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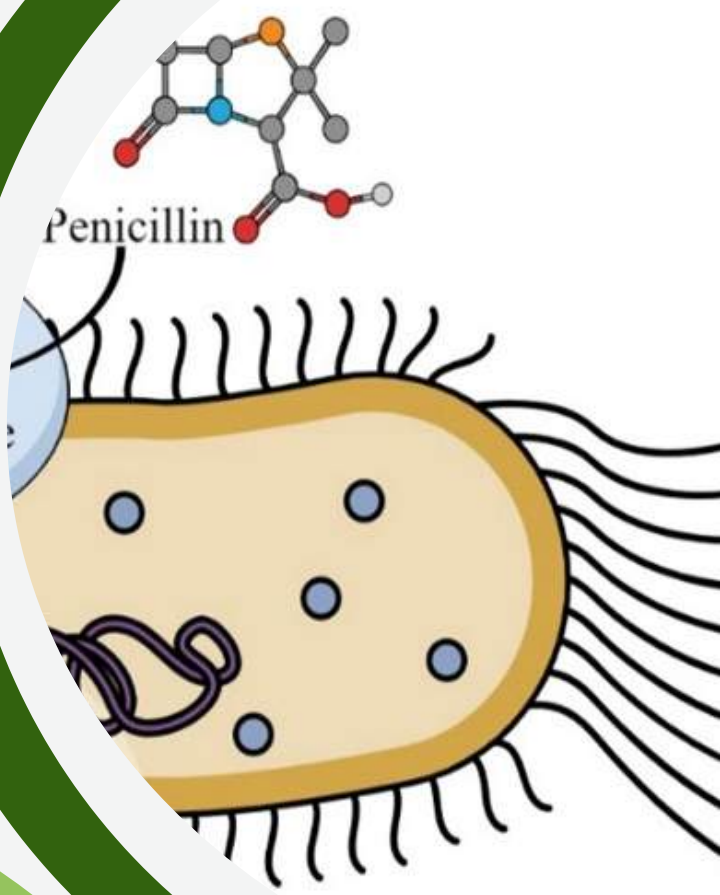

Editorial Note: Journal of Current Pharma Science Research

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Welcome to the first edition of the Journal of Current Pharma Science Research, a dedicated platform to showcase the depth of advances in pharmaceutical science and biotechnology. In this issue, we continue our commitment to foster the dissemination of knowledge across the spectrum of research related to the conceptualization, design, production, characterization, and evaluation of bioactive molecules, both natural and synthetic. Our focus spans a variety of crucial areas, including pharmaceutical biotechnology, medicinal chemistry, microbiology, and a myriad of other subjects that are crucial to advancing our understanding and application of pharmacological sciences. Each article in this edition has been meticulously selected to ensure that it contributes significantly to our ongoing dialogue on innovation and efficacy in the field.

In recent submissions, we have observed a promising trend towards the integration of traditional knowledge with modern scientific practices, particularly in the realms of ethnopharmacology and herbal medicine. This synthesis not only enriches our resource pool, but also opens new pathways for drug discovery and development that are both sustainable and innovative. As the landscape of pharmaceutical research expands, so does the complexity of the challenges that we face. For instance, the emergence of new viral and microbial threats demands rapid and innovative responses. Our section on antiviral and antimicrobial activities addresses these urgent needs by presenting cutting-edge research that explores novel agents and strategies to combat these pathogens.

This edition also highlights the significant advances in natural product drug discovery. Detailed studies on the isolation, characterization, and synthesis of bioactive compounds exemplify the critical role of natural products continue to play in the search for novel therapeutic agents. These studies were complemented by robust *in silico, in vitro*, and *in vivo* examinations, ensuring that the bioactivity of these compounds is well understood and effectively applied.

Moreover, the importance of comprehensive analytical methods cannot be overlooked. Contributions to our sections on chromatography and phytochemical analysis provide insights into the latest techniques that enhance the precision and efficiency of our research. These methodologies pave the way for more accurate assessments and validation of the pharmacological activities of the substances under study. We also take this opportunity to encourage readers and contributors to engage in multidisciplinary collaboration. The intersection of molecular biology, organic chemistry, and pharmaceutical formulations is just one example where cross-specialty collaboration can yield transformative results, leading to breakthroughs that no single discipline can achieve. As we continue to advance in our journey, the journal remains an advocate for innovation, quality, and integrity in research. We are immensely grateful to our contributors, peer reviewers, and readers who uphold these standards and pushed the boundaries of what is possible in the pharmaceutical sciences.

