreign protein by creating methods to transfer feline erythropoietin genes to cats lacking this hormone. Physiologically active feline erythropoietin can be synthesized in vitro using a replication-defective delivery method, according to in vitro research on the transfer of the erythropoietin gene to cells [18]. AAV was not employed as a viral vector by the creators of this genetic factor analysis technique. This was because earlier studies on gene therapy using AAV, which expresses recombinant feline erythropoietin, demonstrated that when given intramuscularly, it raised the hematocrit levels of healthy cats for seven weeks. Conditions Affecting the Skeletal System: Since the produced therapeutic proteins in gene therapy target the specific area without affecting the body as a whole, it may be the perfect way to treat joint problems. The necessity for frequent intra-articular injections of conventional medicinal medicines is also eliminated by gene therapy, which offers transgenes long-term expression [19]. In Osteoarthritis (OA), a chronic inflammatory disease, the pro-inflammatory cytokines interleukin 1(IL-1) and tumor necrosis factor α play a critical role. By limiting the combination and activity of IL-1, a gene therapy based on an adenovirus vector was used to treat OA in horses. The results of this gene treatment in vivo included significant improvements in experimental parameters, a reduction in pain compassion, and the preservation of articular cartilage in horses [20]. Moss and associates expected gene therapy to be used in a different way to treat OA. Since there are now no effective medications that may stop or reverse the progression of OA, the researchers referred to this as courtesy. The proposed strategy is based on the characteristics of interleukin-10 (IL-10). The suggested approach is predicated on interleukin-10's (IL-10) features. Moss and co-researchers created a gene research grounded on the AAV vector with the beneficial equine species particular gene IL-10. It is a cytokine with a strong anti-inflammatory consequence that is predominantly molded by immune cells. In vitro, IL-10 has also been shown to have an apoptotic effect on chondrocytes [21] and to maintain connective tissue homeostasis by inhibiting matrix metalloproteinase movement. By delivering the genetic component via the AAV vector in a mouse model of inflammatory soreness, in vitro revisions have demonstrated a flagging of the seditious cataract and chondroprotective capabilities related with overexpression of IL-10 [22]. It was feasible to caricature the inflammatory responses in the joints of OA-affected horses by administering this medication intra-articularly, which stopped cartilage degradation and improved osteoarthritis symptoms. The feasibility of administering the suggested gene preparation intra-articularly is demonstrated by its chondroprotective potentials [23]. Conditions Linked to Metabolic Disorders: There is presently no cure for diabetes, a chronic illness. Diabetes onset is highly correlated with increasing dog age. According to research by Heeley and associates, dogs older than eight years old are considered to be at risk. However, there was no correlation between this sickness and the animals' sex, which was consistent with prior research [24]. Achieving normoglycemia and preventing hypoglycemia are the objectives of any treatment for insulin-dependent diabetes. Exogenous insulin treatment is unable to completely prevent the illness's consequences, which result in substantial morbidity, a decreased quality of life, and fatality [25]. In canine diabetes studies, AAV serotype 1 vectors containing insulin and glucokinase transgenes have been used as a single intramuscular injection for gene therapy. The enzyme glucosidase, which activates glucose phosphorylation, is the part of this system that reacts to elevated intracellular glucose levels, and the creation of low, consistent levels of insulin has other positive effects on metabolism [26]. The improvement of insulin and gluco-kinase in muscle tissue did not have any adverse effects on the muscles or the rest of the body, allowing for long-term observations (more than two years) of dogs that had established the gene medication inoculations [27]. At the same time, histology investigations showed that a significant amount of the injected AAV vector was found inside the muscle. Even with intense physical activity, studies have shown that this method works well for treating diabetes when consuming large amounts of glucose raises the risk of hypoglycemia episodes. Genome Sequence-Based Modern Drug Discovery: It is impossible to overstate the significance of whole genome sequencing for contemporary drug development methodologies. The majority of human proteins can be categorized into structurally and

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mechanistically related groups based on sequence homology, and researchers now know the whole complement of proteins encoded by the human genome. Using bioinformatics data combining techniques, gene purpose analysis from high throughput investigations of protein-protein connections can be separated into networks and pathways [28]. A thorough components list of all the proteins found in the human body has been provided by the genome's sequence, and high throughput screening methods allow podia to expose these proteins to millions of tiny chemicals [29]. Therefore, it is impossible to overstate the importance of protein and genome sequences in today's drug discovery process. Using Proteomics in Drug Development: Although the number of proteome studies in veterinary medication and animal well-being has grown recently, they still make up a small portion of the extensive corpus of findings in the proteomics canon. In veterinary medicine, the proteomes of animal tissue and biological solutions may be examined for health and disease; in this context, there are similarities to human disease research. Nevertheless, there are other characteristics of comparative proteomics that contribute to the scientific value of proteomics in species like fish, cattle, dogs, poultry, cats, pigs, and horses [30]. These include: Animal proteomics is a separate field of study that applies to the biology and pathology of native species, offering important insights into the basic characteristics of each species. Comparative proteomics provides intriguing insight into the evolution of species' proteomes by comparing the similarities and differences between proteomes in health and sickness across species. Compared to using rodents, investigational proteomics in internal animals has intrinsic rewards. For example, time series studies can employ multiple sampling more frequently, and noninvasive (milk, slobber, urine) or slightly invasive (serum, plasma) samples can be obtained in large enough quantities for multiple analyses. In contrast to human disease research, which can merely be conducted on persistent samples from usual disease, proteomics can be used to study both investigational and natural disease progressions in the same animal [31]. As new medications get closer to controlling approval, species other than rats are frequently better matched as mockups for human bodily processes. For instance, pigs and dogs are needed for drug safety testing. Understanding the populace heredities of species where periods of recognized breeding provide a priceless reserve on proteome–genome interactions can help to facilitate the communication among the proteomic phenotype and inheritances in domestic faunas.

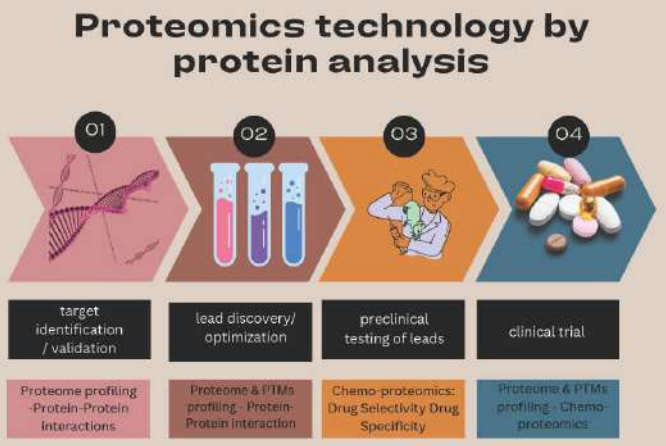

Figure 2: Proteomics and Vetinformatics in Drug Discovery for Animal Health

Proteomics technology used protein analysis to support different phases of the drug discovery process (global protein profiling, protein-protein interaction profiling, PTMs profiling, and chemo-proteomics). Vetinformatics for Drug Discovery: Animals used for livestock are vital to the existence. Public health depends on science-ran revolution in veterinary exploration that helps humans and animals as folks and populations [32-33]. This includes studies on the basic biology and welfare of animals as well as the prevention, diagnosis, and treatment of disease. Numerous chances to enhance both human and animal wellness are presented by this innovation. In addition to the challenges posed by a rapidly expanding human population that needs proper food and nutrition, veterinarians currently face a number of issues made worse by climate change, such as the emergence of new diseases. To decipher the intricate molecular mechanisms of cattle systems, interdisciplinary techniques and veterinary science must be integrated immediately [34, 35]. Research on the operation of livestock systems is vigorous and continuous. Biologists may now learn about biological systems both statistically and qualitatively because to developments in computer science, statistical methodologies, mathematical science, and information technology [36, 37]. Computers play a key role in these scientific advances because they are essential to the research and development industries and have become an important tool for researchers. Although computers can handle big data easily in the "omics" age, the term "bioinformatics" was not coined until the early 1970s by Hogeweg and Ben Hesper, when DNA sequencing was still in its infancy [38]. Prior to 1952, there was a great deal of debate regarding the role of DNA as genetic material, specifically how pure DNA from a virulent bacterial strain may be absorbed by a non-virulent strain to confer virulence. However, their findings were not immediately accepted by the scientific community, as many scientists

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believed that proteins carried genetic information instead of DNA [39]. In 1952, Hershey and Chase established that bacteriophage-infected bacterial cells eat and transmit DNA, demonstrating the role of DNA as a molecule that encodes genetic information. Although the fundamental function of DNA was established at this time, nothing was known about the structure of the DNA molecule. It was only known that its monomers, or nucleotides, were present proportionately [40]. Watson and Crick were ultimately responsible for discovering the double-helix structure of DNA. Despite this achievement, it would take another 25 years to develop the first DNA sequencing techniques and another 13 years to interpret the genetic code. Therefore, the investigation of proteins, whose chemical composition was already better understood than that of DNA, surpassed DNA analysis using computational techniques by around 20 years [41]. Protein analysis served as the foundation for bioinformatics in (Beall CJ *et al.*,) due to notable advancements in the crystallographic determination of protein structures. The first protein sequence to be published was that of insulin, or the arrangement of its amino acids [42, 43].

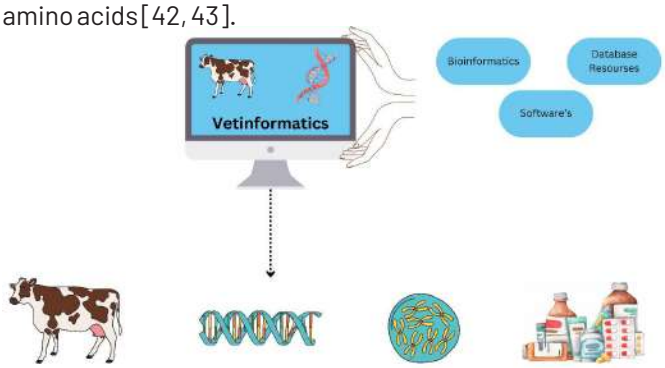

Figure 3: Role of Vetinformatics In Understanding And Improving Livestock Systems

This diagram highlighted the role of Vetinformatics in understanding and improving livestock systems. Vetinformatics combines bioinformatics, database resources, and specialized software tools to analyze genetic information of animals. The diagram illustrates how the genes of livestock, represented by DNA and microorganisms, are studied and modified to develop products such as medicines and other biotechnological advancements. This approach enhances the use of animal genetics for health, productivity, and medical purposes, showcasing the integration of technology in veterinary sciences. Biotechnological Approach in Drug Delivery: Medication distribution is one of the most important components of a drug discovery and development program. The distribution of drug molecules at a certain place and speed may be the goal of the drug-delivery system's design. Targeting medications through a delivery system allows for the development of the capacity to guide a drug to a cellular target area of interest for its efficient use. Giving drug carriers activity by including groups or ligands that are specifically recognized by receptors on the surfaces of the cells of interest is known as active targeting of drug molecules [44, 45]. Numerous drug-carrying systems, including liposomes, dendrimers, nanotubes, polymeric micelles, polymeric conjugates, chitosan-based polymers, lipid crystals, nanoparticles, and drug lead molecules, phytochemicals, and derivatives, are included in an effective drug-delivery system based on biotechnology. When it comes to medicine distribution, these systems are crucial. These systems are better options for drug delivery because of their low toxicity, biodegradability, biocompatibility, derivatization, and immunomodulatory effects. The effectiveness of a medication is also significantly influenced by how it must be taken. Drug delivery and targeting have advanced significantly as a result of academics' increased efforts over the past ten or so years to create drug delivery systems [46]. Using Bacteria to Deliver Drugs: New technologies for manipulating cellular genetic information have proliferated in recent years. Thanks to the engineering skills, we can create cells with synthetic genomes [47, 48], which could lead to the development of therapeutic or drug-producing bacteria. It may be able to give "synthetic" organisms only the genetic information they need to function, leaving out any potentially harmful and interfering genetic and metabolic material. Modular genetic components might be logically added to these cells to produce an organism with the appropriate phenotype. In the future, organisms like robots could be programmed by combining genetic devices. Initial efforts resulted in newly linked genetic circuits with newly combined biosensor modules [49]. For instance, an E. coli strain that responds to light was created by fusing a cyanobacteria phytochrome's chimeric sensor domain with an E. coli signal transduction domain [50]. It is possible to develop additional sensors and rewire downstream signaling at will by extending the engineering of novel bacterial sensors that control genes in response to novel environmental conditions. For instance, promoters could be designed to integrate (many) distinct signals [51, 52]. This strategy can be expanded to create live bacteria as probiotics, anti-tumor medicines, and tailored delivery methods for live immunization. Numerous bacteria infiltrate tumors and are designed to destroy them by secreting cytokines and TNFα, which is a chemotherapeutic prodrug.

CONCLUSIONS

The conclusion of the article is that biotechnology has made significant progress in veterinary drug discovery. The use of biotechnological models is a powerful way to identify

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and develop new therapies for a wide range of animal diseases. These models offer several advantages over traditional methods, including reduced time and cost, a deeper understanding of disease mechanisms, and the development of highly targeted treatments. With significant promise for treating a range of veterinary ailments, gene therapy has become a particularly exciting field of study. Gene therapy's transformational potential in veterinary medicine is demonstrated by its effective use in treating ophthalmic illnesses in dogs, cardiovascular and renal problems in cats, osteoarthritis in horses, and metabolic disorders including diabetes in dogs. Researchers now have a thorough understanding of animal proteomes thanks to developments in genome sequencing and proteomics, which has helped them identify possible drug targets and create more targeted treatments. Utilizing big data analysis and computational tools, the integration of vetinformatics is essential for boosting veterinary science research and development. Overall, the conclusion emphasizes that biotechnology holds great promise for improving animal health and advancing veterinary science as a whole.

Authors Contribution

Conceptualization: OFA, SUR, AS, IUK, MWA, MAS, FGTY, MAA, AR, AB
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All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

All the authors declare no conflict of interest.

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Review Article

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Common Sage (*S. Officinalis*)- A Natural Medicine and Its Health Benefits

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ABSTRACT

Common Sage (*Salvia officinalis*) belongs to the Labiatae/Lamiaceae family Indigenous to the Middle East and Mediterranean regions but today has been world-widely revolutionized. *Salvia officinalis* exhibited an extensive array of functionally bioactive chemical constituents that have been employed in the treatment of multiple chronic health conditions and has been under primarily extensive research documenting its novel biological significance and exploring revolutionary biological impacts on well-being revealing an extensive range of pharmaceutical significance. These investigations demonstrated that besides curing relatively mild illnesses, *S. officinalis* possesses potentially revolutionary natural remedial treatment alleviating chronic health-related life-threatening conditions by possessing anti-carcinogenic, anti-depressive, and anti-diabetic efficacy. This review will shed light on *Salvia officinalis* pharmaco-medicinal biological properties signifying its anti-oxidative, immune-modulatory, anti-tumor, anti-hyperlipidemic, Neuro-cognitive efficacy, Microbicide activities, and its toxicological effects to preserve and restore health by highlighting the significance of this plant as a fantastic herb with multi-factorial health and wellness benefits.

INTRODUCTION

Salvia officinalis L. (Sage) is a perennial spherical shrub that belongs to the family of Labiatae/Lamiaceae containing approximately 900 species worldwide and the flowering plants are mostly aromatic with distinctive color variations [1] globally grown owing to its culinary, pharmacological, and flavour enhancing characteristics [2]. *S. officinalis*' customary application and traditional folk-medicinal utilization are globalized around the world, and the Latinian meaning of this plant *Salvia* is curative demonstrating health-promoting properties signifying the importance of this plant as a pharmacologically therapeutic herbal botanical [3]. The aerial plant parts are strategically employed against the therapeutic treatment of gouty arthritis, rheumatic arthritis, anti-hyperglycemic, and diarrhea throughout Asian and Northern American countries [4]. Additionally, *S. officinalis* possessively employed against the pharmacological treatment of digestive problems such as Indigestion, ulceration, cardialgia, and acid indigestion, along with upper airway complications such as pharygitis and inflammatory triggers [5]. Historically, *S. officinalis* tea infusions are traditionally utilized against treating gastrointestinal and cardiovascular diseases such as angina, chest cold, oral infections, Tonsilitis, depressive disorders, hyperhidrosis,

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skin inflammation, and multiple chronic conditions [6]. Additionally, Research investigations potentiated the utilization of *S. officinalis* oil as a therapeutic remedial approach as a de-flatulent agent, spasmolytic, skin disinfectant, and astringent [7]. *S. officinalis* vitally essential oil-based infusions is a mixture of volatile components utilized for therapeutic screening of anti-carcinogenic, microbicides, and free radical scavenging agents [8]. Sage also has noticeable beneficial effects in age-related cognitive disorders [9]. Investigational analysis exhibited the exploration of *S. officinalis* in inducing irritability, tranquillity, and explicit memory within Alzheimer's patients [10]. *S. officinalis* leafage is customarily utilized within the agri-food sector owing to its flavor enhancer, anti-oxidative, and micro-biocidal-related pharmacological health benefits [11]. Additionally, *S. officinalis* hepato-protection or anti-hepatotoxic remedial effects were outlined by investigational approaches as far as the herbal botanical exhibited therapeutically curative effects about anti-hepatotoxic efficacy and is currently employed as a conventional remedial medicinal approach with potentiated hepato-protective effects around the globe [12, 13].

Pharmacologically Functional Properties

Common Sage (*S. officinalis)* possesses an extensive range of functional bioactive constituents which through a diverse range of extensive investigatory techniques provide an enormous amount of physicochemical constituents which gradually becoming popular for its anti-oxidative, immune-modulatory, anti-tumor, anti-hyper-lipidemic, neuro-protective, microbicide and possessing no toxicological effects with extensive historical medicinally and culinary applications [14].

Anti-Oxidative Effects

Oxidative stress played a crucially pivotal role in the development, progression, and pathogenesis of many chronic illnesses disrupting the pathway between oxidative and anti-oxidative mechanisms within cells and tissues resulting in heart diseases, neurodegenerative disorders, mutagenesis, and metabolic abnormalities [15]. Oxidative stress resulted in Reactive oxygen species (ROS) production through mitochondrial-mediated electron transport chain (ETC) reactions, uncoupling of Nitric oxide synthase (NOS) enzymatic reactions, and Xanthine oxidoreductase activities. *S. officinalis* flavonoids and phenolic components are primarily responsible for its anti-oxidative potential and act as free radical scavengers by substantially improving anti-oxidative defensive activities owing to the presence of catalases, glutathione peroxidases, and superoxide dismutase activities [16, 17]. Multiple clinical analyses demonstrated *S. officinalis* potentiates anti-oxidative activities. *S. officinalis* enriched aqueous water enhances rats' hepatocyte cellular resistive action against oxidative damage [18]. Through an increase in glutathione peroxidase activity, it shielded and preserved hepatocytes' cellular activities by preventing hydrogen peroxide and dimethoxy-naphthoquinone-induced oxidative DNA damage [19]. *S. officinalis* isolated phenolic constituents such as carnosol, carnosic and rosmarinic acids, rosmadial, rosmanol, epirosmanol, methyl carnosate, and luteolin-7-O-β-glucopyranoside exhibited markable anti-oxidative activities [20]. *S. officinalis*-derived carnosol possesses radical scavenger activity comparable to that of alpha-tocopherol [21]. Within Streptozotocin (STZ)-induced diabetic rats rosmarinic acid exhibited demonstration of boosting pancreatic catalases, glutathione peroxidases, and SOD activities, radical scavenging activities are 15 to 20 times greater than the synthetically commercialized water-solubilized Vitamin E "trolox" [22]. Additionally, to the chemical component rosmarinic acid, other flavonoids majorly quercitin and rutin possess stronger anti-oxidative properties [23]. For instance, rutin reverses, suppresses, and mitigates elevated lipid peroxidation levels and causes a substantial reduction in the kidney's thiol concentration [24]. Aqueous isolated extract of *S. officinalis* possesses potent anti-oxidative and virucidal activities. An observational study analyzed that *S. officinalis* decoction tea administration to diabetic rats for fourteen days resulted in improving anti-oxidative defensive mechanisms, improves catalases and superoxide dismutase (SOD) activities [25].