Thank you for your continued support and interest in Journal of Current Pharma Science Research. Together, we are not only witnesses to the evolution of science, but also active participants in shaping the future of healthcare and medicine. Let us continue to collaborate, innovate, and inspire one another, as we contribute to a healthier world.

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Review Article

Green Synthesised Metal Nanoparticles and its Anti-Inflammatory and Anticancer Activity

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ABSTRACT

Background: Metallic nanoparticles have the potential to address various medical challenges including inflammation, cancer and fungal infections. **Aim:** We synthesized metal nanoparticles and evaluated their anti-inflammatory and anticancer activities. **Methodology:** This study examined the biogenic synthesis of metallic nanoparticles and their anti-inflammatory actions. It discusses the mechanisms of their action, including the suppression of the NF-B and COX-2 pathways and emphasizes the importance of stability and specific targeting. The data were uptrained from research articles from PubMed, Research Gate, Google Scholar and other sources. **Results:** Nanotechnology, with its multidisciplinary approach, has opened new avenues for innovative treatments by leveraging the unique properties of nanoparticles. Metallic nanoparticles, such as silver, gold, zinc oxide and titanium, exhibit remarkable anti-inflammatory, anti-cancer and antibacterial activities, which are attributed to their ability to scavenge Reactive Oxygen Species (ROS), inhibit NF-B and cyclooxygenase-2 pathways and induce oxidative stress in cells. Moreover, these nanoparticles hold promise as pharmaceutical carriers, enhancing the efficacy of anticancer medications and offering opportunities for immunotherapy and chemotherapy. **Conclusion:** The review highlighted the importance of metallic nanoparticles in advancing medical research and their potential impact on improving healthcare outcomes.

Keywords: Metallic nanoparticles, Green synthesis, Inflammation, Skin Cancer, Antibacterial.

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INTRODUCTION

Nanotechnology is multidisciplinary, encompassing engineering, physics, chemistry, biology and other fields. Significant advances in science and technology brought about by the introduction of nanotechnology have opened up possibilities for the advancement of medical research and treatment of diseases in healthcare systems.[1] Nanoparticles, also known as ultrafine particles, are small particles with a size range of 1-100 nanometers (nm). Nanoparticles can be synthesized using both natural and synthetic method.[2,3] Nanoparticles have a high surface-to-volume ratio because of their high reactivity, mobility, solubility and strength. Since its formation, soil, dust, water, minerals and nanoparticles have been present on the Earth. Nanoparticles and nanomaterials are used in various industries including food, agriculture and medicine.[4] Nanoparticles are used in the food industry for food processing, preservation and packaging. Nanotechnology has made use of nonfertilizer, insecticides, herbicides and sensors in agriculture. Nanotechnology is also used for communicable and noncommunicable diseases.[5] Nanotechnology is used to detect life-threatening disorders such as cancer in the early stages.[6,7] Nanoparticles are classified into four categories based on their chemical composition: carbon-based, metal-oxide-based, bio-organic-based and composite-based.[8] There are two types of nanoparticles: inorganic and organic nanoparticles. Inorganic materials are composed of metallic nanoparticles, whereas organic nanoparticles are biodegradable. They are used as antibacterial, antifungal and antiviral agents. Nanoparticles have two approaches: top-down and bottom-up. Different physical, chemical and biological methods have been employed for nanoparticle synthesis.[9] Metallic nanoparticles (Figure 1) are eco-friendly synthesis methods that use green synthesis to avoid the formation of dangerous by-products. A good solvent system and natural resources were used to blend the biogenic nanoparticles. The incorporation of different biological components can be achieved by green manufacturing of metallic nanoparticles. On a large scale, plant extracts are important for the synthesis of metallic nanoparticles.[10]

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METHODOLOGY

In this study, we first analyzed studies that explain the biogenic synthesis of metallic nanoparticles and their anti-inflammatory actions.[11] The anti-inflammatory actions of different metal and metal oxide nanoparticles, such as gold,[12] zinc oxide, silver,[13] selenium and titanium dioxide,[14] are discussed in this review based on mechanistic studies. Preventing the expression of proinflammatory ROS and proinflammatory cytokine scavenging mechanisms is caused by the suppression of NF-B and COX-2 pathways by nanoparticles produced through green synthesis.[15] Stability and specific targeting are important factors for the efficacy of anti-inflammatory metabolic nanoparticles.