Immune-Modulatory and Anti-Nociceptive Activities

Inflammation and pain are the two main symptoms in response to tissue damage. Non-steroidal anti-inflammatory drugs are the key pharmacological treatments. However, the clinical administration of these drugs is accompanied by unpleasant side effects such as gastrointestinal and cardiovascular complications [26]. Therefore, investigating new anti-inflammatory and antinociceptive with positive health implications remains an attractive subject. Pharmacological studies have exhibited that *S. officinalis* possesses stronger anti-inflammatory and anti-nociceptive effects [27, 28]. For example, it has been shown that this plant controls and mitigates neuropathic pain in chemotherapy-induced peripheral neuropathy. Among different extracts of *S. officinalis*, the chloroform exhibited potential anti-inflammatory action, while the methanol extract along with the essential oil demonstrated lower mechanistic action [29]. Flavonoids and terpenes potentially contribute to the anti-inflammatory and anti-nociceptive herbal action [30]. Mansourabadi et al reported that flavonoids extracted from *S. officinalis* reduce and mitigate inflammation in the

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mouse carrageenan model by inducing analgesic activities in a dose-dependent manner [28]. Topical application of rosmarinic acid inhibits epidermal inflammation. Manool, carnosol, and ursolic acid are of the terpenes/terpenoids with anti-inflammatory potential [30]. The anti-inflammatory action of ursolic acid is twofold more potent than an anti-inflammatory medication indomethacin [29]. This mechanistic action of *S. officinalis* constituents may also possess stronger anti-nociceptive efficacy in patients with pharyngitis [31]. However, this effect of *S. officinalis* is quite in line as compared to benzamine hydrochloride in controlling postoperative pain after tonsillectomy or adenoidectomy [32].

Anti-tumor and Anti-Mutagenic Effects

Carcinogenesis is characterized by aberrant cellular growth which possesses a propensity to propagate uncontrollably as well as in some instances metastasis spread to various bodily organs. The key component in the development, proliferation, and propagation of cancerous cells is the ability of the tumors to produce a significant proportion of new blood vessels collectively manifested as angiogenesis. Angiogenesis played a significant triggering factor in the development, invasion, and metastasis of the vast majority of primary solid tumors [33]. Investigational research hypothesized that *S. officinalis* extract suppresses angiogenesis within in-vivo analysis at therapeutically pharmacological concentrations which act as a novel innovative strategy that possesses anti-angiogenic mechanisms [34]. *S. officinalis* extracted isolates uphold caspase-mediated cell death and exhibited growth-inhibitory activities on cell lines of Invasive ductal carcinoma (Michigan Cancer Foundation-7 (MCF-7)), mesonephric (HeLa), colon and rectal carcinoma (hematocrit test (HCT)-116, HCT15, CO115, HT29), Islets carcinoma (RINm5F), squamous cell carcinoma (Hep-2), Squamous cell carcinoma (A549), malignant melanoma (A375, M14, A2058, B16) and oropharyngeal carcinoma [35, 36]. Sage's ursolic acid significantly prevents angiogenesis, metastases, and cellular invasion of cancerous cells by preventing pulmonary colonization of B16 melanocytic carcinoma within in-vivo investigational analysis [37]. Macro-phagocytic releasing of Nitric oxidases and Tumor Necrosis factor-alpha is triggered by *S. officinalis* administration owing to the presence of several cytotoxic and anti-carcinogenic components. [38] Metastatic colorectal cancer (CRC) significantly contributes towards mortalities within the Western countries developed as a result of genetic and epi-genetically modifications verily signifying potential capabilities of *S. officinalis* dietary components to viably alter the epigenetic state and playing a significant role in preventing colonic metastatic oncogenes within experimental rats [39]. Another study hypothesized the experimental demonstration of substantially reducing the oxidative H2O2-induced DNA destruction through *S. officinalis* aqueous extract supplementation within in-vitro analysis. A few isolated diterpenoids from the *S. officinalis* roots were found to possess chemotherapeutic and DNA degradation activities within human colonic carcinoma cells Caco-2 and within hepatic cells HepG2 as analyzed through in-vitro analysis [40]. *S. officinalis* driven sesquiterpenoidal proportion encompasses alpha-humelene exhibited stronger cytotoxicity within human prostate cancerous LNCaP cells [40]. Trans-caryophyllene isolated from sesquiterpene fraction from *S. officinalis* possesses stronger cytotoxicity against melanotic malignancy and renal adenocarcinoma cells within human prostate carcinoma LNCaP cells [41]. A diterpenoidal manool causes cytotoxic effects within human cervical mesonephric and Grade IV astrocytoma [42]. Flavonoids such as rosmaricin acid potentially exhibited anti-cancerous protective effects by inhibiting or preventing the growth of skin carcinoma cells within a rat model of dimethyl Benz (a) anthracene and also prevented bone metastasis due to breast carcinoma [43, 44]. Anti-carcinogenic effect of flavonoids possesses inhibitory activity on key signalling pathways involved in the regulation of G1 Phase Progression within proliferating hepatocytes, reactive oxygen species (ROS) generation, NF-κB factor, and exempted probable reduction within pro-inflammatory cyclo-oxygenase genetic expression [45, 46]. *S. officinalis* supplementation potentially inhibited various angiogenic stages (proliferators, migratory, adhesion, and tubular formation phase) within endothelium cells [47]. *S. officinalis* essential oil possesses demonstrated anti-mutagenic action by reducing or preventing UV-induced mutations within *E. coli* and S. cerevisiae [48]. Methyl methane-sulphonate mutations were inhibited and mitigated through the use of S. officinalis infusion tea [49]. *S. officinalis* methanolic isolate exhibited preventative effects against cyclophosphamide-induced gene toxicity within rat models [50, 51]. Anti-mutagenic efficacy of *S. officinalis* is strongly attributable to its mono-terpenoidal components such as thujone, camphor, limonene, and 1,8-cineole potentially exhibiting anti-carcinogenic and anti-mutagenic effects [48].

Anti-Hyperlipidemic Effects

The beneficial effects of various herbal medicinal plants influence regulatory effects on the body's metabolism majorly on adipogenesis, lipolysis, and serum lipids [52]. Pancreatic lipase plays a significant role in lipid degradation with a regulatory effect on fat absorption and metabolism [53]. Various studies on anti-obesity compounds from natural medicine, *S. officinalis*

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characteristics of its natural components, and its mechanistic action on pancreatic lipases and fats digestion were outlined. *S. officinalis* methanol extract signified inhibitory activity on pancreatic lipases and suppressed serum triglyceride elevation within an olive-oil-supplemented rat model [54]. Two diterpenoids such as carnosol and carnosic acid isolated from the methanol extract of *S. officinalis* possess inhibitory action on pancreatic lipases. Moreover, carnosic acid considerably decreased TG percentiles within the olive oil-loaded rat model by reducing body weight gain and accumulation of epididymal fat within the high fat-fed mice model after fourteen days [54]. *S. Officinalis*-derived carnosic acid substantially decreased triglycerides (TG) spike, total cholesterol, low-density lipoprotein (LDL) levels, excess body weight, and abdominal fat mass within diet-supplemented obese rats. *S. officinalis* investigational analysis possesses a beneficial effect on serum lipid profile with hyperglycemic animals which probably exhibited low levels of triglycerides, creatinine, urea, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels within Streptozotocin (STZ)-induced diabetic rats [55]. *S. officinalis* investigational analysis hypothesized significant improvement in TG, total cholesterol, LDL, very-low-density lipoprotein (VLDL), and 2 hours' post-prandial blood glucose levels within hyper-lipidemic and hyper-glycaemic patients [56]. *S. officinalis* flavonoid content has also been shown to exhibit therapeutically advantageous effects in managing dyslipidemia within hyper-glycaemic and hyper-cholesterolemic patients [22]. *S. officinalis* isolated rutin fraction decreases adiposity and body weight gain within high fat diet-induced obese rat model further improving mitochondrial DNA composition and mitochondrial biogenesis-related genetic expression. (e.g., Peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1α, Nuclear respiratory factor (NRF-1), transcription factor A, and nicotinamide adenine dinucleotide-dependent deacetylase) within skeletal muscles [57].

Neurocognitive Development

Several plant-derived botanical extracts from *S. officinalis* are widely recognized for their beneficial effects in treating memory disorders and in ameliorating depressive symptoms [58]. Increasingly supportive evidence apprehended *S. officinali's* potential neuro-cognitive and memory-boosting effects. In the experimental model, it has been demonstrated that ethanolic extract from *S. officinalis* enhances and improves cognitive and passive memory retention learning within rats. Garden Sage (*S. officinalis*) has been prescribed for ages to restore lost or deteriorating mental abilities, such as those triggered by Alzheimer's disease (AD), and boosts immediate world recall efficiency [59]. In AD, the enzyme acetylcholinesterase is degraded and inactivated acetylcholine, which is a neurotransmitter associated with the transmission of signals between synapses. Anti-AChE inhibiting drugs function by counteracting acetyl-choline depletion and enhancing acetylcholine content within the brain cells. *S. officinalis*-derived essential oil inhibited 46% of AChE activities at the concentration range of 0.5mg/ml [60]. *S. officinalis*-derived hydro-alcoholic extract and its main flavonoid rosmarinic acid enhance cognitive learning within healthy rats by preventing diabetes-related memory impairments [61]. *S. officinalis*-derived hydro-alcoholic isolate attenuated morphine-induced memory impairments [62, 63]. Additionally, *S. officinalis* supplementation enhances cognitive learning and memory retention and possesses positive effects on mood disorders with an increase in alertness, peace, and contentment levels with an increase in significant dosage relationship [64]. Ethanol-extracted isolates of this plant enhance and boost cognitive memory and ameliorate attention deficits within over-aged participants [65]. Plant essential oil aromas possess positive effects on neuro-cognitive development and mood disorders as investigated through experimental analysis. Moss *et al*, in the year 2010 analyzed that *S. officinalis* essential oil supplementation enhanced and improved long-term memory restoration and word recall possessing a noticeable improvement that influenced memory recollection as analyzed through the Cognitive Drug Research (CDR) Systematic Approach within healthy individuals [66]. *S. officinalis* supplementation benefited long-term protective effects against the emergence and progression of dementia by possessing long-term anti-oxidative and anti-inflammatory mechanisms [64] Sage's probable cytoprotective effect against amyloid beta plaques toxicity within nerve cells provides a pharmacological framework for conventionally using S. officinalis against Alzheimer disease. *S. officinalis*-derived rosmaricin acid exhibits neuroprotection, and antioxidant activities and causes caspase-mediated cell death which enhances its utilization in the treatment of AD [67, 68]. By demonstrated experimental trials, *S. officinalis* therapeutically supplementation exhibited potentially protective neurocognitive and memory-enhancing effects by acting as a promising therapeutically herbal remedial approach against Alzheimer's disease.

Microbicide Activity

S. officinalis potential anti-microbial and microbicide implications were supported through a wide array of experimental trials. *S. officinalis* derived ethanolic essential oil exhibited potential germicidal anti-septic and possesses high sensitivity as a bacteriostatic agent

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against gram-negative and gram-positive bacterial pathogenic strains (*B.cerus, L.monocytogens,* and *S. auerus*) and its mechanistic action primarily dependent on the type of extract used *S.officinalis* essential oil exhibited a substantial inhibitory effect on the growth of bacterium hydrophilum, Aeromonas Septicemia (MAS), Burkholderia pseudomallei and "Group D Shigella" possessing a weaker attraction for *E.coli* and *S. enteriditis* [69]. Furthermore, the bactericidal effect of *S. officinalis* has reportedly been claimed to induce fungicidal, and viricidal and act as an anti-parasitic chemical agent [56]. *S. officinalis* exhibited anti-fungicidal activity against *B.cinera, C.albicans,* and *C.parapsilosis* as analyzed through experimental analysis [70]. The bactericidal activity of *S. officinalis*-derived water-based extract within *in-vitro* analysis against specific food-decomposing bacterial species indicated substantial bactericidal effects against *B. mycoides, B. subtilis,* and *Proteus spp* [71]. The experimental analysis demonstrated that *S. officinalis* extracted essential oil acts as a potential substitute in line with alternative therapeutically anti-bacterial [72]. *S. officinalis*-driven terpenoidals, camphor, and thujonoic acid also exhibited substantial microbicide activities against *A. hydrophila, A. sobria, B subtilis,* and *klebsiella oxytoca* as analyzed through experimental trials [73]. Another analysis by Horiuchi K *et al.*, in the year 2007 hypothesized that oleanic acid and ursolic acid derived from *S. officinalis* exhibited growth inhibitory action against antibiotic-resistive microscopic bacteria such as enterococcus exhibited resistive action against vancomycin drug, Penicillium resistive *S. pnuemonia* and methicilin resistive *S. aureus*. The experimental analysis demonstrated that urosolic acid derived from *S. officinalis* exhibited stronger microbicidal activity against entero-bacterial *E. faceium* and multi-drug resistive bacterial species in comparison with ampicillin. Another experimental analysis showed that carnosolic acid, a dieterpenoidal from *S. officinalis* exhibited bactericidal properties potentiating the mechanistic action of antibiotic aminoglycosides within methicilin resistive *S. aureus* [74]. *S. officinalis* viricidal activity is probably intervened by safficinolide content which primarily features in its aerial sections [75]. Experimental analysis hypothesized probable efficacy and growth-inhibiting action of a hydro-alcoholic isolate from *S. officinalis* against certain dental diseases aggravated by *S. mutans, L. rhamnosus,* and *A. viscosus*. Given replacing herbal therapeutically conventional treatment with pharmacological therapies, *S. officinalis* anti-bacterial properties might be an alternative remedial approach for treating multiple diseases, particularly affecting the oral cavity [76]. Research revealed that Common Sage (*S. officinalis*) in combination with certain other herbal botanicals was analogous to synthetic preservative agents thereby demonstrating that water-soluble isolated extract can be employed in biotechnological research as a natural preservative component within the food sector [71].

Toxicological Effects

Multiple sub-clinical research investigations on animal trials have hypothesized that *S. officinalis* administration possesses no adverse consequences and does not trigger any negative adverse reactions [77]. However, prolonger administration and subsequent over-dosage of isolated ethanol extract and volatile oil (equivalent to greater than 15 g of the leaf), several undesirable outcomes including acro-cyanosis, sputum production, arrhythmias, light-headedness, vasomotor symptoms, hyper-sensitivity and in extreme condition seizures might occur. *S. officinalis* oil's mediated pro-convulsion action is possibly owing to its immediate effect on the peripheral neurological nerves [78]. Research trials hypothesized that *S. officinalis*-derived terpenoidal ketones such as camphor and thujonoic acid when taken in extremely high dosages might considered a potentially hazardous compound that could pose detrimental effects on fetuses and newly born infants. Consequently, *S. officinalis* consumption is not recommended for pregnant and lactating mothers [79]. Experimental research trials within animals demonstrated that the LD50 value for *S. officinalis oil* (oral administration) and methanol's isolated extract (intraperitoneal administration) is considered to be 2.6g/kg and 4g/kg correspondingly [59].

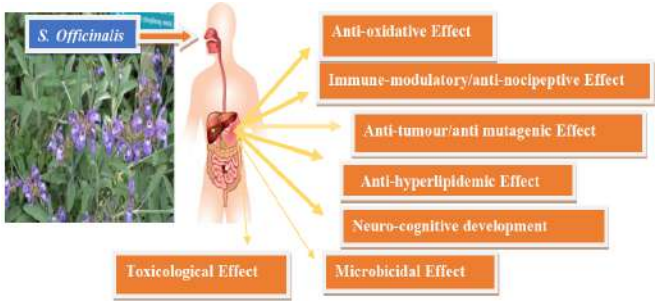

Figure 1: Pharmacological Beneficial Functional Properties of Common Sage (*Salvia Officinalis*)

CONCLUSIONS

The objective of this article is to undermine and explore the current advancements by probing the therapeutic significance and pharmacological effectiveness of Common Sage (*S. officinalis*) as a conventional medicinal herb gaining a lot of popularity in Today's World. Common Sage (*S. officinalis*) is rich in phytochemicals and antioxidants and possesses a wide range of bioactive components such as alkaloids, carbohydrates, fatty acids, glycosides derivatives, phenolic compounds, and poly-acetylenes exhibiting extensive biological effects, have proven to be exceptionally effective in the development of

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innovative natural pharmaceuticals regulating and preventing a variety of acute medical health problems as well as more serious and chronic health problems exhibiting anti-oxidative, immune-modulatory, anti-nociceptive activities, anti-tumor, anti-mutagenic effects, anti-hyperlipidemia effect, neurocognitive development, microbicide activities alongside toxicological investigational analysis of *S. officinalis* exhibited LD50 dosage for oral administration and methanol isolated extract (intraperitoneal administration) considered to be 2.6g/kg and 4g/kg respectively. Owing to *S. officinalis* therapeutically pharmacological significance, probable biochemical and medicinal experimentation along with human metabolically investigations ought to be the primary objective of our forth-coming studies and the prospective potential of *S. officinalis* has to be used in novel therapeutic medications.

Authors Contribution

Conceptualization: SI
Methodology: NA, SH, FH, SR, WA, AUK
Formal analysis: SI
Writing review and editing: SI, MKN

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

All the authors declare no conflict of interest.

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Impact of Zinc Phosphide on Hematology, Behaviour and Proximate Composition of *Oreochromis niloticus*

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ABSTRACT

Zinc phosphide is a rodenticide, crystalline compound of dark grey color. It is present in many pesticides and when sprayed on plants it gains entry into food and water. **Objective:** To evaluate the effect of zinc phosphide on the hematology, behavior and proximate composition of *Oreochromis niloticus*. **Methods:** In present research, fish were given a dose of zinc phosphide in aquarium for twenty days. Fish were divided into two groups, control, and treatment, each with three replicates. Treatment group was exposed to 1mg/ml of Zinc phosphide given to treatment group twice a day for 20 days continuously. When the experiment was completed fish were anaesthetized, dissected and blood was drawn to evaluate the hematological parameters. Fish activities swimming, gill movement, mortality and morbidity were recorded. **Results:** Zinc Phosphide exhibited variable impact on proximate composition. There was a significant decrease in values of crude fat and increase in value of crude protein and total ash in comparison to control group. Fish were active during the trial period they exhibited fast movement, no rubbing against the wall, and fish didn't harm each other in the treatment group. **Conclusions:** According to these results, zinc phosphide have harmful impacts on fish health. As a result, its usage must be carefully regulated to reduce environmental contamination and safeguard aquatic ecosystems.