Inflammation

Inflammation is the body's reaction to injury or infection and involves the immune system's protective response to remove harmful stimuli and initiate the healing process. It typically involves swelling, redness, heat and pain in affected areas. Inflammation is a protective mechanism of the human body. These originate from different sources, such as infectious agents (bacteria and viruses), Radical Oxygen Species (ROS), physical agents and metabolic stress (hypoxia). Acute respiratory distress is an example of an unfavorable reaction that can result from COVID-19. Other manifestations linked to infection include a powerful cytokine storm, viral sepsis and uncontrolled systemic inflammation. Depending on the origin of the agent, inflammation can be categorized based on whether it is an endogenous abnormal reaction or exogenous agent. Inflammation can be either acute or acute, depending on its duration.[16] The two main host defense mechanisms that mediate these responses are the innate and adaptive immune responses. The innate immune response is the initial host reaction to any foreign substance, whereas the adaptive immune response involves granulocytes, phagocytes and other cells.[17] Acute inflammation is thought to be the body's defense against infection or other injuries, whereas chronic inflammation can coexist with pathological conditions even in the absence of an illness or injury, such as obesity.[16] Tissue damage, wounds and infections cannot heal without inflammatory reaction.[17]

Prolonged inflammation may harm the body. Damaged areas require more blood because tiny artery branches supply blood to the damaged area.[17] Inflammation lasts for a specific period, depending on how much harm the infection has caused. High concentrations of cytokines and coagulation factors stimulate the production of prostaglandins and acute phase proteins, such as C-reactive protein, by hepatocytes, affecting the CNS and resulting in discomfort, fatigue and fever, which are additional systemic effects that prolonged inflammation can have. Acute inflammation, which is less severe and localized and chronic inflammation, which develops in the pathogen that causes acute inflammation and is not eradicated or destroyed, are the two categories of inflammation. The sickness can then progress into an autoimmune condition, where healthy, normal host cells are attacked. In certain cases, chronic inflammation can lead to malignancy and the onset of pathologies such as rheumatoid arthritis. The systemic production of TNF by macrophages is characteristic of both acute and chronic inflammation; TF subsequently triggers the central innate immune response, particularly in microglial cells. Acute inflammation increases the innate immune response and leads to the production of cytotoxic inflammatory mediators, which worsen neurodegeneration in cases where chronic neurodegenerative changes have already activated microglial cells. Inflammatory mediators hasten the development of inflammation by altering endothelial permeability, neutrophil extravasation, excess plasma containing complement components and antibodies at the site of inflammation. The NF-B and COX-2 pathways are the most significant systems involved in increasing inflammation.[18-25]

Figure 1: Different types of metal nanoparticles.

Skin Cancer

The most prevalent cancer globally is skin cancer and its incidence is rising without any signs of slowing down. UVB rays cause DNA damage via an inflammatory process and UVA rays play an important role in the carcinogenesis of skin stem cells. According to estimates from the American Cancer Society, there were skin cancer-related fatalities and more than 1.6 million newly reported cases of skin cancer in 2012. Skin cancer, which is not melanoma, accounts for the Majority of Newly Diagnosed Cases (NMSC). An overview of skin cancer types, pathophysiology, normal skin design, malignant melanoma, Squamous Cell Melanoma (SCC), Basal Cell Carcinoma (BCC), risk factors and comorbidities and physiological variables are given in this article.