INTRODUCTION

The aquaculture industry is flourishing rapidly and production of aquaculture is important. It is rapidly expanding as it provides protein-based food for human consumption [1]. This expansion necessitates sustainable practices to ensure the health and productivity of cultured species, such as Nile tilapia *(Oreochromis niloticus)*. It will approximately reach to 62% of total worldwide production by 2030 [2]. About 8,563,820 km2 area of Pakistan is covered with water which includes rivers, ponds, lakes, and water lodging areas [3]. Aquaculture also contributes approximately 1% of national GDP [4]. Pesticides control pests by killing, destroying, or mitigating their ratio. About 1.8 billion people globally are involved in agriculture. Europe has the utmost contribution (31.7%) followed by Latin America, Asia, Africa, and North America, which contribute 47.6% of the worldwide pesticide trading [5]. Herbicides are the major category followed by the Fungicide (17.5%), Insecticide (29.4%) along with others (5.5%) [6, 7]. The global trading forecast was predicted to reach $59 billion in the year 2016, pursuant to World Agricultural Pesticides [8]. Particularly, 5% of the rodent species are considered serious pests. The authorized state agencies as well as the United States Environmental Protection Agency (EPA) manage rodenticides along with their usage [9]. Zinc phosphide is a crystalline compound having a dark gray color. Zinc phosphide is considered a rodenticide because

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it is used to kill small mammals such as rats, mice, squirrels, and field mice [10]. Zinc phosphide can cause acute poisoning indirectly by accidental inhalation of phosphine gas which is generated during its usage and directly by ingestion [11]. Zinc phosphide is poisonous to non-targeted mammalian organisms, wild birds as well as freshwater fish [12, 13]. Food is sprayed with pesticides, particularly fruits and vegetables. It is absorbed through seepage into the groundwater and soil, where it may end up in drinking water. They also have the potential to disperse and pollute the air [14]. Tilapia is transported all over the world and is native to Africa. Tilapia does not harm other native species [15]. Tilapia belongs to Cichlidae family which is a freshwater fish. It is wholly connected with Africa along with the Middle East [16, 17]. One of the first fish species to be cultivated in the globe is the Nile tilapia *(Oreochromis niloticus)* [18]. Tilapias grow quickly, have thick, white meat, can tolerate unfavorable water environments, consume a variety of food kinds, reproduce readily without the use of advanced hatchery equipment, and forage at the foundation of the aquatic food chain. Recent research has demonstrated that insecticides, notably zinc phosphide, may adversely affect Nile tilapia *(Oreochromis niloticus)*. In Nile tilapia, acute poisoning from zinc phosphide has been associated with death and serious health problems, such as gill tissue destruction, liver dysfunction, and oxidative stress brought on by the production of reactive oxygen species (ROS) [19, 20]. Furthermore, it was discovered that sub-lethal levels of zinc phosphide reduced the survival rates of tilapia in contaminated habitats by affecting immunological function and causing histopathological alterations [21, 22]. Fish health is seriously threatened by the bioaccumulation of zinc phosphide in tilapia tissues, and eating contaminated fish can have negative health effects on humans. The purpose of this study was to determine the possible toxicity of zinc phosphide to fish populations and the possible hazards it presents to the aquaculture sector by investigating its effects on the hematological, behavior, and proximate composition of *Oreochromis niloticus*.

METHODS

Zinc phosphide (Zn_3P_2) is a crystalline powder and is grey in color, it is accessible in 2% to 10% assemblage as sugar-based or grain baits in a pellet, powder, tablet form, or paste [23]. Zinc phosphide was purchased from Sigma Scientific Lab, Lahore, Punjab, Pakistan, and it contained ≥ 19% active phosphor (P) basis, powder (CAS- No:1314-84-7) (Sigma-Aldrich). *Oreochromis niloticus* commonly known as Nile tilapia was purchased from Al-Madina Fish Hatchery Kasur, division Lahore, Punjab and transported to fish laboratory at Department of Fisheries and Aquaculture, University of Okara. Live fish was transported through sterilized plastic oxygen-filled bags and specimens with average lengths and weights of 9.31 ± 0.59cm and 21.4 ± 2.5g respectively were shifted to aquariums. Fish were acclimatized to lab conditions for two weeks in an aquarium containing tap water. Fish were fed with commercial fish feed twice a day. Waste products were siphoned daily and 30% of water was renewed. This study was conducted according to the declarations of Helsinki. After acclimation for 15 days, fish were divided into two groups and moved into six aquariums of equal size and shape. To recover against stress management, fish were kept for 24 hours with regulated oxygenation and temperature. Three aquariums were labeled as a control group and the other as treatment group for zinc phosphide. Each aquarium was stocked with 10 fish per 50L of water respectively. Zinc phosphide impact on *Oreochromis niloticus* was observed for 20 days and water-borne dose at 1 mg/ mL (0.5 mg/g) in distilled water according to the body weight. The 0.5 mg/g dose of zinc phosphide employed in this investigation was chosen in light of earlier research that investigated the harmful effects of this chemical on animals. Hinds, Henry reported the acute oral toxicity of zinc phosphide on wild house mice (Mus musculus), with LD50 values ranging from 25 to 50 g/Kg. However, for fish, the LD50 value is typically lower due to their aquatic environment and unique physiology. The World Health Organization (WHO) states that 0.1 mg/L of zinc phosphide is the highest amount that can be present in water [24, 25]. However, depending on the fish's body weight, we used a dose of 0.5 mg/g in this investigation, which is equal to 1 mg/mL in distilled water. This dosage was selected to reduce the chance of death while guaranteeing that the fish were exposed to enough zinc phosphide to cause a hazardous reaction. Water was continuously aerated artificially. Water quality parameters, total dissolved solids (144.3 ± 1.81), Dissolved oxygen (7.87 ± 0.86), Temperature (37-39°C), Electrical Conductivity (284.5 ± 0.75), and pH (8.08 ± 0.60) were monitored at regular intervals during experimental period. Proximate analysis of commercial feed was conducted at the Poultry Research Institute (PRI) Rawalpindi, Punjab. Fish were anesthetized before being dissected and sampled, and feeding was discontinued 24 hours before the experiment's conclusion. *Oreochromis niloticus* caudal vein was punctured with a 3 ml medical syringe, which was then washed with EDTA solution (as an anticoagulant) and gently shaken to minimize hemolytic anemia of blood utilized for hematological analysis. The process of collecting blood samples was completed within 30 to 40 seconds. Samples of blood were collected in EDTA tubes. Hematological parameters include white blood cells (WBC), lymphocytes (LYM), mid cells (MID), granulocytes (GRA), LYM%, MID%, GRA%, red blood cells (RBC), hemoglobin (HGB), hematocrit

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(HCT%), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), Red blood cell distribution width (RDW-SD), Red blood cell distribution width (RDW-CV%), Platelet (PLT), Mean platelet volume (MPV), platelet distribution width (PDW%), plateletcrit percentage (PCT%) and Platelet larger cell ratio (P-LCR%). WBCs were counted in blood smears which are stained with routine panoptic stains LYM, MID, GRA, LYM%, MID%, GRA%, RBCs were examined manually with a Neubauer hemacytometer and using a solution Natt-Herrick's as a diluent stain, Hgb was obtained by the cyanmethemoglobin process according to method of Hrubec et al., 2000 [14]. HCT was evaluated by micro centrifugation in a capillary tube. RDW-SD, PLT, RDWCV%, MPV, PCT%, PDW%, P-LCR. MCV, MCH, MCHC, were calculated by using the formula. The ash content, moisture, dry matter, crude fats, digestible, and crude protein from muscles were evaluated according to method of AOAC (2000) at the Pakistan Poultry Research Institute (PPRI) Islamabad. Crude protein and crude fat were determined by using micro Kjeldahl method along with Soxthlet appliances respectively according to method of For moisture determination, evaporating dish was cleaned, washed in addition to this it was then placed in a forced air circulating oven at temperature105ºC for 10 to 15 minutes. After 15 minutes it was taken out and cooled in desiccators then properly weighed on digital balance. Fry after a facet on filter paper and placed on a pre-weighed evaporating dish and weighed once again then these samples were placed in a forced air circulating oven for 24 hrs. at 98ºC until the sample is dried.

Dry matter was determined by using formula:

$$\%\ \text{Dry Matter} = \frac{\text{Weight of sample + Weight of china dish after drying}\ \times 100}{\text{Weight of sample + Weight of china dish before drying}}$$

Moisture (%) = 100 - Dry Matter (%)

After being thoroughly cleaned and rinsed, a crucible was put in a muffle oven set to 90°C for an hour, after which it was cooled and weighed. Following sample weighing, 5.0g of the sample was deposited in a crucible, and it was once more heated at 600°C in a muffle furnace for 24 hours. This was then put into a dryer, quickly cooled, and along with weighed to stop moisture absorption.

The formula used to determine crude ash %.

$$\text{Crude ash}\ (\%) = \frac{weight\ of\ ash \times 100}{weight\ of\ sample}$$

For three weeks, behavior was monitored between the hours of 9:00 am and 4:00 pm. For capturing behavior visually, a stopwatch, a multifunctional counter, an Android mobile camera, and a notebook were utilized. Fish behavior was observed again and again after short intervals. Survival, morbidity, and mortality rates were observed. Fish swimming movement was observed and recorded. Fish behavior was observed by instantaneous sampling method. Experimental data was recorded and processed using MS Excel Software. Data were subjected to 'T-test' followed by Post hoc LSD test. Statistical analysis was performed with SPSS IBM V.22. (Chicago, USA). Data significance was established at a $P<0.05$. The results were displayed in the form of tables and figures in addition to graphs with elaborated values as mean ± SD.

RESULTS

Zinc phosphide exhibited profound effects on hematological parameters of *Oreochromis niloticus* exposed for 20 days (Table 1). T-test showed a significant effect on MID, MCHC, GRA%, MCH, PLT, PCT, MPV,and P-LCR values of both groups. Moreover, Treatment group showed significant ($p<0.05$) decrease in MID, GRA, MID%, GRA %, MCH, MCHC, PLT, MPV, PCT, and P-LCR values as compared to control group. However, treatment group exhibited significant ($P<0.05$) increase in the LYM%, RBC, HGB, MCV, RDW- SD, HCT, RDW-CV, values as compared to control group. Furthermore, no significant effect was observed on WBCs, LYM, and PDW% values of both treatment and control groups. Data is presented as mean ± SD (n=3). Alphabets a and b indicated values that were significantly (confidence level 95 %) different among control and treatment group.

Table 1: Impact of Zinc Phosphide On Hematological Parameters of *Oreochromis niloticus* on exposure for 20 Days

Variables	Control	Treatment	T-Value	DF	p-Value
WBC (109/L)	17.9 ± 0.57ns	14.8 ± 3.12ns	1.000	2	0.42
LYM (10^9/L)	9.51 ± 0.57ns	7.97 ± 1.14ns	2.69	2	0.11
MID (109/L)	3.00 ± 0.51a	0.43 ± 0.28b	10.52	2	0.009
GRA (109/L)	5.39 ± 0.60a	0.46 ± 0.27b	15.1	2	0.004
LYM (%)	53.1 ± 0.57b	91.9 ± 1.13a	-70.0	2	0.000
MID (%)	16.7 ± 0.72a	3.20 ± 0.55b	67.5	2	0.000
GRA (%)	30.2 ± 0.59a	4.73 ± 0.78b	133.1	2	0.000
RBC (1012/L)	0.43 ± 0.05b	1.68 ± 0.21a	-4.59	2	0.04
HGB (g/dL)	4.39 ± 0.60b	6.65 ± 0.81a	-8.43	2	0.01
HCT (%)	3.00 ± 0.28b	24.6 ± 0.88a	36.05	2	0.001
MCV (fL)	77.3 ± 0.57b	136.5 ± 1.45a	-67.2	2	0.000
MCH (pg)	112.1 ± 0.63a	37.03 ± 0.88b	263.5	2	0.000
MCHC (pg)	144.6 ± 0.88a	25.4 ± 1.85b	119.2	2	0.000
RDW-SD (fL)	21.7 ± 0.57b	109.2 ± 3.84a	-26.2	2	0.001
RDW-CV (%)	20.6 ± 0.57b	21.6 ± 0.88a	-3.20	2	0.08
PLT (109/L)	634 ± 0.57a	434 ± 1.52b	200.0	2	0.000
MPV (fL)	7.3 ± 0.57a	3.69 ± 0.81b	14.3	2	0.005
PDW (%)	9.00 ± 0.57ns	10.3 ± 1.27ns	-1.74	2	0.22
PCT (%)	0.46 ± 0.005a	0.15 ± 0.02b	14.03	2	0.005
P-LCR (%)	14.2 ± 1.15a	0.89 ± 0.11b	12.7	2	0.006

The figure 1 illustrated the impact of zinc phosphide on the hematological parameters of *Oreochromis niloticus*. Data are presented as Mean ± SD (n = 3). Significant differences between the control and treatment groups are indicated by the letters a and b, representing a 95% confidence level.

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Hematological parameters measured include key indices such as Red Blood Cell count (RBC), hemoglobin (Hb), hematocrit (HCT), White Blood Cell count (WBC), and others, highlighting the physiological changes induced by zinc phosphide exposure. Figures are represented as Mean ± SD (n=3). Letters a and b indicates the values that were significantly (confidence level 95 %) different among control and treatment group.

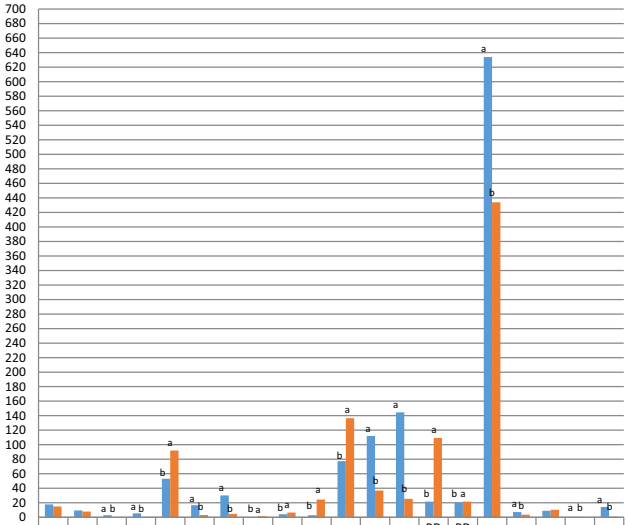

Figure 1: Impact of Zinc Phosphide on Hematology of *Oreochromis niloticus*

Zinc Phosphide exhibited variable impact on proximate composition of *Oreochromis niloticus* exposed for twenty days (Table 2). A highly significant impact was observed on crude protein (CP), crude fat (CF), and ash components in fish muscles of control and treatment group. Moreover, treatment group resulted significant ($p< 0.05$) decrease in CF, increase in CP and ash content.

Table 2: Impact of Zinc Phosphide on Proximate Composition of *Oreochromis niloticus* on Exposure for 20 Days

Proximate (%)	Control	Treatment	T-Value	DF	p-Value
CP	61.2 ± 0.71b	63.8 ± 0.80a	-26.7	2	0.001
CF	8 ± 0.80a	6.6 ± 0.75b	24.2	2	0.002
Ash	20 ± 0.63b	22 ± 0.69a	-34.6	2	0.001

A behavioral index is considered a reliable indicator to monitor response of fish. Zinc phosphide showed some impact on the behavior of *Oreochromis niloticus* on exposure for 20 days (Table 3). Fish became very aggressive after 10 days of exposure and irregular swimming was observed. Due to anemia surface agitation was observed in treatment fish.

Table 3: Impact of Zinc Phosphide on Behavior of *Oreochromis niloticus* on Exposure for 20 Days

Variables	Control	Treatment
Erratic Swimming	No	Yes
Coloration	No Change	Shiny
Opercular Movement	Remained Same	Fast
Gill Movement	Remained Same	Fast
Feeding	Active	Active
Rubbing Against Wall	No	Yes
Aggression	No	Yes
Inappetence	No	No
Flared Opercula	No	Yes
Loss of Equilibrium	No	Yes
Hanging Head Up In Water	Yes	No

DISCUSSION

Pesticide exposure to organisms over an extended period poses a constant risk to public health. This study mimics environmental fish toxicity by administering a waterborne dose of zinc phosphide (1 mg/mL or 0.5 mg/g), which represents real-world contamination conditions in aquatic ecosystems. Pesticides and rodenticides found in agricultural runoff frequently end up accumulating in freshwater systems.The aquactic animals come into contact with this contaminated water and eating these contaminated aquatic animals puts a large portion of the human population at risk [27]. In most cases, pesticides quickly changed the hematological fish characteristics [28]. Hematological parameters are good indicators of the severe impacts of many toxic compounds mainly pesticides and industrial effluents containing heavy metals so these parameters are signs of internal homeostasis and the physiological condition of exposed organisms. Significant drops in hemoglobin, hematocrit, and red blood cells were seen mostly after contact with fipronil, which highlights the anemic state of fish. Possible causes of the reduced hemoglobin include its oxidation to methemoglobin, a reduction in gas exchange, and damage brought on by free radicals. Reduced blood parameter levels also indicate ineffective hematopoietic tissue function, improper osmoregulatory mechanisms, and increased RBC damage in blood-forming organs. However, after 20 days of exposure to zinc phosphide, *Oreochromis niloticus* showed a significant increase in values of red blood cells, hemoglobin, hematocrit, lymphocytes, mean corpuscular volume, red blood cell distribution width-SD, and red blood cell distribution width -CV when compared to the control group. Red blood cells aid in cellular respiration and transport oxygen from the gills to tissues. Pesticide stress is responsible for a large increase in these metrics. According to Far, Roodsari there is momentous escalation

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in the value of MCV and reduction in MCH on dosing Oncorhynchus mykiss with diazinon [29]. David, Sangeetha showed that there is a significant increase in values of MCV and MCH on dosing Cirrhinus mrigala with Deltamethrin [30]. Conversely, there is significant increase in Red Blood Cell calculation has been explained in Prochilodus lineatus showing a herbicide clomazone. Ghaffar, Hussain exposed Labeo rohita to 0.03-0.15mg/L of fipronil for almost nine days [31]. Directories of lymphocytes, erythrocytes in addition monocytes are reduced while total leukocyte counts, as well as neutrophils augmented prominently. Erythrocytes displayed variety of nuclear abnormalities. Furthermore, Ghaffar, Hussain discovered the toxic effects of fipronil on Cyprinus carpio treated with different concentrations (0 - 0.10mg/L) for just about 12 days [32]. Fish in groups that were given inappropriate doses showed significant abnormalities in both biochemical and clinical-hematological markers. Hematocrit, hemoglobin, and Red blood cell counts were reduced generally total leukocyte count, mean corpuscular volume, neutrophils, lymphocytes, and monocytes were mainly amplified. Organophosphate-induced anemia caused by a significant drop in Hb level has been reported in Barbonymus gonionotus subjected to quinolphos in the modern era [32]. Present research showed that treatment of *Oreochromis niloticus* with zinc phosphide resulted significant decrease in MID, GRA, MID%, GRA%, MCH, MCHC, PLT, MPV, PCT, P-LCR. There is a substantial reduction in MCH, MCHC and substantial increase in MCV on treating Channa punctatus with Deltamethrin [33]. According to studies, contaminated water systems of zinc phosphide and other pesticides, can have a major effect on aquatic life [34]. Comparable hematological abnormalities have been shown in similar experimental setups, such as exposing fish to organophosphate pesticides as deltamethrin, diazinon, and fipronil [29, 31, 32]. Future studies on zinc phosphide should concentrate on long-term exposure studies, ecotoxicological assessments across species, and an understanding of its molecular mechanisms of toxicity. Investigating its environmental destiny, bioaccumulation impacts on fish behavior and physiology, as well as the impact of co-contaminants, will aid in determining its overall ecological risks. Furthermore, a study on remediation tactics and their possible effects on non-target species is critical for enhancing environmental management and regulatory procedures.

CONCLUSIONS

In conclusion, this study focused on how zinc phosphide contamination affects hematological parameters, flesh, and behavior of *Oreochromis niloticus*. This study also highlighted possible ecological runoff related concerns posed by zinc phosphide and other toxicants, adding to the understanding of their effects on aquatic ecosystems and human health through fish eating.

Authors Contribution

Conceptualization: IS, SY, FK
Methodology: IS, FK, MF, MIH
Formal analysis: IS, SY, FK, MF, MIH
Writing, review and editing: IS, SY, NI, MSS, MH

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

All the authors declare no conflict of interest.