Types of skin cancer

Skin cancer is typically classified into two main categories: malignant melanoma and non-malignant melanoma (NMSC),

which include BCC and SCC as the key subtypes. Because instances of BCC and SCC are not compelled to be reported to national cancer registries, it is impossible to estimate the true number of NMSC cases.[26,27] The general upward trend in NMSC incidence is between 3% and 8%.[28-31] Most cases of NMSC are treatable, especially if discovered early in malignant melanoma, which is the most severe and unpredictable form of skin cancer, specifically when detected at an advanced stage.

Anatomy of normal skin

Clinicians need to have a fundamental understanding of the skin to properly comprehend skin cancer. The layers of the epidermis, reticular dermis, papillary dermis and subcutaneous fat constitute the normal skin (Figure 2). Four major cell types and four sub-layers were used to define the epidermis. These sublayers show the various phases of maturation that the actively divided cells or keratinocytes undergo over the course of 30 days. Keratinocytes build the stratum basale, the lowest sublayer, pushing other cells upwards.

The stratum corneum, which is composed of many laminated and loosely linked keratinized cells, is the most superficial among these sublayers. It protects the layers underneath and acts as a vital barrier. The pigment melanin, which shields the skin from UV rays, is produced by melanocytes found in the stratum basale. The dermis lies beneath the epidermis and provides support and nourishment. In addition to specialized cells such as sebaceous glands (oil glands), hair follicles, eccrine glands (sweat glands) and apocrine glands (scent glands), the dermis comprises ground substances and fibers. Blood vessels and nerves that permit touch, temperature and pain perception are also found in the dermis. Fibroblasts in this area produce collagen and elastin. Each has a different thickness of subcutaneous tissue, which is made up of nerves, connective tissue, fat and larger blood vessels. It aids in the storage of fat, control of body and skin temperature and absorption of shock.

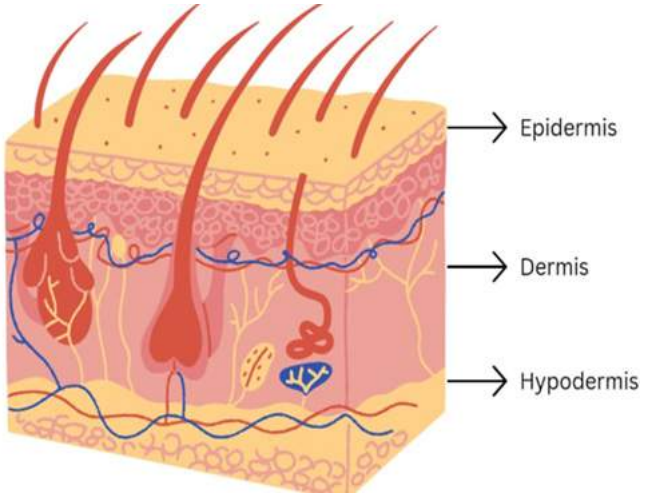

Figure 2: Anatomy of normal skin.

Skin cancer pathophysiology

Skin cancer has a multifactorial etiology. The primary causative factor for the formation of NMSC and malignant melanoma is Ultraviolet (UV) radiation from sunlight. UVA and UVB radiations are the principal subtypes of UV radiation. Compared to UVB rays, UVA can cause deeper cutaneous injuries, such as elastosis, because it can penetrate the skin more deeply. Sunburn and erythema are the main effects of UVB irradiation. UVR causes oxidative stress, immunosuppression, DNA damage, gene alterations and inflammatory reactions, all of which are critical for skin photoaging and development of skin cancer. UVB radiation directly harms the DNA. UVA photons cause indirect DNA damage, which is mediated by cellular membrane damage and production of free radicals. Research suggests a link between skin cancer growth and immunosuppression caused by UVR. UVR is a carcinogen that stimulates tumor growth. Tumorigenesis begins in addition to mutations in tumor suppressor genes. UVB rays damage DNA by inducing tumors and inflammatory responses and UV rays are a major contributor to skin stem cell carcinogenesis (Figure 3).