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Isolation of Endospore-Forming Bacteria from Milk Collected from Selected Cities of Pakistan

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ABSTRACT

Human nutritional needs are linked to milk production, processing, and consumption, as determined by the current study, which involved the microbiological analysis of milk samples from various Pakistani cities. **Objective:** To investigate the isolation of endospore-forming bacteria from milk collected from selected cities of Pakistan. **Methods:** Several milk samples were collected from Lahore, Gujranwala and Sheikhupura for microbiological evaluation and antibiotic susceptibility patterns. Isolation and characterization employing different morphological and biochemical tests was done which showed a heavy load of Bacillus endospore-forming bacterial species. **Results:** The results revealed that all the samples were contaminated with endospores-forming bacteria. Highly effective drugs in this study included Azithromycin, Rifampicin and Chloramphenicole resulted in 97%, 95.5% and 95.5% bacterial sensitivity respectively whereas Novobiocin was found to be 88.8% and Amoxilin showed 84.4% efficacy against bacterial Isolates. Metronidazole and Cefaxitin were found to be the least sensitive up to 44.4% and 17.7% respectively. Cefaxitin and Metronidazole were the most resistant medicines recorded at 83.3% and 66.6% respectively. In the case of metals, the bacterial sensitivity was found to be much lower i.e. at 1% Zn concentration, the highest recorded sensitivity was 51.1%. Bacterial Isolates were found to be highly resistant against Fe and Pb (1% metal concentration) and showed 0% and 2.2% sensitivity respectively. **Conclusions:** It was concluded that for the eradication of harmful endo-sporogenic bacteria in drinking milk, samples should be obtained employing efficient and safe standard antibacterial protocols for milk processing.

INTRODUCTION

High-quality milk and dairy products have been linked to human health and are regarded as nature's perfect food for mammals. Many active antimicrobial agents found in freshly milked milk temporarily stop the growth of contaminating microorganisms, but these agents are dependent on the temperature at which raw milk is stored. A certain amount of raw milk contamination is practically unavoidable, even in the best possible circumstances for routine milk collection. Microorganisms grow to high populations and cause milk rancidity, even if handled and stored suitably [1, 2]. The animal hide that are contaminated, the milking equipment and the animal feed, are just a few of possible sources of contamination of raw milk [3, 4]. Contaminants of milk include diverse types of microorganisms but some microorganisms produce spores in a non-vegetative form, resistant to heat, pressure and desiccation. Two main genera of endospore-forming bacteria are genus Bacillus and Clostridium. Bacillus are aerobic, facultative and anaerobic but the clostridium is strictly anaerobic. The bacteria form endospores are thick-walled structures that are released from vegetative cells up one cell lysis. Due to their dehydrated state, the endospore bacteria contain less moisture than the vegetative cell and are also more resistant to temperature, disinfectants, dehydration, and other environmental stresses as compared to the vegetative cell [5, 6].

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Commonly present members of the genus Bacillus in milk are the *B. cereus* group, *B. subtilis* group and *B. licheniformis*. Other aerobic endospore-forming bacteria isolated from milk included the genera e.g. *Paenibacillus, Oceanobaqcillus, Brevibacilolus, Lysinbacillus, Ureibacillus, Ornithinibacillus* and *Sporosarcina* [7, 8]. Clostridium species are anaerobic spore-formers that are widespread in dairy environments just like the aerobic endospore-forming bacteria. They are isolated from dairy farms from different sources such as the soil, silage, forage, hay and other raw milk and its products, silage is the major source of contamination of these clostridium species like *C. disporicum, C. tyrobutyricum* and *C. sporogenes.* Clostridium bacteria in milk convert the lactic acid into butyric acid and grow during fermentation and in cheese maturation. These are also the causative agent of the late blowing in butyric acid fermentation causing defects in chees during long fruiting time. The primary cause of late blowing is *C. tyrobutyricum* and another source of chees defects is *C. sporogenes.* Clostridium species are very important like *C. perferinges* particularly linked with food poisoning poisoning starts after heat treatment the spore-germinate and cell go through to the proliferation of vegetative cells. Some Clostridium spp. are pathogens e.g., *C. botulinum* of the food-borne botulism, Clostridium spp. baratii and butyricum are associated in a burst of food-borne, Type-E botulism. In the raw milk clostridium is low due to high RH value but during milk supply, botulinum neurotoxin is the most toxic substance that deliberately contaminates the milk. *C. botulinum* complexes and their neurotoxin types A and B are inactivated by pasteurization at high temperatures [9]. Milk processing units comprising milk loading cavities, pipelines, and filling machines also play a pivotal role in milk spoilage due to biofilms produced by various microbes [10]. Mostly the sporogenic bacteria create biofilms that are highly resistant to most of the contamination processes. Due to its resistance, it causes an additional obstacle for the dairy industry. Some strains of sporogenic bacteria can proliferate in low temperatures such as under the condition of refrigeration (4°C) foremost to the production of diverse lipolytic and proteolytic enzymes that will degrade the main constitutes of the milk, consequently affecting the sensory features [11]. Thorough investigations that have been conducted, involving the microbiological examination of multiple samples from different cities including Lahore, Gujranwala and Sheikhupura indicated that all the samples were contaminated with bacterial endospores. Numerous factors, such as management practices, sampling stage (farm vs. dairy bulk tank), farm hygiene, season, and milking practices, have been linked to the diversity and quantity of endospore-forming bacteria in raw milk. This information was not for strict legislation to save the measures from consumption of milk contaminated with bacterial endospores of a diverse nature. As it is known that bacterial endospores survive the boiling temperature of the water, these products prepared from such milk, even after boiling may pose health issues including food pasteurization to the consumers.

This study aims to examine the quality of raw milk by assessing the bacterial content resistant to antibiotics and heavy metals in three cities.

METHODS

Observational and prospective research was conducted at the Microbiological and biotechnology lab at the University of the Punjab, Lahore, Pakistan, from the period of September 2017 to 25 June 2018. Several milk samples were collected from Lahore, Gujranwala and Sheikhupura for microbiological evaluation and antibiotic susceptibility patterns. A total of 20 milk samples were collected in glass vials and transported to the laboratory immediately and maintained at 4°C, after heating at 80°C for 10 minutes 10-1, 10-2, and 10-3 dilutions were made. 100ul from 10^{-3} dilution of each sample was spread on the plates of nutrient agar and incubation was done at 37°C for 24 hours. 24 hours incubated plates containing only 2-3 bacterial colonies were Isolated. Four nutrient media (Nutrient agar media, Blood agar media, Tryptophan broth and Simmons citrate agar) were used for the separation of bacterial Isolates. After spreading of diluted sample (100ul) on the nutrient agar plates and incubation was done at 37°C for 24 hours, different types of bacterial colonies were obtained. The isolated colony was picked up by loop and inoculated in nutrient broth for biochemical testing. Also, slants were made of each bacterial strain for further work. Incubated Slants and nutrient broth bottles were kept at 37°C for 24 hours. After isolation, bacterial isolates were purified by using quadrant streaking. From the pure cultured plates, the unknown bacteria were identified by performing characterization colony morphological, and biochemical tests and different antibiotics and metals susceptibility patterns were measured. Bacteria identification succeeded through multiple biochemical analyses. The examinations analyzed motility movement of bacteria in combination with cellular observation using Gram staining and endospore staining and bacterial enzyme evaluations through conductance of oxidase and catalase testing. The tests for indole production and citrate utility were conducted to examine further metabolic traits. The disc diffusion method on nutrient agar medium detected the sensitivity of antibiotics to bacteria. A test included novobiocin (5 µg), metronidazole (5 µg), rifampicin (5 µg), azithromycin (15 µg), chloramphenicol (30 µg),

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streptomycin (10 µg), cefoxitin (30 µg), amoxicillin (25 µg) and ticarcillin-clavulanic acid (85 µg) used at their stated concentrations. The investigators placed their antibiotic discs gently onto prepared surfaces of nutrient agar where bacterial cultures rested. Plates were placed in an incubator at 37°C for 18-24 h to which the radial zones of inhibitions measured. Cu, Pb, Zn, Cr and Fe were used to test the susceptibility of bacterial Isolates i.e. sensitivity or resistance to these metals. For this purpose, two concentrations of each metal were formed one was 1% and the second was 0.5%. Filter paper discs were poured with 6ul solution of each metal in two concentrations. Sterilization was performed by autoclaving the disc placed in a vial containing plastic caps. Nutrient agar plates were formed and each bacterial Isolate was spread on the agar plates by a sterilized glass spreader under aseptic conditions. These metal discs were placed on the agar plates with sterilized forceps and incubated at 37°C for 24 hours. The CFU data corresponding to the cities of Sheikhupora, Lahore and Gujranwala were statistically analyzed using the Kruskal-Wallis Test as a non-parametric, alternative to one-way ANOVA. Results showed significance at $p<0.05$.

RESULTS

Bacterial growth was observed after 24 hours of incubation, two to three types of colonies appeared one type of colony is small just like puncta form, transparent, convex, shiny and smooth. The other type of colony was opaque white in medium size with a rough texture and flat and curled edges. The third type of colony on the nutrient agar was filamentous, large, flat, opaque grey and with rough texture. Further morphological details of bacterial colonies are given in table 1.

Table 1: Colony Morphology and Configuration Analysis of the Procured Bacterial Isolates from Different Milk Samples

Sr. No.	No. of obs.	Morphology of Bacterial Colonies					
		Name of Strain	Texture	Elevation	Margin	Appearance	Size
1	S1	A	Rough	Raised	Curled	Opaque white	Moderate
		A	Smooth	Convex	Entire	Transparent	Punctiform
2	S2	A	Rough	Raised	Curled	Opaque white	Moderate
		A	Smooth	Convex	Entire	Transparent	Punctiform
3	S3	A	Rough	Raised	Curled	Opaque white	Moderate
		A	Smooth	Convex	Entire	Transparent	Small
4	S4	A	Rough	Raised	Curled	Opaque white	Moderate
		A	Smooth	Convex	Entire	Transparent	Punctiform
5	S5	A	Rough	Raised	Curled	Opaque white	Moderate
		A	Smooth	Convex	Entire	Transparent	Small
6	L6	A	Rough	Raised	Curled	Opaque white	Moderate
		A	Smooth	Convex	Entire	Transparent	Punctiform
7	L7	A	Rough	Raised	Curled	Opaque white	Moderate
		A	Smooth	Convex	Entire	Transparent	Punctiform
8	L8	A	Rough	Raised	Curled	Opaque white	Moderate
		A	Smooth	Convex	Entire	Transparent	Punctiform
9	L9	A	Rough	Raised	Curled	Opaque white	Large
		A	Smooth	Convex	Entire	Transparent	Punctiform
10	L10	A	Rough	Raised	Curled	Opaque white	Large
		A	Smooth	Convex	Entire	Transparent	Punctiform
11	G11	A	Rough	Raised	Curled	Opaque white	Large
		A	Smooth	Convex	Entire	Transparent	Small
		Fg	Rough	Flat	Filamentous	Gray	Large
12	G12	A	Rough	Raised	Curled	Opaque white	Large
		A	Rough	Convex	Entire	Transparent	Punctiform
13	G13	A	Rough	Raised	Curled	Opaque white	Large
		A	Smooth	Convex	Entire	Transparent	Punctiform
14	G14	A	Rough	Raised	Curled	Opaque white	Large
		A	Smooth	Convex	Entire	Transparent	Punctiform
15	G15	A	Rough	Raised	Curled	Opaque white	Large
		A	Smooth	Convex	Entire	Transparent	Punctiform
16	G16	A	Rough	Raised	Curled	Opaque white	Large
		A	Smooth	Convex	Entire	Transparent	Punctiform
		B	Rough	Flat	Filamentous	Gray	Large
17	G17	A	Rough	Raised	Curled	Opaque white	Large
		A	Smooth	Convex	Entire	Transparent	Punctiform
		B	Rough	Flat	Filamentous	Gray	Large
18	G18	A	Rough	Raised	Curled	Opaque white	Large
		A	Smooth	Convex	Entire	Transparent	Punctiform
19	G19	A	Rough	Raised	Curled	Opaque white	Large
		A	Smooth	Convex	Entire	Transparent	Punctiform
20	G20	A	Rough	Raised	Curled	Opaque white	Large
		A	Smooth	Convex	Entire	Transparent	Punctiform
		B	Rough	Flat	Filamentous	Gray	Large

All of the purified bacterial strains (n=45) were identified based on cultural characteristics, microscopic morphology with Gram's reaction and biochemical profiles. All the bacterial isolates were endospore-forming gram-positive bacilli, oxidase-positive and negative, indole-positive, citrate-positive and motile are presented in table 2.

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Table 2: Microscopic and Biochemical Characterization of Bacterial Isolates Procured from Different Milk Samples

Sr. No.	Samples from Different Cities	Strains Derived from Each Sample	Morphological Characteristics			Biochemical Tests			
			Gram Staining	Endospore Staining	Motility	Catalase	Oxidase	Citrate	Indole Production Test
1	S1	A	+	+	+	+	+	+	+
		a	-	+	+	+	+	+	+
2	S2	A	+	+	+	+	+	+	+
		a	+	+	+	+	+	+	+
3	S3	A	+	+	+	-	+	+	+
4	S4	a	+	+	+	-	+	-	+
		A	+	+	+	+	+	+	-
		a	+	+	+	+	+	+	+
5	S5	A	+	+	+	+	+	+	+
		a	+	+	+	+	+	+	+
6	L6	A	+	+	+	+	+	-	+
		a	+	+	+	-	+	-	+
7	L7	A	+	+	+	-	+	-	+
		a	+	+	+	+	+	-	-
8	L8	A	+	+	+	+	+	-	+
		a	+	+	+	-	+	-	-
9	L9	A	+	+	+	+	+	+	+
		B	+	+	+	+	+	-	+
10	L10	A	+	+	+	+	+	-	+
		a	+	+	+	+	-	-	+
11	G11	A	+	+	+	+	+	+	+
		a	+	+	+	+	+	+	+
		Fg	+	+	+	+	+	+	+
12	G12	A	+	+	+	+	-	-	+
		a	+	+	+	+	+	+	+
13	G13	A	+	+	+	+	+	+	+
		a	+	+	+	+	+	+	-
14	G14	A	+	+	+	+	+	+	+
		a	-	+	+	+	-	+	+
15	G15	A	+	+	+	+	-	+	+
		a	+	+	+	+	+	+	+
16	G16	A	+	+	+	+	+	-	+
		a	+	+	+	+	+	-	+
		B	+	+	+	-	+	-	-
17	G17	A	+	+	+	+	+	-	+
		a	+	+	+	+	+	+	+
		B	+	+	+	-	-	-	+
18	G18	A	+	+	+	+	+	+	+
		a	+	+	+	+	+	+	+
		B	+	+	+	+	+	+	+
19	G19	A	+	+	+	+	+	+	+
		a	+	+	+	+	+	+	+
20	G20	A	-	+	+	+	+	+	+
		a	+	+	+	-	+	+	+
		B	+	+	+	+	+	+	+

The inhibition zone produced by antibiotics was measured in mm from the 24 hours post-incubated plates, a clear large zone of inhibition was produced when bacterial Isolates showed sensitivity against antibiotic discs, whereas resistant bacteria don't produce a zone of inhibition. The results were recorded and interpreted in(Table 3).

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Table 3: Against 45 Bacterial Isolates from Milk Samples Diameter of Zone of Inhibition in Mm of Different Antibiotics

Sr. No.	Milk Samples from Different Cities	Bacterial Isolates from Each Sample	Inhibition Cell Wall			Inhibition Protein Synthesis			Inhibition Nucleic Acid Synthesis		
			Inhibition FOX Zone in Mm	Inhibition TIM Zone in Mm	Inhibition AML Zone in Mm	Inhibition S Zone in Mm	Inhibition AZM Zone in Mm	Inhibition C Zone in Mm	Inhibition RD Zone in Mm	Inhibition NV Zone in Mm	Inhibition MTZ Zone in Mm
1	S1	A	7	18	16	22	28	30	20	16	7
		a	R	14	18	R	16	26	20	16	R
2	S2	A	R	R	R	20	26	22	8	R	R
		a	R	12	14	22	28	26	16	16	R
3	S3	A	8	14	14	8	24	28	20	18	14
		a	R	14	14	R	20	25	18	16	R
4	S4	A	R	14	18	R	20	30	18	20	R
		a	R	14	12	16	28	28	20	14	R
5	S5	A	R	14	18	16	14	28	18	22	R
		a	R	14	18	16	14	28	22	20	R
6	L6	A	R	R	R	8	10	R	R	18	R
		a	R	R	R	8	R	R	R	R	R
7	L7	A	R	8	9	19	14	18	11	12	R
		a	R	8	10	6.2	14	18	15	14	10
8	L8	A	9	8	9	7	13	17	11	15	6.2
		a	7	8	R	R	9	22	10	12	10
9	L9	A	R	9	10	R	15	22	12	14	7
		B	7	7	12	R	15	19	9	8	8
10	L10	A	R	9	10	21	15	18	14	13	7
		a	R	8	9	R	14	15	11	13	R
11	G11	A	R	13	11	8	17	25	14	16	7
		a	8	9	9	15	14	21	11	13	9
		Fg	R	8	9	R	19	21	13	12	R
12	G12	A	R	9	10	19	17	16	12	10	R
		a	R	8	9	26	14	19	13	13	6.2
13	S13	A	R	6.2	9	R	13	14	11	10	R
		a	6.2	9	10	7	18	21	16	14	7
14	S14	A	R	R	R	16	24	11	8	11	R
		a	R	8	9	R	17	20	16	9	R
15	S15	A	R	R	9	R	17	22	16	10	R
		a	8	10	9	18	14	21	16	16	9
16	L16	A	R	7	9	17	16	21	13	16	R
		a	R	7	10	16	11	20	16	11	7
		B	R	R	R	12	20	25	9	R	9
17	L17	A	R	R	9	17	10	24	11	16	10
		a	R	8	9	R	15	22	12	12	6.2
		B	R	12	13	21	16	25	22	18	6.2
18	L18	A	R	12	14	7	18	22	15	14	6.2
		a	R	R	10	22	24	22	21	15	R
		B	R	6.2	7	18	18	22	15	16	R
19	L19	A	R	R	7	R	18	18	10	14	R
		a	R	R	8	18	35	34	14	R	R
20	L20	A	R	R	7	R	14	11	16	11	R
		a	R	8	9	R	13	16	10	10	R
		B	R	R	8	R	10	14	8	12	R

R=Resistance, S=Sensitivity, FOX=Cefaxitin, AML=Amoxillin, TIM=Clavulanic acid, AZM=Azithromycin, C=Chloramphenicole, S=Streptomycin, NV=Novobiocin, MTZ=Metronidazole, RD=Refampicin

Antibiotic susceptibility patterns of the bacterial Isolates (n=45) derived from milk samples (n=20) of Lahore, Gujranwala and Sheikhupura against different antibiotics. The results were recorded and interpreted in Figure 1.

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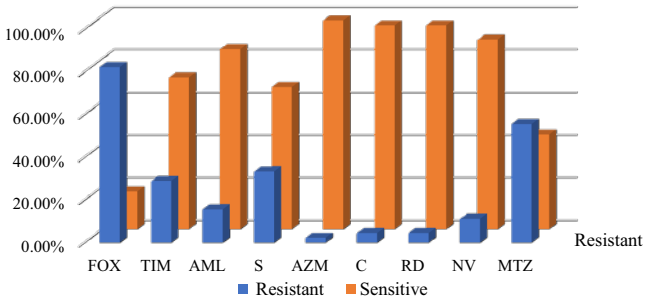

Figure 1: Antibiotic Susceptibility Patterns

R=Resistance, S=Sensitivity, FOX=Cefaxitin, AML=Amoxicillin, TIM=Clavulanic acid, AZM=Azithromycin, C=Chloramphenicole, S=Streptomycin, NV=Novobiocin, MTZ=Metronidazole, RD=Refampicin

The antibiotic susceptibility analysis of bacteria strains received interpretation in figure 2.

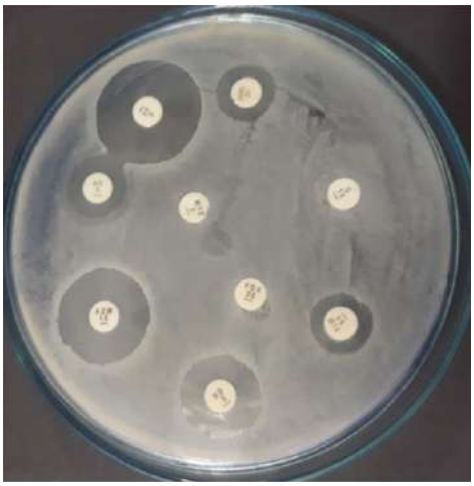

Figure 2: Bacterial Strain analysis

The zone of inhibition produced by the heavy metals Cu, Pb, Zn, Cr and Fe was measured in mm from the 24-hour incubated plates, the sensitive bacterial Isolates showed a large zone of inhibition.Resistant bacteria did not produce a zone of inhibition.The results were recorded and interpreted in table 4.

Table 4: Diameter of Zone of Inhibition in Mm of Different Metals Against 45 Bacterial Isolates from Milk Samples

Sr. No.	Samples from different cities	Strains derived from each sample	Copper (Cu)		Ferric (Fe)		Chromium (Cr)		Zink (Zn)		Lead (Pb)	
			Zone of inhibition at Cu1%	Zone of inhibition at Cu0.5%	Zone of inhibition at Fe1%	Zone of inhibition at Fe0.5%	Zone of inhibition at Cr1%	Zone of inhibition at Cr0.5%	Zone of inhibition at Zn1%	Zone of inhibition at Zn0.5%	Zone of inhibition at Pb1%	Zone of inhibition at Pb0.5%
1	S1	A	20	R	R	R	26	22	30	30	R	R
		a	25	20	R	R	28	22	32	26	R	R
2	S2	A	R	R	R	R	40	36	38	36	R	R
		a	28	20	R	R	R	R	22	18	R	R
3	S3	A	R	R	R	R	R	R	28	26	R	R
		a	22	18	R	R	16	18	18	16	R	R
4	S4	A	R	R	R	R	R	R	18	R	R	R
		a	24	R	R	R	R	R	26	R	R	R
5	S5	A	24	16	R	R	R	R	R	R	R	R
		a	22	18	R	R	R	R	24	22	R	R
6	L6	A	R	R	R	R	46	40	48	46	38	40
		a	R	R	R	R	R	R	R	R	R	R
7	L7	A	24	R	R	R	R	R	28	26	R	R
		a	R	R	R	R	28	22	20	18	R	R
8	L8	A	R	R	R	R	R	R	R	R	R	R
		a	R	R	R	R	R	R	R	R	R	R
9	L9	A	R	R	R	R	R	R	28	26	R	R
		B	R	R	R	R	36	34	R	R	R	R
10	L10	A	R	R	R	R	28	22	30	26	R	R
		a	R	R	R	R	36	28	R	R	R	R
11	G11	A	R	R	R	R	28	26	R	R	R	R
		a	26	14	R	R	R	R	R	R	R	R
		Fg	26	20	R	R	28	24	30	26	R	R
12	G12	A	28	R	R	R	20	18	28	R	R	R
		a	R	R	R	R	28	26	R	R	R	R
13	G13	A	R	R	R	R	R	R	R	R	R	R
		a	R	R	R	R	R	R	18	16	R	R
14	G14	A	R	R	R	R	R	R	R	R	R	R
		a	24	R	R	R	R	R	R	R	R	R

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15	G15	A	R	R	R	R	26	30	R	R	R	R
		a	R	R	R	R	R	R	R	R	R	R
16	G16	A	R	R	R	R	30	30	28	26	R	R
		a	R	R	R	R	R	R	18	16	R	R
		B	R	R	R	R	20	R	R	24	R	R
17	G17	A	R	R	R	R	28	26	R	R	R	R
		a	R	R	R	R	32	28	R	R	R	R
		B	R	R	R	R	R	R	18	16	R	R
18	G18	A	R	R	R	R	R	R	28	16	R	R
		a	R	R	R	R	R	R	R	24	R	R
		B	R	R	R	R	R	R	R	R	R	R
19	G19	A	R	R	R	R	R	R	R	R	R	R
		a	R	R	R	R	R	R	R	R	R	R
20	G20	A	R	R	R	R	R	R	R	R	R	R
		a	R	R	R	R	R	R	R	R	R	R
		B	R	R	R	R	28	26	26	22	R	R

R=Resistance, S=Sensitivity, Cu1%, Cu0.5%, Fe1%, Fe0.5%, Cr1%, Cr0.5%, Zn1%, Zn0.5%, Pb1%, Pb0.5%

Heavy metals susceptibility pattern of the bacterial strains (n=45) isolated from milk samples (n=20) of Lahore, Gujranwala and Sheikhupura in contradiction of diverse Metals. R=Resistance, S= Sensitivity, Cu1%, Cu0.5%, Fe1%, Fe0.5%, Cr1%, Cr0.5%, Zn1%, Zn0.5%, Pb1%, Pb0.5%. The results were recorded and interpreted in Figure 3.

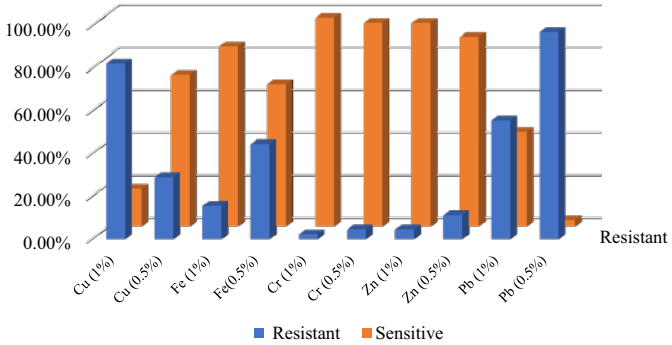

Figure 3: Heavy Metals Susceptibility Pattern of the Bacterial Strains (n=45) Isolated from Milk Samples (n=20)

Heavy Metals Susceptibility analysis of the Bacterial Strains was recorded in Figure 4.

Figure 4: Heavy Metal Susceptibility Analysis of the Bacterial Strains

Following the significance Post Hoc Test was applied, test results are shown in which shows that milk samples from Gujranwala and Sheikhupora were significantly different from each other while the comparison of Sheikhupora, Lahore and Gujranwala-Lahore showed to be non-significant-respectively at $p<0.05$. The results were recorded and interpreted in Figure 5.

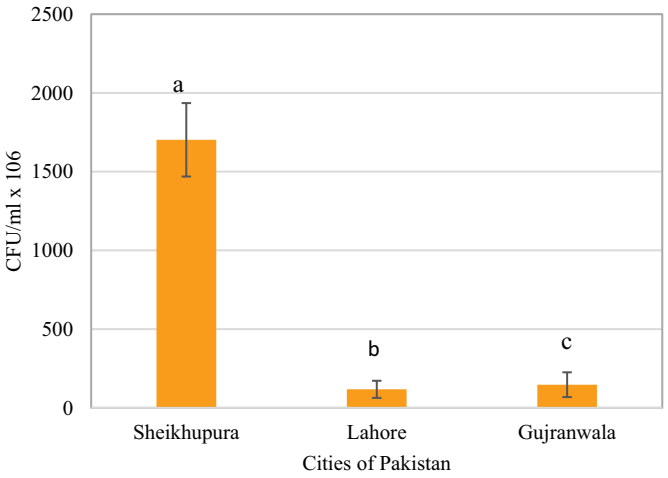

Figure 5: CFU\ml median values of Gujranwala, Lahore and Sheikhupora Letters Showing Significance at $p<0.05$

CFU\ml count for coliform in fresh milk samples collected from Gujranwala, Lahore and Sheikhupora. The results are shown in Table 5.

Table 5: CFU\ml Count for Coliform in Raw Milk Samples

Sr. No	Samples from Different Cities	CFU\ml
1	S1	$2.11x10^9$
2	S2	$2.41x10^9$
3	S3	$1.37x10^9$

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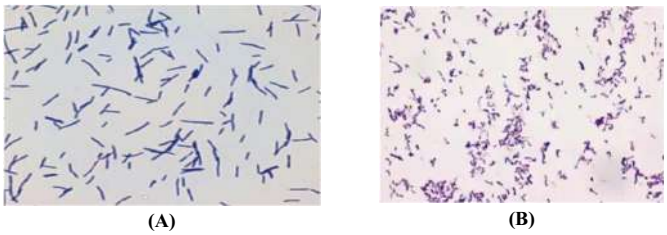
4	S4	$1.27x10^{9}$
5	S5	$1.35x10^{9}$
6	L6	$2.3x10^{8}$
7	L7	$2.7x10^{8}$
8	L8	$2.98x10^{7}$
9	L9	$2.95x10^{7}$
10	L10	$2.88x10^{7}$
11	G11	$7.2x10^{6}$
12	G12	$6.0x10^{6}$
13	G13	$3.1x10^{7}$
14	G14	$4.6x10^{6}$
15	G15	$3.9x10^{6}$
16	G16	$7.4x10^{8}$
17	G17	$3.3x10^{8}$
18	G18	$3.1x10^{6}$
19	G19	$3.4x10^{8}$
20	G20	$4.8x10^{6}$

To check the number of endospore-forming bacteria in the milk samples endospore staining was done at forty-eight hours' culture broths employing the Schaeffer Fulton method. The slide was observed under a bright field light microscope at 40x objective lens magnification [12]. The endospore-forming bacteria were motile when microscopically checked, at twenty-four hours of incubation. Gram's reaction, shape, size and arrangement of bacteria and released spores were also observed microscopically in Figure 6.



(A) (B)

Figure 6: Gram-positive Bacteria Are in A and Endospore-Forming Bacteria in B

DISCUSSION

In this study, twenty milk samples were collected from three different cities of Pakistan Lahore, Gujranwala and Sheikhupura from which forty-five bacterial isolates were obtained. Following isolation characterization of these Isolated bacteria was done based on their different morphological, and biochemical profiles and antibiotic and metal susceptibilities and their results were recorded. Samples were collected in glass vials under aseptic conditions and transported to the lab by maintaining temperature of 4°C. Heat treatment (80°C for 10 minutes) was applied to kill the non-sporogenic bacteria. Some samples were collected from dairy farms directly whereas other samples were collected from the shops. Interestingly the samples from dairy farms contained more bacteria than the samples from the shops. This low bacterial count in different samples obtained from milk shops was probably due to the addition of chemicals that on one hand maintain the milk for a long shelf-life. On the other hand, highly dangerous to human health. But in dairy farms, these chemicals were not added so a high number of bacteria were observed. When the heat treatment was applied, the majority of no spore-former bacteria died but as the sporogenic bacteria survived formed vegetative cells showed resistance to this high temperature, these spore-forming bacteria caused the milk spoilage even after the pasteurization process [14]. It has been inferred from the research that milk samples collected from Sheikhupura contain a higher number of spore-forming bacteria than Lahore. Morphology of the different isolated bacteria was presented which are separated from different samples. Three types of colonies were observed in Lahore and Sheikhupura's milk samples while two types of colonies were present in Gujranwala's samples. The colony morphology was noted by observing the texture, elevation, margin and size of the bacterial colonies. In Table. 2 the biochemical test results of the indole test, citrate utilization test, catalase test and oxidase test were described [15, 16]. In the oxidase test different bacteria have variable abilities to produce cytochrome oxidase enzyme by addition of the following test reagent p-amino dimethyl, aniline oxalate, the development of pink then dark purple coloration on the surface of colonies showed production of cytochrome oxidase whereas no colour change on colonies specified the absence of oxidase activity [17]. Table 2 shows the results of the catalase test that converts the hydrogen peroxide into oxygen and water. In the positive test bubbles were formed which indicated the presence of Bacillus spp. and negative results may show the presence of Clostridium spp according to [18]. An indole synthesis assay was done to determine the ability of the bacterial isolates to convert the amino acid tryptophan into indole, which revealed the presence of the tryptophanase enzyme. For this purpose, Kovac's reagent was added to 4 ml of tryptophan broth culture and a ring was observed for a positive indole test [18]. To check the ability of bacterial strains to utilize sodium citrate as a sole source of carbon and inorganic ammonium salts as a nitrogen source, a citrate utilization test was performed. Each bacterial Isolate was inoculated on Simon's citrate agar and incubated at 37°C for 18-24 hours. The appearance of a blue colour indicated a positive citrate test [12]. The bacterial susceptibility to various antibiotics was determined by the Kirby-Bauer disc diffusion technique (9) using commercially available antibiotic discs on the nutrient agar plates. Variable zone of inhibition produced by bacterial Isolates susc eptible to different antibiotics: Novobiocin (5 µg), Metronidazole (5 µg), Rifampicin (5 µg), Azithromycin (15 µg), Chloramphenicol (30 µg),

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Streptomycin (10 µg), Cefoxitin (30 µg), Amoxil (25 µg), Ticarcillin Clavulanic acid (85 µg) resulted in that were measured, Clear large zones of inhibition were produced when bacterial Isolates were found to be sensitive against antibiotic discs, whereas resistant bacteria didn't produce a zone of inhibition. Highly effective drugs included Azithromycin, Rifampicin and Chloramphenicole showed 97%, 95.5% and 95.5% sensitive results respectively, Novobiocin was found to be 88.8% and Amoxilin showed 84.4% sensitivity, results in (Table 3). Metronidazole and Cefaxitin were found to be the least sensitive 44.4% with and 17.7% sensitivity. Further, Cu, Pb, Zn, Cr and Fe were used to test the susceptibility of bacterial Isolates i.e. sensitivity or resistance to these metals. Zone of inhibition produced by the heavy metals Cu, Pb, Zn, Cr and Fe were measured in mm scale. The sensitive bacterial Isolates showed a large zone of inhibition whereas, resistant bacteria didn't produce a zone of inhibition. The results were recorded and interpreted in Table 4. But in the case of metals the bacterial sensitivity was found to be much lower Zn (1%) had the highest recorded that was 51.1% while others had low sensitivity i.e. <50%. Fe and Pb were found to be highly resistant and showed 0% and 2.2% sensitivity respectively as shown in Table 4. The resistance of metals was probably due to the presence of heavy metals in the water and the silage of animals which were drunk. The research infers that the population of the endospore-forming bacteria did not decrease till the end of milk processing and even after heat treatment (10 minutes at 80°C). Therefore, the endospore-bacterial population causes additional contamination during milk processing [19, 20]. Further studies on the identification of endospore-forming bacterial Isolates may unveil the human health risk associated with the consumption of such contaminated milk and milk products. It is suggested that such milk be consumed after endospore processing recommended for such products by international standards.

CONCLUSIONS

It was concluded that the population of the endospore-forming bacteria did not decrease till the end of milk processing and even after heat treatment (10 minutes at 80°C). As it is known that bacterial endospores survive the boiling temperature of the water, these products prepared from such milk, even after boiling may pose health issues to the consumers. Therefore, the endospore-bacterial population causes additional contamination during milk processing. Further studies on the identification of endospore-forming bacterial Isolates may unveil the human health risks associated with the consumption of such contaminated milk and milk products. It is suggested that such milk be consumed after endospore processing is recommended for such products by international standards.

Authors Contribution

Conceptualization: JIQ
Methodology: SJ[1], SJ[2], AH[2]
Formal analysis: MAR, SA
Writing review and editing: AH[1]

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

All the authors declare no conflict of interest.

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Green Synthesis of Copper Nanoparticles from Artemisia Maritima: Characterization and Evaluation of Antibacterial Properties

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ABSTRACT

Copper nanoparticles (Cu NPs) attracted many researchers due to their potential biomedical and pharmacological activities that depend on the shape and size of the nanoparticles. **Objective:** To extract and characterize nanoparticles from the aqueous extract of the *Artemisia maritima* plant. **Methods:** UV-V spectroscopy indicates the presence of Cu NPs with unique optical characteristics. FTIR analysis identified functional groups and chemical bonds in the Cu NPs. XRD analysis revealed a hexagonal crystal structure for the Cu NPs. Antibacterial activity of the synthesized Cu NPs was evaluated against *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Bacillus subtilis,* and *Staphylococcus aureus.* **Results:** The Cu NPs exhibited varying zones of inhibition (ZOI) against different bacterial strains, with the largest ZOI observed against *Staphylococcus aureus* (20 mm), followed by *Bacillus subtilis* (19 mm), *Pseudomonas aeruginosa* (17 mm), *Klebsiella pneumoniae* (16 mm), and *Escherichia coli* (11 mm). These results highlight the potential of *Artemisia maritima*-synthesized Cu NPs as antimicrobial agents, particularly against Gram-positive bacteria. **Conclusions:** Artemisia maritima-mediated Cu NPs offer a promising, green alternative for antimicrobial development, warranting further research for clinical and environmental applications.

INTRODUCTION

Medicinal plants describe a broad group of natural herbs and botanicals which have been traditionally employed in herbal medicine. Research teams investigate the healing potential of these plants, which medical communities value for medicinal purposes. The natural compounds in medicinal plants serve as the principal factors for their health benefits, which enable the treatment of various medical conditions, including infectious diseases [1]. The rising interest in herbal medicines stems from their reputation for being safe while remaining affordable as well as environmentally friendly. The natural remedies have grown in popularity because they reportedly produce fewer serious adverse effects than conventional pharmaceutical medicines [2]. Plants, including Artemisia maritima, contain medicinal phytochemicals that enable their application in herbal medicine manufacturing and in producing natural dyes and beverages and flavorings, as well as herbal teas and cosmetics. The scientific community found anti-malarial properties in Artemisia japonica alongside Artemisia maritima and Artemisia nilegarica through laboratory testing [3]. Scientific research has demonstrated that extracts derived from Artemisia japonica and Artemisia maritima, along with Artemisia nilegarica, possess promising anti-malarial properties [3]. The culinary herb tarragon exists among numerous Artemisia genus species, with Artemisia

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dracunculus as its widely recognized variety. Many chefs use tarragon in various dishes, including meat preparations and cheese blending, as well as pickled foods and both vinegar-based condiments and mustards and decorative use for meats and fruits. Tarragon serves nutritional purposes for the kitchen, yet its phytochemical compounds could support cardiovascular health and provide pain treatment to teeth and open wounds [4]. Science has identified the plant Artemisia capillaris under its alternative name capillaristhunb, which contains multiple active compounds, including capillarisin and apigenin and hesperidin and coumaric acid. Experimental studies have proven that these substances exhibit anticancer effects and antimicrobial properties [5, 6]. The Asteraceae family includes Artemisia maritima, which serves essential purposes in both traditional medicine and contemporary pharmaceutical industries. Many known names exist for this plant, including wormseed and Santonica, along with Drooping Sea wormwood, See mugwort, and Old Woman. Artemisia maritima plant expands throughout multiple global areas, which include France, the UK, Italy, Belgium, Germany, Denmark, Pakistan, China, India, Sweden, Bulgaria, Russia, Afghanistan and the Himalayan range [7, 8]. The development of new therapeutic agents depends on antimicrobial property exploration, and silver nanoparticles demonstrate strong antibacterial and antifungal, and antiviral actions [9]. Research demonstrates that Artemisia maritima exhibits vital anticoagulant capabilities [10]. Research shows that A. maritima extracts can block enzymatic activities involved in blood clotting processes [11]. Research evidence supports A. maritimia's effectiveness in cancer treatment [12]. Research demonstrates that A. maritima extract-derived nanoparticles demonstrate dual efficacy by fighting bacterial infections and cancer cells [13]. Nanoparticles engineered with Artemisia ciniformis have shown their ability to activate apoptosis-programmed cell death as an essential mechanism to destroy cancerous cells, according to research [14]. Studies reveal that Artemisia-based nanoparticles disrupt parasite life cycles because they block the developmental progress of the parasites [15]. Scientists have discovered that silver nanoparticles excel at killing bacteria yet maintain low toxicity levels toward human cells [16]. For centuries, people have utilized Artemisia annua to treat malaria, while its active component artemisinin, remains fundamental to World Health Organization-approved combination therapies for this disease [17]. Researchers have identified multiple physical and chemical methods for creating nanoparticles. Bio-based methods receive increased attention because of their negligible effect on the environment [18]. Medicinal plant extracts proved efficient for noble metal nanoparticle synthesis, especially for silver, gold, platinum and palladium nanoparticles [19]. The well diffusion method demonstrated that synthesized nanoparticles exhibited strong antimicrobial effects against multiple disease-causing pathogens [20]. The exploitation of Artemisia maritima for biological nanoparticle synthesis represents an innovative approach because there is no documented literature regarding its utilization in producing copper nanoparticles. The current work utilizes this plant in green synthesis to generate valuable knowledge about sustainable nanotechnology while developing eco-friendly substitutes for conventional chemical-intensive syntheses. We chose Artemisia maritima because it contains rich phytochemicals alongside abundant endogenous copper that could help make efficient Cu NPs and boost their activity levels. A unique study emerges because no previous research has fully examined Artemisia maritima as a method for environmentally friendly copper nanoparticle manufacturing.