UV rays that reach the skin are mostly absorbed by the DNA of the epidermal keratinocytes. DNA is assumed to be the skin photoreceptor and research indicates that UVR-induced cyclobutane pyrimidine dimer synthesis is the first biochemical step leading to immune suppression. UVR-induced damage results in skin cancer owing to its intricate mechanics. UVR causes mutations in genes that suppress tumor p53, which participates in DNA repair or the death of cells with DNA damage. Consequently, p53 genes can no longer assist in DNA repair if their expression is altered. Skin cancer begins to proliferate as a result of this imbalance in apoptosis, which allows keratinocytes to divide uncontrollably. UVR-induced free radical damage is a significant cause of carcinogenesis and patients may be predisposed to skin cancer based on their genetic makeup and their capacity to metabolize free radicals. Glutathione S-transferase (GST) enzymes have antioxidant functions because of their ability to reduce the adverse effects of ROS. Skin cancer may be mediated in part by the Glutathione S-transferase Polymorphism (GSTP) enzyme, which is abundantly expressed in the dermis and epidermis of the skin. In animal experiments, deletion of the GSTP gene significantly enhanced vulnerability to the formation of skin tumors. Changes in the size, shape, color and texture of moles or other skin lesions, as well as the emergence of new skin growth, are significant clinical indicators of cutaneous cancer. A healthcare professional should be consulted if the alteration worsens over a month or more and alterations that occur over a few days are not cancerous.

Basal cell carcinoma

BCC accounts for over 80% of NMSC. Predisposing factors include intermittent UVR exposure and UVR exposure in the

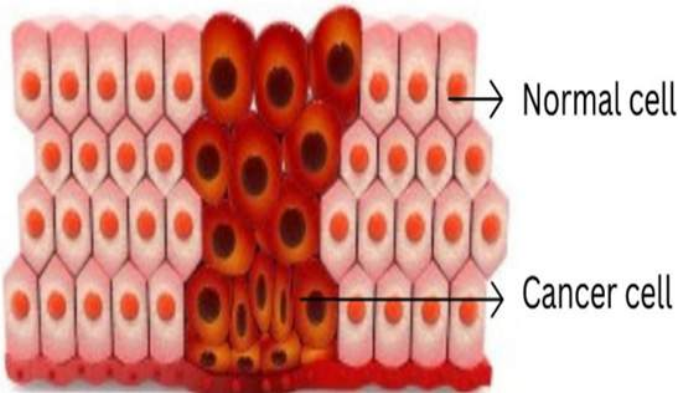

Figure 3: Cancer cell formation.

youth. The head and neck account for 80% of all BCC cases and clinical diagnosis is not difficult. BCC is a malignant tumor that originates from basal cells. In contrast to SCC, BCC typically manifests without antecedent lesions. BCC typically manifests as small papules that gradually enlarge over months or years. BCC also manifests as pearly bordered, shiny papules with a core ulcer and noticeable engorged vessels (telangiectasias) on the surface. Variants resembled scar-like, flat, yellowish-white patches. Repeated bleeding or crusting is a common occurrence. Ambiguous symptoms include sensitivity and itchiness. Clinically, BCC can be challenging to differentiate from benign growth and patients frequently confuse it for acne. BCC can occasionally involute and seem to heal, which could ease anxiety regarding the extent of the injury. Although metastasis is uncommon, local expansion can cause a great deal of harm.

Squamous cell carcinoma

Approximately 16% of skin cancer cases have SCC.[32] The incidence of SCC is highly correlated with cumulative lifetime sun exposure. SCC is a dermal invasion caused by a malignant tumor of the epidermal keratinocytes. There could be significant local tissue loss and advanced stages of metastasis through hematogenous or lymphatic dissemination are possible. Based on factors such as cancer location, underlying medical problems, cell differentiation and size, the predicted total metastasis rate ranges from 3% to 10%. SCC can manifest in a variety of ways clinically, but any lesions that do not heal after exposure to the sun should raise suspicions. Papules, nodules, plaques and smooth, erosive and hyperkeratotic (crusty) lesions are examples of clinical symptoms. The tumor may initially appear as a rough, scaly, erythematous patch or papule and may later develop into a nodule that occasionally has a plaque or warty surface. Tumor bleeding may occur without significant provocation. The tumor eventually becomes ulcerated and infiltrates the surrounding tissue. Most lesions may occasionally be located below the surrounding skin. When tiny SCC lesions are appropriately and promptly removed, prognosis is usually good. Noninvasive and invasive tumors are examples of SCC variations. The tumor first disperses locally to the skin and lymph nodes in the area and then moves to adjacent organs. SCC is more prone to spread and may require major surgery if it develops in scars, behind the ears, or along the vermilion border of the lip. Before diagnosis, almost one-third of lingual or mucosal malignancies spread.

Malignant melanoma

Although it only accounts for approximately 4% of cases, malignant melanoma is responsible for 65% of skin cancer-related deaths. Malignant melanoma can arise in any tissue that contains epidermal melanocytes, as this is where cancerous cells originate. Malignant melanoma develops as a result of several circumstances, including repression of the immune system of the skin, accelerated division of melanocyte cells, production of free radicals and damage to the DNA of melanocytes. Identification of the p16 melanoma susceptibility gene has provided insight into the genetic relationship between heredity and malignant melanoma. When p16 mutant cells are exposed to UVR, injured melanocytes proliferate unchecked. Numerous random mutations in p16 cause sporadic (non-familial) melanoma. The primary technique for the identification of malignant melanoma is eye inspection, as the majority of cases begin on the skin's surface. The clinical characteristics of malignant melanomas may vary widely. The ABCDE rule describes the clinical appearance and warning signals of most melanomas. The letter "A" and "B" denotes asymmetry (one half of the mole does not match the other half), "C" denotes diameter greater than 6 mm (roughly the size of a pencil) and "E" denotes evolution, elevation and/or enlargement of lesion. Some, but not all, of these features are present in many lesions, suggestive of melanoma. It has been observed that 2-8% of melanomas lack pigments.

Method for the synthesis of metallic nanoparticles

There are several methods to synthesize metallic nanoparticles, with biological and chemical methods being the most popular methods among them. The three most crucial variables for the synthesis of NPs are the eco-benign solvent, reducing agent and substance for stabilization. Chemical techniques use poisonous and hazardous chemicals and are expensive. To create nanoparticles the biosynthetic pathway uses plants and bacteria for biomedical applications. Fungi, algae, bacteria, plants and several other organisms can be used for nanoparticle synthesis. Phytochemicals are present in the extracts of plant parts such as roots, fruits, leaves and seeds, which act as stabilizing and reducing agents. Top-down and bottom-up approaches are two types of biological and chemical methods.

Top-down approach

Using this procedure, bulk materials are crushed, divided and milled to create nanoparticles using lithographic materials. The plant extract content, pH, temperature, incubation time and metal salt solution concentration all affect the stability, shape and size of the nanoparticles.

Bottom-up technique

In this method, small components such as molecules and atoms are self-assembled into new nanoparticle nuclei, which are then further developed into complete particles using various biological and chemical methods.

Metallic nanoparticle production by green synthesis

The concept of environmentally friendly green chemistry for the synthesis of metallic nanoparticles involves green synthesis. To produce clean and eco-friendly metallic nanoparticle uses, bacteria, fungi, actinomycetes and other species are used. Actinomycetes are good sources for producing nanoparticles with distinctive surface and size characteristics owing to their wide variety of secondary metabolites. Using either extracellular or intracellular methods, actinobacteria can produce metallic nanoparticles. The external production of actinomycetes is heavily reliant on polydispersity, which offers greater economic benefits than intracellular production. Fungi are widely used in the production of nanoparticles because they can produce a variety of nanoparticles with high efficiency. Organisms are green alternatives for creating nanoparticles with useful properties for the manufacturing of metallic nanoparticles. Plant extracts can be easily combined with a room-temperature metal salt solution to create nanoparticles. They require minimal maintenance and are frequently free.[33-35]

Heavy metals can be dangerous even at low concentrations. Environmental contamination can be handled by plants through increased potential for heavy metal detoxification and accumulation. The use of microbes or plant extracts for the synthesis of nanoparticles requires basic laboratory techniques to maintain microbial colonies. Plant-assisted nanoparticle synthesis has the advantage of significantly faster kinetics than other biosynthetic methods. The green synthesis of nanoparticles using plant extracts has more benefits than utilizing microorganisms because it is a one-step procedure that is nonpathogenic, affordable and generates a high number of metabolites, making it an economical and environmentally friendly method. For the formation of nanoparticles, the color of the solution begins to change after pressing and filtering, which may separate. The huge potential of the biological synthesis of nanoparticles for the safe and effective removal of poisons and contaminants from waste has attracted a lot of interest. It is an inexpensive and environmentally friendly synthesis process that does not require intermediate chemicals or specialized instruments.[36]