This study aims to synthesize and characterize Cu NPs using A. maritima leaf extract and evaluate their antimicrobial efficacy against selected bacterial strains. We hypothesise that A. maritima-derived phytochemicals will enable the eco-friendly synthesis of Cu NPs and confer significant antimicrobial activity.

METHODS

For extract preparation, 20g of fine powder of dried plant was mixed in 200 ml of distilled water in a flask and homogenized on a magnetic stirrer at 70°C for 1 hour. Later, the mixture with filtered with a muslin cloth to remove large particles to obtain a liquid solution. The obtained mixture was centrifuged at 3000 rpm for 15 minutes. After centrifugation, the supernatant was filtered by Whatman filter paper No. 1 three times to obtain a clear *Artemisia maritima* plant extract. The extract was stored at -4°C for further use as a reducing and stabilizing agent in the synthesis of Cu NPs. Sterility was maintained throughout the experiment. An aqueous solution of 1 mM Copper sulfate (CuSO4) was prepared in a 250 ml Erlenmeyer flask and used for the synthesis of Cu NPs. *Artemisia maritima* extract was added drop-wise into 50 ml of 0.4M copper nitrate solution at lab temperature for 1 hour with continuous stirring. The colour of the nanoparticle solution changed to a dark brown precipitate, indicating the formation of copper nanoparticles. The entire reaction took place in the dark. The complete reduction of CuSO4 to Cu+2 ions was confirmed by the change in color from colorless to colloidal brownish yellow. The colloidal mixture

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was then sealed and stored properly for future use. To isolate the nanoparticles, either unloaded or Ampicillin loaded, the solution was centrifuged (15 minutes; 15,000 rpm) and the pellet was kept for lyophilization. Copper ions and plant extract residue were removed from the Cu-NPs-containing pellet by washing it three to four times with deionized water. After drying in the hot air oven, the obtained powder was stored in Eppendorf tubes for future analysis. The nanoparticles were further confirmed spectroscopically. To check the antibacterial activity of Cu NPs synthesized by *Artemisia maritima* against bacterial strains (such as *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, and *Bacillus subtilis*), the serial dilutions of Cu NPs (4 mg, 2 mg, 1 mg, and 0.5 mg) were prepared in 1ml of DMSO (dimethyl sulfoxide). For bacterial growth, nutrient broth and nutrient Cuar media were prepared. The nutrient broth is a liquid medium that is used for the expansion of bacterial growth. For the preparation of the nutrient broth, 3g of the nutrient broth powder was taken and added to 100 ml of distilled water in a conical flask. After mixing and dissolving, the media were sterilized in an autoclave at 121°C for 15 minutes. For nutrient Agar preparation, 28g of nutrient Agar media was dissolved in 1000 ml of distilled water and autoclaved at 121.5°C and approximately >15 psi. Media was poured into plates and then left the solidify. When the gel was completely solidified then poured 200 microliters of the bacterial strains mentioned above were poured into each plate. After, pouring bacterial strains were streaked over the plates with sterile cotton swabs. For the preparation of antibiotic dilution, 4 mg of powder of Ampicillin was taken, and 1ml of distilled water in an Eppendorf tube. The solution was sonicated in an ultrasonic bath for 3 minutes to mix it properly. The dilutions were stored in the refrigerator at 4°C for further use. Cu NPs made from *Artemisia maritima* aqueous extract were prepared for dilution by adding 1 milliliter of dimethyl sulfoxide to an Eppendorf tube. To thoroughly mix the solution, it was sonicated for three minutes in an ultrasonic bath. Twenty microliters of the stock's bacterial strains were added to culture tubes with one milliliter of nutrient broth medium to create inoculate. For a whole day, the culture tubes holding the different bacterial strains were kept in an incubator. The bacterial inoculum was ready after a day. Once turbidity was detected, the inoculum was applied to nutrient agar plates. After being autoclaved, the nutrient agar medium was allowed to cool to room temperature. After being transferred onto Petri dishes, the liquid agar medium was allowed to set. Using a micro-pipette, the 200 µl inoculum was transferred into agar plates. With the use of a sterile cup propagator, the inoculum was dispersed throughout agar medium until it was entirely digested under laminar flow. The Well Diffusion assay is primarily used to determine bacterial strain susceptibility to antibiotics, with a clear zone around the well reflecting bacterial antibiotic sensitivity. We used nutritional Agar plates spread with bacterial strains instead of discs. Then, 10mm diameter wells were prepared in the gel and named A, B, C, D, +ve, and -ve. A has the highest concentration (4 mg/ml), and D has the lowest concentration (0.5 mg/ml). DMSO was used as a negative (-ve) control, and Ampicillin as a positive control (+ve). Cu NPs synthesized by Artemisia maritima were injected into the wells and incubated for 24 hours at 37°C to determine the zone of inhibition (ZOI). Both gram-positive and gram-negative bacterial strains were used to examine the ZOI. After an incubation period of 24-48 hours, the plates were examined for the efficacy of antibacterial samples by measuring the area of the barrier (i.e., ZOI) in millimeters (mm).

RESULTS

In the present UV characterization, the broad peak suggests that the Cu NPs synthesized by *Artemisia maritima* have a distribution of sizes, which could be due to various factors such as the synthesis method, reaction conditions, and stabilizing agents used during the synthesis process (Figure 1).

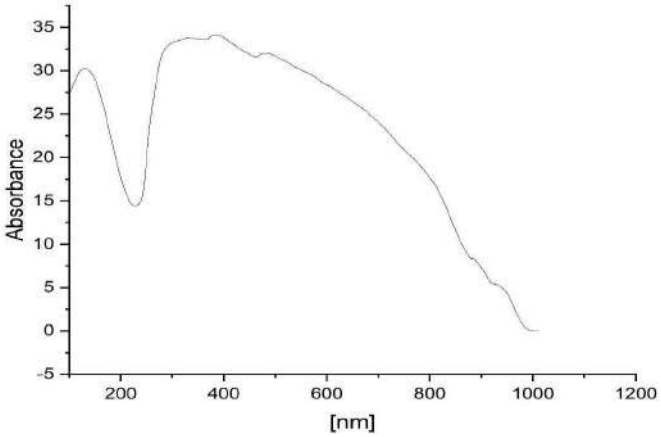

Figure 1: UV-Vis Representing the Absorbance Peaks of Artemisia Maritima-Mediated Cu NPs

The FTIR spectrum of *Artemisia maritima*-mediated CuNPs exhibited characteristic bands at 3593.38 cm-1, 2877.79 cm-1, 2349.29 cm-1, 1680 cm-1, 1502.5 cm-1, and 430.13 cm-1. FTIR analysis shows a strong broad stretching of the free–OH functional group at 3593.38 cm-1. The sharp peak at 2877.79 cm-1 is attributed to C-H asymmetric stretching. Strong C=O stretching is involved in the absorption band at 1680 cm-1. The C-O bond of carbonate ions, assigned to the oxy-carbonate structure (Figure 2).

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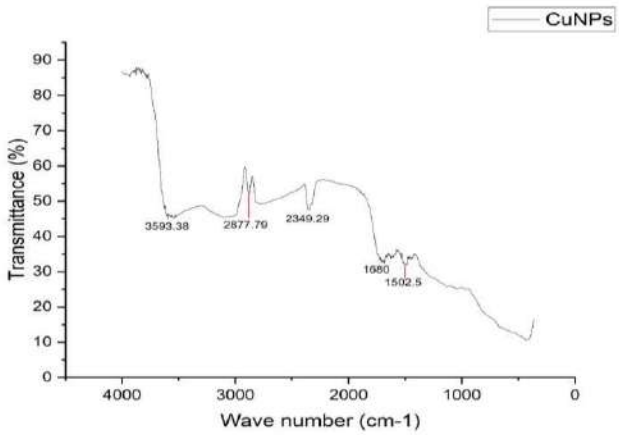

Figure 2: FTIR Spectrum of Cu NPs Synthesized from Artemisia Maritima

The XRD diffraction pattern shows the XRD pattern of Cu NPs of Artemisia maritima, which manifests various angles at 2θ, several sharp diffraction peaks at positions (2θ) are 20.7647°, 38.2520°, 44.4898°, 44.5963°, 64.8751°, 65.0540°, 78.0168°, and 78.2430°. The peak at 20.7647° could correspond to a copper phase with a particular crystal structure. The peaks at 44.4898° and 44.5963° might indicate a separate copper phase or a possible impurity (Figure 3).

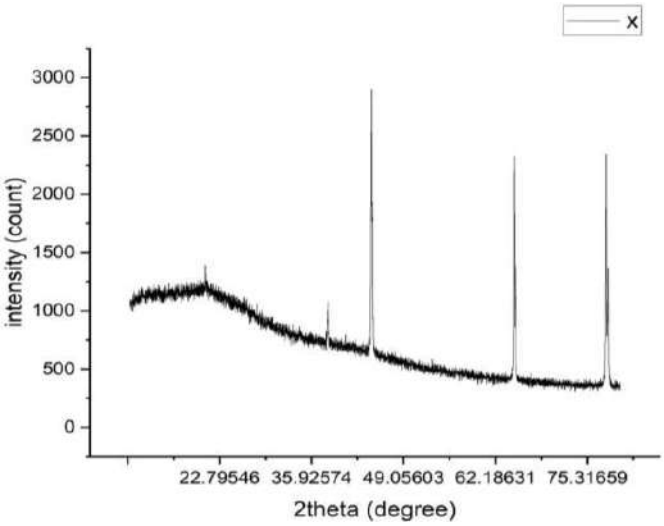

Figure 3: XRD Pattern of Cu NPs Synthesized from Extract of Artemisia Maritima

At a concentration of 40 µg/ml, Cu NPs demonstrated notable antimicrobial activity against all tested bacterial strains. The largest inhibition zone was observed for *Staphylococcus aureus* (20 mm), followed by *Bacillus subtilis* (19 mm), *Pseudomonas aeruginosa* (17 mm), *Klebsiella pneumoniae* (16 mm), and *Escherichia coli* (11 mm). These findings suggest that Cu NPs are more effective against Gram-positive bacteria than Gram-negative bacteria at this concentration. Conversely, at the lowest tested concentration (5 µg/ml), the antimicrobial activity of Cu NPs was significantly reduced. No inhibition zones were observed for the Gram-negative Bacteria E. coli and K. pneumoniae, indicating a lack of activity at this dosage. However, *P. aeruginosa* showed a moderate inhibition zone of 12.5 mm, while B. Subtilis and S. aureus exhibited zones of 12mm and 13mm, respectively. Despite reduced efficacy, these results confirm that Cu NPs retained some degree of antimicrobial activity even at low concentrations (Table 1).

Table 1: Zone of Inhibitions (mm) Demonstrating the Antibacterial Activity of Artemisia Maritima-Synthesized Cu NPs Against Selected Bacterial Species

Concentrations of Cu-NPs	Zone of Inhibition (mm) Against Bacterial Species				
	Gram Negative			Gram Positive	
	Escherichia coli	*Klebsiella pneumonia*	*Pseudomonas aeruginosa*	*Bacillus subtilis*	*Staphylococcus aureus*
40 µg/ml	11	16	17	19	20
20 µg/ml	10	12	16	16	17
10 µg/ml	-	11	13.5	15	14
5 µg/ml	-	-	12.5	12	13
Positive Control	44	45	40	45	39
Negative Control	0	0	0	0	0

As expected, the negative control did not show any zone of inhibition for any of the tested bacterial strains, confirming the validity of the results and excluding the possibility of external contamination. Overall, the results affirm that Cu NPs possess concentration-dependent antimicrobial activity, with greater effectiveness observed against Gram-positive bacteria (Figure 4).

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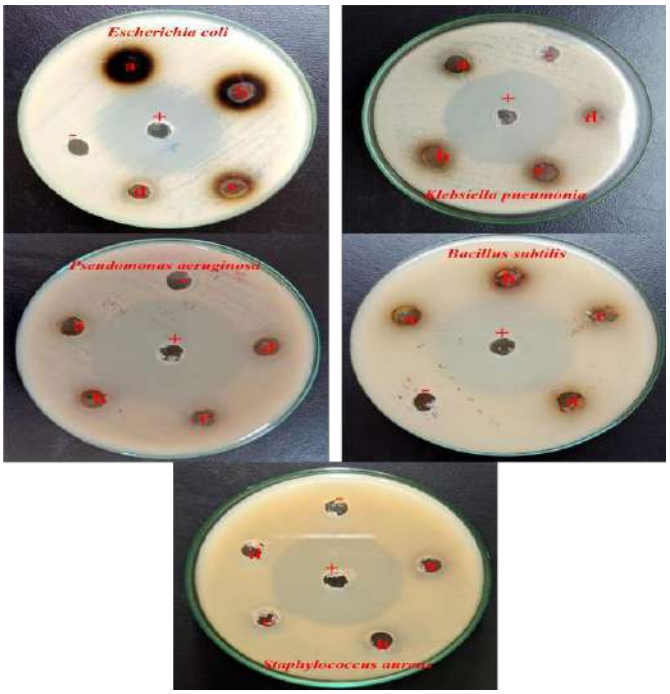

Figure 4: Zone of inhibition (mm) Demonstrating the Antibacterial Activity of Artemisia Maritime-Synthesized Cu NPs Against Selected Bacterial Species

DISCUSSION

This study highlights the promising potential of Artemisia maritima as a sustainable source of the green synthesis of copper nanoparticles (Cu NPs), an area still underexplored. The plant's phytochemical richness and inherent copper content make it a viable candidate for eco-friendly nanoparticle production. The successful synthesis and characterization of Cu NPs from A. maritiima mark a new contribution, as limited studies to date have evaluated their application in nanoparticle fabrication. MIC values indicated a higher inhibitory effect against Bacillus subtilis, Klebsiella pneumonia, and Staphylococcus aureus. The enhanced antibacterial performance may be attributed to the ability of Cu NPs to disrupt bacterial membranes, generate reactive oxygen species (ROS), and release copper ions that interfere with intracellular functions [21, 22]. These multitarget mechanisms enhance their antimicrobial efficacy while reducing the chances of resistance development. At 5 µg/mL, Cu NPs still showed moderate inhibition against *P. aeruginosa*, *B. subtilis*, and S. aureus, strengthening their potency at even low concentrations. The UV-Vis spectroscopy confirmed nanoparticle synthesis, with a surface plasmon resonance (SPR) peak observed between 370-419 nm, typical for copper nanoparticles. FTIR analysis revealed functional groups such as hydroxyl, carbonyls, and aromatic rings-suggesting the involvement of plant phytochemicals in reduction and stabilization. Though flavonoids are known to contribute to these processes, their broader biological relevance is well established and need not be elaborated further here. The XRD analysis provides information about the diffraction pattern of Cu NPs synthesized from the extract of *Artemisia maritima*. The XRD pattern exhibits several sharp diffraction peaks at different angles [2θ]. The specific peaks mentioned in the results are at 20.7647°, 38.2520°, 44.4898°, 44.5963°, 64.8751°, 65.0540°, 78.0168°, and 78.2430°. The peak at 20.7647° suggests the presence of a copper phase with a particular crystal structure. The peaks at 44.4898° and 44.5963° indicate the existence of either a separate copper phase or a potential impurity. These results indicate that Cu NPs at this concentration effectively inhibited the growth of Gram-positive bacteria to a greater extent compared to Gram-negative bacteria. The antimicrobial activity reduced substantially when the Cu NPs concentration reached the lowest tested level of 5 µg/ml. Antibiotic susceptibility testing demonstrated that Pseudomonas aeruginosa developed a 12.5 mm zone of inhibition. The data shows that Cu NPs at 5 µg/ml displayed reduced antimicrobial activity when compared to concentrations above it. The negative control without antimicrobial agents showed no inhibitory effect which proves that the observed zone of inhibition was directly caused by the presence of Cu NPs and not affected by external elements. The results from this study show solid evidence to support the antimicrobial properties of Cu NPs. This study demonstrates that Cu NPs synthesized from Artemisia maritima show promise as an antimicrobial agent. The antimicrobial activity of Cu NPs showed greater inhibition against Gram-positive bacteria while also producing strong effects against both bacterial strains tested. Results show that increased concentrations of Cu NPs lead to more potent bacterial growth inhibition. Future research must study how Cu NPs work and investigate their possible uses in fighting bacterial diseases. Future studies need to broaden their investigation of bacterial strains to determine the full antimicrobial spectrum of Cu Nps.

CONCLUSIONS

It was concluded that artemisia maritima-mediated Cu NPs offer a promising, green alternative for antimicrobial development, warranting further research for clinical and environmental applications.

Authors Contribution

Conceptualization: SA
Methodology: SA, MA, MNA, UUR
Formal analysis: SA
Writing review and editing: HAA, MNA

All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

All the authors declare no conflict of interest.

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Original Article

Antioxidant Profiling of Rice Varieties for Use as Therapeutic Diet

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ABSTRACT

Nowadays, despite the production of a large number of new rice cultivars with improved yield and enhanced climate tolerance, the average person's nutritional needs are not being fulfilled. Although rice has higher antioxidant activity, which significantly improves human health. **Objectives:** To assess secondary metabolite composition and antioxidant potential of various local and mutant rice varieties. **Methods:** The Nuclear Institute of Agriculture (NIA) in Tandojam, Sindh, Pakistan, provided thirteen different rice cultivars. All experiments were carried out three times to find out the total phenolic content (TPC), total flavonoid content (TFC) and to analyze the antioxidant potential of rice extracts with various solvents by DPPH, FRAP, Fe2+ chelating activity and OH- radical scavenging activity. Graph-Pad Prism 7.0 was applied for analysis of the data. **Results:** It was found through the study that mutant rice cultivars differ significantly ($p<0.05$) from local rice varieties. Rice varieties such as Sonehri Sugdasi, Shandar, and Shua-92 had the highest TPC, while Jajai-77, IRI-6, and Shandar had the highest TFC. Shua-92, Mehak and Shadab were found to be best at their antioxidant potential. **Conclusions:** It was concluded that mutant rice varieties showed a significant difference from their parent varieties. The study highlights the antioxidant potential as phenolics known for their antioxidant properties, are of interest, with higher consumption linked to reduced cardiovascular and cancer risks. Notable varieties included Sonehri, Sugdasi, Shandar, Shua-92, Jajai-77, RI-6, and Shandar for the highest TPC and TFC, respectively. It was found through the study that mutant rice cultivars differ significantly ($p<0.05$) from local rice varieties.

INTRODUCTION

Rice is the most popular and nutritious staple food of not only Pakistan but also approximately 50% of the world's population. In Pakistan, rice is one of the main exports and the second most important cereal crop with a total production of 7.4 million metric tons [1, 2]. Rice also has a diverse range of antioxidant, anti-allergic, anti-inflammatory and anti-cancerous properties. Rice contains vitamins, fiber, minerals, and bioactive polyphenols and flavonoids (like ferulic acid, tocopherols, phytic acid)[2, 3]. These phytochemicals play an important role in inhibiting cellular oxidation via free radical scavenging and thus maintaining the proper ratio between antioxidants and oxidants [4]. In several ways, tissues and cells are being damaged by Oxidative stress, which is made possible by a disequilibrium in the balance due to the change in concentration of antioxidants and oxidants. Different mechanisms may be used by phenolic compounds to exhibit their antioxidant action [5]. As chain-breaking antioxidants, some reactive species, such as superoxide and hydroxyl radicals, are directly scavenged by them. Lipid peroxidation can be controlled or reduced by recycling more antioxidants, such as tocopherol. Some polyphenols are pro-oxidants which can bind with metals such as copper and iron, hindering the production of free radicals from these pro-oxidants while still conserving their ability to scavenge free radicals [6, 7]. Certain polyphenols are also associated with a rise in the activity of antioxidant enzymes and the activation of some antioxidant proteins. Phenols and flavonoids have been explored to have therapeutic potential for cancer and

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inflammation due to their extraordinary antioxidant capacity [8, 9]. The ions such as hydroxyl radical, superoxide ion and hydrogen peroxide, among them, can all be promptly scavenged by flavonoids, which serve as the most predominant plant secondary metabolites in rice [10, 11]. Therefore, some mutant rice varieties (such as Shadab, Shandar, etc.) with genetic variations have been developed to get higher yield, and these may have comparatively better nutrition in terms of polyphenols and flavonoid content.

This study aims to explore the therapeutic potential of rice varieties (in terms of their secondary metabolite content and antioxidant potential) for combating life-threatening diseases like cancer.

METHODS

The experimental study design was carried out for a comparative nutritional assessment and antioxidant potential of various mutant as well as local rice cultivars in Sindh province, Pakistan. The study duration was 2022-2023. Thirteen varieties of rice cultivars were procured from the Nuclear Institute of Tandojam, which include Sadagulab, Sugdasi Ratria, and Sonehri Sugdasi, IRI-6, Shadab, Shandar, Sarshar, IRI-8, Khushboo-95, Jajai-77, Shua-92, uper Basmati, and Mehak. Among them, IRI-6, Jajai-77, IRI-8 and Super Basmati were parent cultivars, whereas Shandar and Shadab were included as mutant varieties of IR6, Shua-92 and Sarshar as mutants of IRI-8, Khushboo-95 as a mutant of Jaijai-77 and Mehak as a mutant of Super Basmati. Three rice cultivars were selected as references, i.e., Sada gulab, Sugdasi Ratria and Sonehri Sugdasi. To justify a chosen sample size for rice research, a power analysis was conducted before the study, considering the effect size, desired power level (typically 80%), and significance level (usually 5%) to ensure sufficient statistical power to detect meaningful differences. The total phenolic and flavonoid content in rice varieties was analyzed using a control randomized sampling. Each sample was extracted with 20 times its weight of methanol 80% (v/v) that was acidified with 1% (v/v) hydrochloric acid. This was conducted by sonication at 25°C for 2 hours. After centrifugation at 2000 rpm (15 minutes), the supernatant was used to determine the total content of phenolics and flavonoids. A previous procedure [3] was followed to assess the TPC; for this, 200 µL supernatant, 1.5 mL of Folin-Ciocalteu's reagent, an incubation for five minutes and the addition of 1.5 mL of Sodium Carbonate (6% w/v) were used. The contents were incubated for one hour at 25°C, and the data were gathered at 725 nm. Gallic acid equivalent (GAE) was used to measure the total polyphenol content (mg per gram protein). To identify total flavonoid content, the colourimetric technique was used but with minor changes [4]. 5% sodium nitrate of 75 µL was mixed with the sample of 250 µL and distilled water of 1.25 mL. Six minutes after the water was introduced, 150µL of AlCl3.H2O (10%) was added, and the solution remained untouched for 5 minutes. 2.5 mL of the solution was made up using 500µL of NaOH. The absorbance was determined on a UV-Visible spectrophotometer at 510nm. The catechin equivalents (CE, mg (+)-catechin/g sample) indicated the total flavonoid content. To test for antioxidant activity, de-husked rice was ground into flour. 1 gram of each flour was measured and soaked in 10 milliliters of distilled water for 5 minutes, after which a centrifuge (LXJ-IIC made by Shanghai Anting Scientific Instruments) was used to spin the solution at 14000 rpm for 15 minutes to get the supernatant. The DPPH, Fe2+ reducing power, OH radical scavenging and FRAP assays were performed using this supernatant [5]. For the DPPH assay, Rice samples (3 mL) were mixed with 150 µl DPPH solution (0.1 mM in 95% ethanol), then incubated in the dark for 30 minutes at 270°C. The absorbance at 517 nm was studied for every sample [3]. As blanks, ethanol and ascorbic acid were used in the experiment, and the results were compared with those. The percent of scavenging was calculated by the following formula. DPPH scavenging is measured as 100 times (1- the absorbance of the sample/the absorbance of the blank). To measure the ferrous ion-chelating activity [6], a 500 µl sample was mixed with 1 ml of Ferrozine (0.5 mM), vigorously shaken for 30 minutes at room temperature and the absorbance was measured at 562 nm when the shaking was completed. The experiment included EDTA to act as a positive control. % Binding Activity (Absorbance of blank – Absorbance of the sample) x 100. The sample (100 µl) was placed into 3 ml of FRAP reagent, vortexed and at this point, absorbance was measured immediately. The water bath was heated to 370C °C for 4 minutes, and the second reading was taken at 593 nm. FRAP value of the Sample (µM) = (The change in sample absorbance divided by the change in reference absorbance) ×2/absorbance of blank. When the FRAP value of the standard (ascorbic acid) is found to be 2. 0.2 ml of 10 mM FeSO4. 7H2O was incorporated with 10 mM EDTA, and 0.2 ml of deoxyribose solution (10 mM) was added to the mixture. After that, 0.1 M phosphate buffer (1 mL; PH 7.4) was mixed with the above solution, and 0.2 mL each of the sample and H2O2 10 mM were added to the mixture in a screw-capped tube. The mixture was then incubated at 37°C for one hour. When the incubation was over, 1 ml of trichloroacetic acid (2.8%) and 1 ml of Thiobarbituric acid (1.0%) were added to the mixture. What was left in the cuvette was boiled for 10 minutes, and the absorbance was collected at a wavelength of 520 nm after cooling. A positive control in the investigation was BHA. OH-Scavenging Activity (Absorbance of blank – Absorbance of the sample) x 100. Measure the absorbance of a blank. Data

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were shown using mean and standard deviation. Both ANOVA and T-test were applied to the data in Graph-Pad Prism 7.0 and Excel 2016. Any p-value less than 0.05 was considered to be significant.

RESULTS

Estimation of total phenolic content depicted that mutant cultivars, including Shua-92 and shandar, have high phenolic content as compared to their local varieties. Whereas among standard rice varieties, Sonehri Sugdasi showed highly significant phenolics content as compared to other reference varieties. The findings showed that overall polyphenol content varied considerably ($p<0.05$) amongst the rice cultivars (Figure 1).

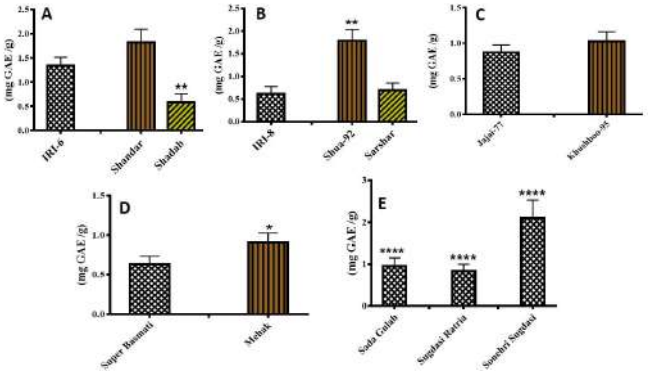

Figure 1: Comparison of Total Phenol Content in Local and Mutant Rice Varieties

****=p<0.001, *** =p<0.005, ** =p<0.01. *=p<0.05

Determination of total flavonoid content revealed that Shua-92 and Mehak had higher total flavonoid content as compared to their local varieties, whereas Khushboo-95 and Shadab differed significantly from their parental varieties. All rice varieties taken as reference showed highly significant flavonoid content (Figure 2).

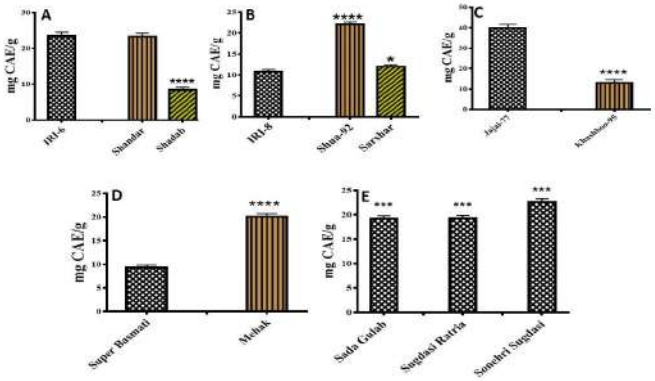

Figure 2: Comparison of Total Flavonoid Content in Local and Mutant Rice Varieties

****=p<0.001, *** =p<0.005, ** =p<0.01. *=p<0.05

Results showed high DPPH in Sugdasi Ratria and Sonehri Sugdasi in comparison to Sada Gulab but three varieties were found significant. Mehak variety showed significantly high DPPH radical scavenging activity as compared to the parent variety. Moreover, OH scavenging results revealed that local line varieties Sugdasi Ratria were significantly higher than the Sada Gulab and Sonehri Sugdasi. Among mutant cultivars, shadab and khusboo-95 showed significantly high OH scavenging as compared to their local varieties (Table 1).

Table 1: DPPH Radical Scavenging Activity in Local and Mutant Rice Varieties

Sr. No	Rice Varieties	DPPH Radical Scavenging	p-value	OH Scavenging	p-value
1	IR-6	79.06 ± 2.83	<0.005	38.47 ± 1.71	<0.005
2	Shandar	79.78 ± 2.71		38.04 ± 1.61	
3	Shadab	50.57 ± 2.63		51.62 ± 1.92	
4	IR-8	54.57 ± 2.68	<0.005	38.91 ± 1.76	<0.005
5	Shua 92	79.02 ± 2.96		35.84 ± 1.56	
6	Sarshar	52.88 ± 2.79		38.41 ± 1.64	
7	Jajai-77	77.99 ± 2.67	>0.05	33.04 ± 2.54	<0.01
8	Khushboo-95	78.73 ± 2.57		55.98 ± 3.94	
9	Super Basmati	62.09 ± 2.46	<0.001	49.12 ± 2.38	>0.05
10	Mehak	82.33 ± 2.82		55.97 ± 3.59	
11	Sada Gulab	68.17 ± 2.43	<0.01	49.25 ± 1.76	<0.01
12	Sugdasi Ratria	77.78 ± 3.65		52.46 ± 1.85	
13	Sonehri Sugdasi	79.89 ± 3.52		44.39 ± 1.64	

Comparison of FRAP among rice varieties revealed that Mehak and Shua-92 had significantly higher FRAP activity, respectively, as compared to their local counterparts. Sugdasi Ratria showed increased FRAP values as compared to the other two local rice varieties, Sonehri Sugdasi and Sada Gulab. While comparing the Fe2+ chelating activity, the mutant cultivars. Local varieties like Sada Gulab, Khushboo-95 and Sarshar had Fe2+ chelating activity values (Table 2).

Table 2: FRAP and Fe (II) Chelating Activity in Local and Mutant Rice Varieties

Sr. No	Rice Varieties	FRAP	p-value	Fe (II) Chelating Activity	p-value
1	IR-6	15.77 ± 0.51	>0.05	78.11 ± 0.95	>0.05
2	Shandar	17.79 ± 0.62		79.92 ± 1.77	
3	Shadab	17.51 ± 0.53		81.14 ± 2.95	
4	IR-8	11.96 ± 0.97	<0.01	71.89 ± 1.71	>0.05
5	Shua 92	17.78 ± 1.22		74.09 ± 1.76	
6	Sarshar	12.59 ± 0.99		83.46 ± 2.89	
7	Jajai-77	16.41 ± 1.21	>0.05	70.63 ± 1.99	<0.01
8	Khushboo-95	17.78 ± 1.81		81.34 ± 2.35	
9	Super Basmati	14.49 ± 1.23	<0.005	79.18 ± 1.99	<0.01
10	Mehak	26.32 ± 1.34		69.38 ± 1.71	
11	Sada Gulab	16.41 ± 0.91	<0.001	80.78 ± 1.98	<0.01
12	Sugdasi Ratria	28.41 ± 1.51		72.58 ± 1.78	
13	Sonehri Sugdasi	18.62 ± 1.12		71.93 ± 1.97	

DISCUSSION

Rice polyphenols have received prominent attention in combating cellular oxidative damage due to their ability to scavenge free radicals and quench singlet oxygen [8]. In our study, mutant varieties showed a higher TPC (total phenolic content) than the local varieties. High Phenolic

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content is vital in oxidative stress regulation for the prevention of cancer, nervous, cardiovascular and other acute and chronic disorders, as evident from Various epidemiological research [10]. Shua-92 and Mehak are mutants that contain a lot of flavonoids. This varied flavonoid content in rice cultivars may be due to the genetic diversity between the different kinds of rice [12, 13]. Local varieties such as IRI-6SonehriSugdasi and Jajai-77 exhibited an increased flavonoid content; therefore, these varieties can be used for the treatment of coronary heart disease, gastrointestinal ulcers, cancer and rheumatic illnesses as flavonoids act together with the molecules involved in the cell growth signalling pathways and apoptosis [14, 15]. DPPH is a stable free radical, and its degree of radical scavenging is frequently associated with a sample's better antioxidant activity [16, 17]. Following the exclusion of hydrogen from antioxidants in the DPPH assay, DPPH's purple colour is lessened to a pale yellow colour. In our study, rice varieties were found to be different in terms of secondary metabolite composition and DPPH free radical scavenging capability. Differences in the amount of important chemicals in crops may be the result of genetic variation and changes in the environment [18]. The ferric reducing antioxidant power assay has been proposed to evaluate the capability of various dietary antioxidants to scavenge active free radicals. In our study, Mehak and Shua-92 had higher FRAP activity, possibly due to their higher flavonoid content, which helped them boost ferric ion reducing power [19]. A Fe2+ chelating agent caused a reduction in the red hue that appeared in the ferrous ion-chelating test. Hence, the ability of the chelator to bind metal ions could be seen by the reduction of colour. Chelating activity varied significantly among the species due to the difference in their plant secondary metabolite composition. Mutant cultivars such as Khushboo-95, Mehak and Shadab showed significant OH-scavenging activity. The free radicals, such as hydroxyl radicals, hydrogen peroxide and oxygen species, can cause DNA damage. In the OH scavenging assay, the Fenton reaction was observed releasing the hydroxyl radicals by cleavage of H2O2(due to electron transfer from ferrous ions), and these free OH radicals are reported to cause oxidative DNA damage[20].

CONCLUSIONS

It was concluded that mutant varieties such as Shua-92, Mehak and Shandar may be chosen as a high phenolic and flavonoid diet. Among local varieties, SonehriSugdasi, Jajai-77 and IRI-6 were found best in terms of phenolics and flavonoids content. Additionally, Khusboo-95 and Shadab displayed the highest chelating and OH scavenging power, respectively. Whereas, Shua 92 and Mehak were found best in terms of DPPH scavenging and FRAP. The current research may prove a useful tool for producing new rice varieties with substantial secondary metabolite composition for boosting their nutritive qualities.

Authors Contribution

Conceptualisation: ITA
Methodology: K, MAS
Formal analysis: BK, ZAA
Writing review and editing: ITA, BK, FNM

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

All the authors declare no conflict of interest.

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Original Article

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Comparative Evaluation of Phenotypic Assays for Detecting *mcr*-Mediated Colistin Resistance in *Acinetobacter baumannii*

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ABSTRACT

Acinetobacter baumannii that is resistant to colistin has proven to be a significant problem in neonatal intensive care units (NICUs), where neonates are highly susceptible to multidrug-resistant pathogens. The introduction of plasmid-mediated mobile colistin resistance (mcr) genes has added to the global panic, but the prevalence of these genes in neonatal isolates is limited in South Asia. **Objectives:** To establish the frequency of *mcr-1* to *mcr-5* genes in colistin-resistant *A. baumannii* isolates of NICU patients and to compare the phenotypic assay of colistin susceptibility to establish a dependable and affordable diagnostic tool. **Methods:** Sources of thirteen *A. baumannii* isolates were blood samples, urine samples, and respiratory samples of NICU patients. Colistin resistance in carbapenem-resistant isolates was determined by disc diffusion, broth macro-dilution, disc elution, colistin agar, and minimum inhibitory concentration (MIC). *Mcr* gene screening was done genotypically using PCR and gene-specific primers. **Results:** Eight (61.5) isolates had the *mcr-1* gene with no detection of *mcr-2* to *mcr-5*. Isolates were all colistin resistant, with MICs ranging from 16 -32 μg/ml. Colistin agar method gave similar results to the broth assays, and it was found that it is a viable and low-cost alternative to parallel screening. **Conclusions:** This research is the first reported case of *mcr-1*-mediated colistin resistance in clinical isolates of *A. baumannii* in a neonatal unit in Pakistan. Enhanced antimicrobial stewardship and resistance monitoring of neonatal care are needed. In resource-limited settings, the colistin agar method is suggested to be used as a routine laboratory screening.

INTRODUCTION

Acinetobacter baumannii is a gram-negative bacillus that is non-motile and aerobic, and has become one of the most feared nosocomial pathogens with the reputation of multidrug resistance (MDR) and resistance to eradication in hospitals. *A. baumannii* is a highly adaptable species to the environment, which has helped it to thrive in healthcare-associated infections [1]. It is closely linked with the occurrence of device-related infections, ventilator-associated pneumonia, bacteremia, urinary tract infections, and meningitis. The genotoxic acquisition of exogenous resistance genes in the pathogen, in addition to inherent resistance determinants, has contributed to its worldwide dissemination during hospital outbreaks. The spread of the opportunistic pathogen, especially in medical institutions of low and middle-income countries, is a major challenge to the survival of neonatal lives [2]. In the most recent update of the WHO Bacterial Priority Pathogens List, carbapenem-resistant *A. baumannii* (CRAB) has been designated with the critical priority, highlighting its severe nature as a threat to human health

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[3]. Antibiotics, even though they revolutionized medicine initially, have been slowed down by MDR bacteria, which has been an indication that the antibiotic era may soon come to an end. The diffusion of MDR *A. baumannii* all over the world has created a more vulnerable situation for healthcare systems. Medical devices that cause infections include ventilators, catheters, and other risk factors such as surgical operations, burns, long-term hospital stay, and immunosuppressive treatments [4]. Neonatal Intensive Care Units (NICUs) constitute a very delicate system, and newborn babies housed in them are very vulnerable to infections. Helped by these special units, a broadening of the multidrug-resistant opportunistic organisms, which in most cases are harmless to healthy people, is possible. MDR *A. baumannii* infections among neonates are linked to a high mortality rate, common use of invasive support (e.g., ventilation, vasoactive medications), and few treatment options. Neonatal sepsis due to *A. baumannii* may result in complications that need vasoactive treatment, mechanical ventilators, and poor survival outcomes [4]. Therefore, treatment opportunities are becoming limited, which is a major challenge to contemporary medicine. Colistin is a polymyxin E antibiotic, which has been regarded as a last-resort treatment of life-threatening MDR bacterial infection [5-7]. In the past, it was assumed that colistin resistance could only be caused by chromosomal mutations (e.g., pmrA/pmrB). The plasmid-mediated colistin resistance that is credited to the *mcr-1* gene and revamped the concept of colistin resistance [6, 7]. Up to the present day, ten mcr gene variants have been reported in various bacterial species [8]. At present, *A. baumannii* is treated with colistin when it occurs in neonates. Colistin resistance has become a current epidemic of increasing concern in the field of public health, and is particularly alarming in Pakistan. Recent research observed 7.3% resistance of 150 isolates from five hospitals, and another study reported that 75.6% of *A. baumannii* and 23.7% XDR in a tertiary care hospital [9, 10]. It is of urgent need to identify colistin resistance by rapid and cost-effective phenotypic approaches. Although the disc diffusion technique is not advised by CLSI and EUCAST [11] because the large colistin molecules are not well diffused, broth microdilution (BMD) and macrodilution techniques are both labour-intensive and technically challenging. Up to now, there is limited information regarding colistin resistance in *A. baumannii* isolates that inhabit Pakistani hospitals in the neonatal unit.

This study aims to focus on gathering neonatal A. baumannii isolates, determining the prevalence of *mcr* genes (*mcr-1* through *mcr*-5), and finding a consistent and fast way of testing colistin susceptibility and the determination of MIC by comparing agar- and broth-based tests.

METHODS

This cross-sectional laboratory-based study was conducted in the Department of Microbiology, BJ Micro Lab, Rawalpindi, Pakistan (March to August 2023). Clinical samples (blood, urine, and respiratory secretions) were collected from neonates admitted to the NICU of a tertiary hospital in Islamabad, Pakistan. Written informed consent was taken from all participants. Samples were obtained using aseptic techniques, transported within 2 h at 2–8°C, and processed immediately in the microbiology laboratory. Urine and respiratory specimens were grown on MacConkey and blood agar, and blood samples were put into automated blood culture bottles. Standard biochemical tests were used to identify colonies that suggested the presence of Acinetobacter, and the API-20E system (bioMérieux, France) was used to validate the results. Verified isolates of *A. baumannii* were kept at -20°C in a broth made with 20% glycerol. The study complied with the ethical standards and regulations of the Department of Life Sciences at Abasyn University's Islamabad Campus, as well as BJ Micro Lab's Gulzar Quaid, Rawalpindi. According to our institution's ethical guidelines and national regulations. The phenotypic assays were performed using different colistin concentrations to determine the resistance pattern of all bacterial strains. The appearance of turbidity in broth indicated colistin resistance Mueller Hinton (MH) agar (HiMedia, India) and MH broth (SBio, Singapore) were prepared with cationic adjustment by adding 2mg CaCl2 (Sigma, Germany) and 1mg MgCl2 (Sigma, Germany) in 100 mL media as per CLSI guidelines to prepare Cationic Adjusted Mueller Hinton Agar/Broth (CAMHA/CAMHB) [12]. The pH of the media was adjusted between 7.2-7.4. Various antibiotics, used in standard clinical practice and encompassing different antimicrobial groups were tested for susceptibility testing via disk diffusion assay as per CLSI version 2022. Only those bacterial samples were selected that were resistant to carbapenems [13]. Colistin stock solution (37.45 µg/mL colistin base equivalent) was serially diluted in CAMHB to obtain concentrations of 1–4 µg/mL. Tubes were inoculated with 10 µL of bacterial suspension (0.5 McFarland) and incubated at 37°C for 18–24 h. Turbidity indicated resistance. CLSI breakpoints were applied (S ≤ 2 µg/mL; R ≥ 4 µg/mL) [14]. *E. coli* ATCC 25922 (susceptible) and *E. coli* NCTC 13846 (*mcr-1* positive control) were included as reference strains to validate test accuracy. The disc elution method was performed by preparing 100 mL CAMHB. The inoculum was prepared in 0.5 mL of normal saline. The turbidity of this suspension was adjusted to 0.5 McFarland standards. As one colistin disc is of 10µg strength so

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colistin concentrations of 10- 40µg/mL were prepared by adding 1, 2, 3, and 4 colistin discs, respectively, in each test tube containing 1ml autoclaved distilled water, followed by incubation at room temperature for 30-40 min to elute disc components. Tubes were inoculated and incubated as above. Minimum inhibitory concentration (MIC) was assessed in broth and agar-based systems, using two-fold serial dilutions (4–128 µg/mL) in separate test tubes. The inoculum was prepared by dissolving a bacterial colony in normal saline, and 10 µL of the inoculum was added to each test tube. For the growth control, 1 mL of Mueller-Hinton (MH) broth was added to a test tube, followed by the addition of 10 µL of the inoculum. For the negative control, 1 mL of MH broth was added to a separate test tube without inoculum. The prepared test tubes were incubated at 37°C overnight. A growth control (inoculated CAMHB) and a negative control (uninoculated broth) were included for each assay [15]. Eluted colistin solution (prepared by dissolving colistin discs in autoclaved distilled water at room temperature for 30-40 min) was incorporated into CAMHA to prepare final concentrations of 2–32 µg/mL. Ten microliters of bacterial suspension were spotted onto plates and incubated at 37°C for 24 h. Growth indicated resistance. Parallel testing with *E. coli* NCTC 13846 and P. aeruginosa ATCC 27853 was performed for validation [16]. The DNA was extracted from all strains using the CTAB method with slight modifications. Briefly, a loop full of overnight-grown bacterial culture was taken into an Eppendorf containing freshly prepared CTAB lysis buffer (500 µL), proteinase K (10µL) (BioShape, Canada), 10% SDS (40µL) (Sigma Aldrich, Germany), and β-mercapto-ethanol (2 µL). The mixture was incubated at 950°C/2 hours, and finally, chloroform: isoamyl alcohol (500 0L) was added to the mixture, and centrifugation was done at 13000 rpm, and three layers were formed. A new Eppendorf tube was dried by vortexing it, and 20 mL of aqueous solution was transferred into it. Chilled isopropanol was added to the supernatant and incubated at room temperature. The pellet was centrifuged, washed with 70% ethanol, and dissolved in low TE buffer and kept at -20°C. On agarose gels stained with ethidium bromide, extracted DNA was stained [17]. Gradient PCR was used to verify the annealing temperature of each gene. The 25 µL of PCR reaction mixture was put together, and in it, 12.5 µL of master mix (Ab clonal), 2 µL of forward and reverse primers, 3.5 µL of PCR water, and 3-5 µL of template DNA were incorporated. The amplification conditions were: 25 cycles with initial denaturation at 94°C 10 min, denaturation at 94°C 30s, primer annealing at 51°C, 56°C, 57°C, 58°C and 58°C, respectively, extension at 72°C 60s, and final extension at 72 o C 10 min in theromocycler (Life-ECO). The presence of *mcr* genes was detected on a 1.5% agarose gel. *E. coli* NCTC 13846 (*mcr-1* positive) served as a positive control, while PCR-grade water was used as a negative control. To strengthen assay validation, at least one representative amplicon per target was purified and sequenced commercially to confirm gene identity via BLAST analysis. Descriptive statistics were used to summarize phenotypic and genotypic results. MIC values from broth and agar-based methods were compared using Spearman's rank correlation and Cohen's kappa (κ) for agreement analysis. Statistical analysis was done by SPSS version 27.0, and $p<0.05$ was considered significant.

RESULTS

Confirmed *A. baumannii* isolates were preserved in 20% glycerol broth at –20°C (Figure 1).

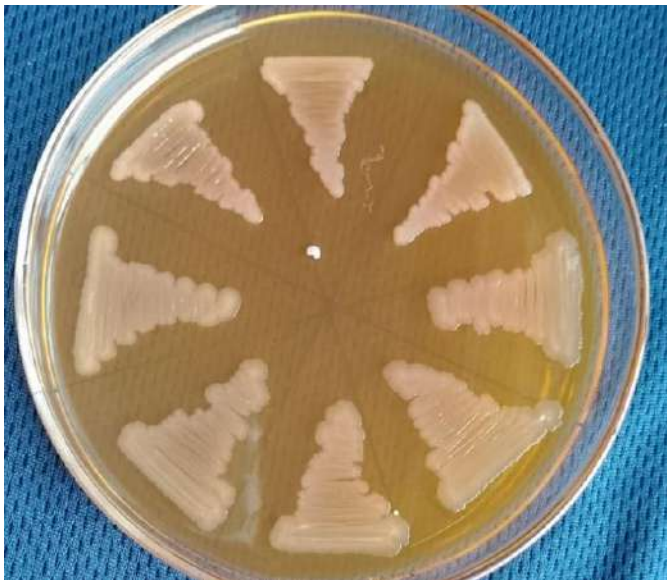

Figure 1: Growth of *A. baumannii* on MacConkey Agar

Extracted DNA was visualized on 1.5% agarose gels stained with ethidium bromide (Figure 2)

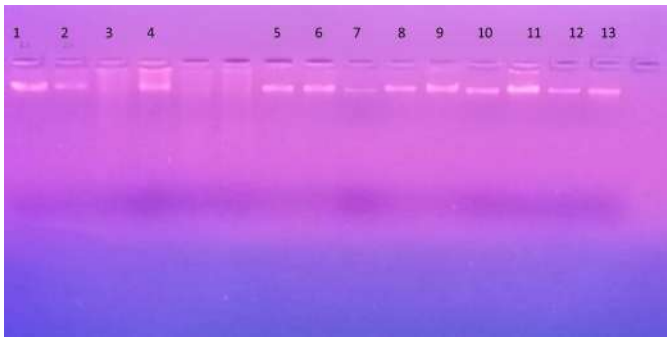

Figure 2: Agarose Gel Electrophoresis for Detection of Mcr Genes in *A. Baumannii* Isolates

The amplification of mcr1-5 genes was carried out by using the following sets of primers. Gene names, forward and reverse primer sequences, and their annealing temperature, expected product sizes, and annealing temperatures used for the amplification of mcr 1-5 genes in this study (Table 1).

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Table 1: The Amplification of *mcr1-5* Genes

Gene	Forward Primer	Reverse Primer	Product Size	Ta
mcr-1	AGTCCGTTTGTTCTTGTGGC3'	AGATCCTTGGTCTCGGCTTG	320 bp	51°C
mcr-2	CAAGTGTGTTGGTCGCAGTT3'	TCTAGCCCGACAAGCATACC	715 bp	56°C
mcr-3	AAATAAAAATTGTTCCGCTTATG	AATGGAGATCCCCGTTTTT	>929 bp	57°C
mcr-4	TCACTTTCATCACTGCGTTG	TTGGTCCATGACTACCAATG	1116 bp	57°C
mcr-5	ATGCGGTTGTCTGCATTTATC	TTGGTCCATGACTACCAATG	1644 bp	58°C

Confirmed *A. baumannii* isolates were preserved in 20% glycerol broth at –20°C (Figure 1).

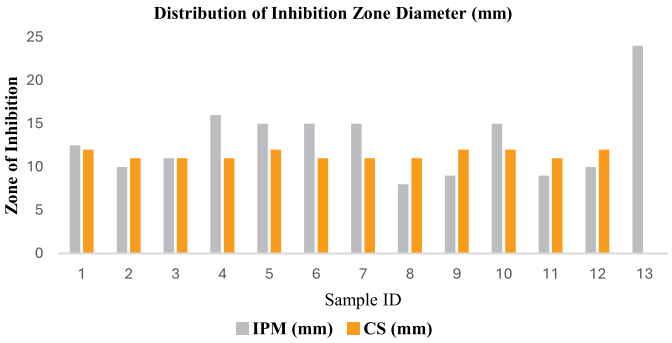

Figure 2: Zone of Inhibition Produced by *A. baumannii* Isolates

The recommended CLSI (2023) cutoff for colistin by broth macro-dilution is S ≤ 2 µg/mL and R ≥ 4 µg/mL. No statistical difference was observed in optical density among replicates ($p>0.05$), indicating consistent assay reproducibility. In the disc elution method, the results were comparable to the broth macro-dilution method; all strains showed resistance against colistin-eluted solutions at concentrations of 1, 2, 3, and 4 µg/mL. This consistency further validated the reliability of broth-based methods over disc diffusion for detecting colistin resistance. For MIC determination, two methods were compared colistin agar test (CAT) and the BMD. Comparable results were obtained using both methods. All strains showed resistance to colistin at concentrations ≥ 4 µg/mL. Results show high-level resistance across all isolates (MIC ≥ 16 µg/mL) (Figure 3).

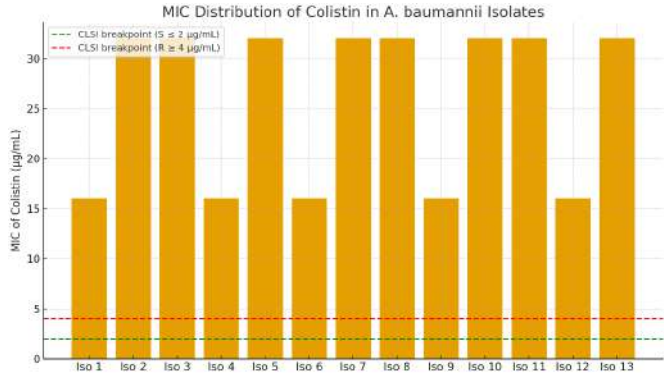

Figure 3: MIC of Colistin against *A. baumannii* Isolates in CAMH Broth

These results follow the colistin agar assay, where: (a) 8 µg/mL showed growth of all isolates; (b) 16 µg/mL showed inhibited growth in strains 1, 7, and 9; and (c) 32 µg/mL showed complete growth inhibition. Scale bar = 20 mm (Figure 4).

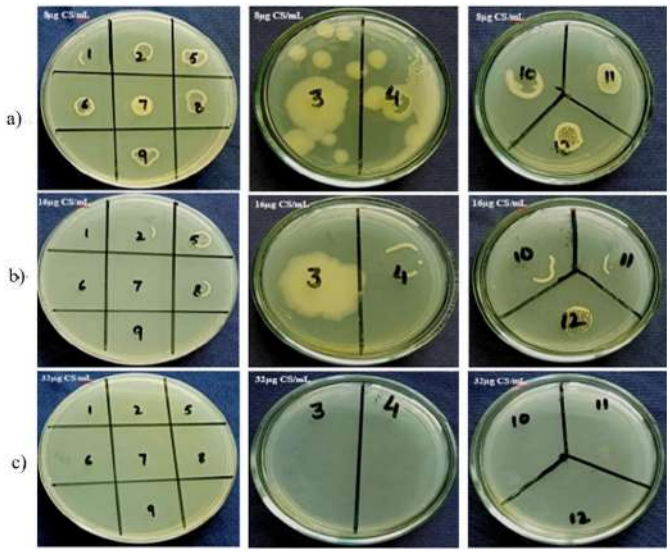

Figure 4: Evaluation of Colistin Resistance on Agar Plates Containing Different Colistin Concentrations

All the colistin-resistant strains of *A. baumannii* were screened for the presence of mcr1 to 5 genes. Only the *mcr-1* gene was detected in strains 1, 2, 3, 5, 6, 7, 8, and 9; however, other resistance determinants, including *mcr-2*, *mcr-3*, *mcr-4*, and *mcr-5*, were not found in any of the strains. Lane M: 100 bp DNA ladder; Lanes 1–9: *A. baumannii* isolates; Lane C+: positive control; Lane N: negative control. Bands confirmed the presence of mcr-1 in eight isolates (1, 2, 3, 5, 6, 7, 8, and 9) (Figure 5).

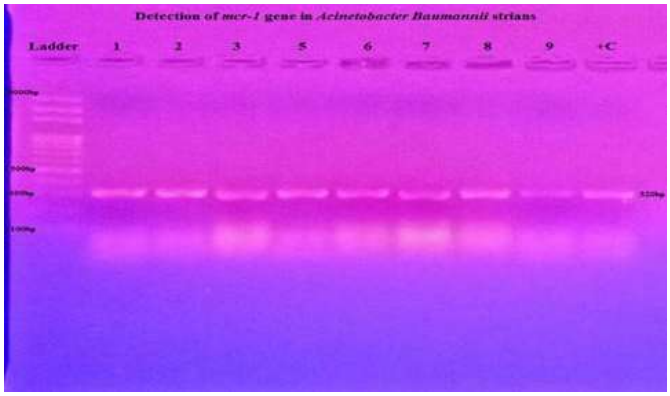

Figure 5: Visualization of PCR-Amplified *mcr-1* Gene (320 bp) Bands on 1.5% Agarose Gel Electrophoresis

DISCUSSION

One of the most widely spread mcr determinants, the *mcr-1* gene, has been reported in different bacterial strains in more than 60 countries [18, 19]. This paper presents the initial findings of the *mcr-1* gene in *A. baumannii* isolates in the NICU of a tertiary hospital in Islamabad, Pakistan. The

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study found that 8 of 13 (61.5) of the isolates had genotypic resistance to colistin since they had *mcr-1*, and the rest (38.5) of the isolates were resistant to colistin, but that could be due to other mcr variants (like *mcr-6* to *mcr-10*) or could be because of chromosomal modes of resistance. Such mechanisms are the lipid A modification through the addition of L-phosphoethanolamine (PEtN) or 4-amino-4-deoxy-L-arabinose (L-Ara4N), impairment of lipopolysaccharide biosynthesis genes, or expression of efflux pumps [20, 21]. On the contrary, 37 carbapenem-resistant *A. baumannii* (CRAB) isolates were analyzed by Germ *et al.* who detected no *mcr-1* to *mcr-5* gene [22]. In our investigation, all isolates showed an inhibition zone that did not exceed 12 mm in the disc diffusion test. Nevertheless, another study in a similar manner has stated that the disc diffusion technique is not very reliable in detecting colistin resistance because of poor diffusion in agar media [23]. There was a comparative analysis of phenotypic assays performed in this research that showed significant methodological understanding. The five methods tested included disc diffusion, broth macro-dilution, disc elution, MIC on colistin agar, and broth MIC broth and agar-based MIC assays; all had the highest concordance with genotypic results (100%), although disc diffusion demonstrated low agreement. Both disc elution and broth macro-dilution assays were consistent in their categorical interpretation, meaning high reliability and diagnostic accuracy. Phenotypic approaches that have been validated in terms of their performance are still necessary where there is no molecular assay, especially in low-resource laboratories. The research establishes colistin resistance in most of the isolates through phenotypic and genotypic methods, and the use of reliability in in-house methods is crucial. The colistin agar technique turned out to be reproducible as well as practical, and less equipment is required; parallel testing can be done, which is an advantage to NICU surveillance in resource-constrained hospitals. The finding of *mcr-1*-positive isolates in a neonatal facility is also very disturbing, because neonates are extremely sensitive to blood-borne infections and they do not have many treatment options. Reports of this nature demonstrate the need to improve infection control measures that include hand hygiene, decontamination of reusable equipment, and frequent monitoring of antimicrobial resistance [24]. Our findings underscore the urgent need for antimicrobial stewardship in Pakistan's healthcare system, particularly in NICUs, where horizontal gene transfer and clonal spread of mcr-mediated resistance can occur rapidly. The study also reinforces the importance of continuous surveillance of *A. baumannii* and judicious use of colistin in both clinical and veterinary contexts. Although the sample size (n=13) was limited, it represented all carbapenem-resistant *A. baumannii* isolates collected during a six-month NICU surveillance period. This exploratory dataset provides baseline evidence to guide larger multicenter validation studies and the development of cost-effective diagnostic frameworks for colistin resistance monitoring in Pakistan.

CONCLUSIONS

This study provides the first evidence of *mcr-1*-mediated colistin resistance in *A. baumannii* from a Pakistani NICU. Colistin agar and broth-based MIC assays were the most reliable phenotypic tests, while disc diffusion performed poorly. Findings underscore the need for low-cost, validated diagnostics, routine molecular surveillance, and strengthened infection control. Despite a small sample size, the study highlights the urgent need for careful colistin use in clinical and veterinary settings.

Authors Contribution

Conceptualization: LJ, RR, BJ
Methodology: MR, MFM, RR, NA
Formal analysis: RA, SS
Writing review and editing: MR, SJK, RA, SS

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

All the authors declare no conflict of interest.

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