Characterization of nanoparticles

Size, surface area and dispersion are the three main characteristics of nanoparticles. In many applications, the homogeneity of the attributes is crucial. Numerous metallic nanoparticles created using environmentally friendly methods have been characterized using a range of techniques, including Raman spectroscopy, spectroscopy methods (UV, FT-IR), zeta sizer and zeta potential, transmission electron microscopy (SEM, Scanning Electron Microscopy) (SEM), Atomic Force Microscopy (AFM) and energy dispersion analysis of XRD (X-ray diffractometer).[37-40]

Silver nanoparticles

The biomedical industry is experiencing great success with AgNPs, primarily because of their use as antibacterial agents, medical equipment coatings and chemotherapeutic medication delivery systems. Although they have been well studied, additional work is needed to develop more bio-sustainable synthesis procedures and identify the mechanisms underlying their toxicological effects. AgNPs have applications in biotechnology, electronics, optics and environmental science. Green synthesis is one of the best techniques for producing silver nanoparticles is green synthesis.[41]

Zinc oxide nanoparticles

ZnO nanoparticles are extensively employed in a variety of industries because of their special chemical and physical characteristics. The rubber industry uses zinc oxide nanoparticles to improve the polymer toughness and resistance, as well as to provide wear resistance and anti-aging properties of rubber composites. Zinc oxide nanoparticles are widely employed in personal care products, including sunscreens and cosmetics, owing to their strong UV-absorbing capabilities. Because of their capacity to stimulate ROS production, additional characteristics, such as antibacterial and anticancer effects, have also been investigated. ZnO nanoparticles are also good drug carriers.[42]

Gold nanoparticles

Colloidal or clustered gold nanoparticles have a gold core surrounded by an inert, biocompatible component. The ability to manipulate the size, shape and surface characteristics of these particles is one of their advantages, owing to their synthetic plasticity. Additionally, its coating can be altered to affect its stability, environmental interactions and particle solubility. Gold nanoparticle-based PA imaging has shown potential for supporting treatment procedures by offering sequential monitoring of tumor functional features, such as modification of the tumor vasculature before, during and after therapeutic procedures.[43]

Titanium nanoparticles

It has been shown that bio-mediated titanium nanoparticles have anti-inflammatory, anti-fungal, anti-microbial and other biological properties. Their biological activity is enhanced by their photo-semiconductor properties, which cause microorganisms to disintegrate.

Selenium nanoparticles

Selenium is an interesting substance mixed with anti-inflammatory medications; it is a dietary component that plays a major role in

biological systems. A trace element, selenium, is required for the continued growth and well-being of the body. Elemental selenium has drawn much interest because it is the least toxic form of the element. Selenium nanoparticles are important for anticancer and antioxidant actions, according to current research.

The anti- inflammatory action of metallic nanoparticles

A localized physical condition known as inflammation occurs when an injury or infection causes a body component to become red, painful, or swollen. In the absence of an anti-inflammatory response, infections, wounds and tissue damage cannot heal. Over the last few decades, nanoparticles have gained attention as possible drugs to reduce inflammation. Because of their extensive surface area, nanoparticles are more effective at preventing the release of inflammatory mediators, such as cytokines and enzymes, that foster inflammation. Many metal and metal oxide nanoparticles, such as gold, zinc oxide, silver, selenium, copper, titanium dioxide, zinc peroxide, nickel and iron oxide, have been reported to have anti-inflammatory properties. When a pathogen injures or attacks tissue, an inflammatory response is triggered. Based on the pattern of damage, macrophages, killer cells and stem cells are drawn into the affected tissue to aid in the response. Macrophages are important for the regulation of inflammatory responses.[44,45]

Several anti-inflammatory strategies are commonly used by nanoparticles, including scavenging ROS, suppressing NF-B and blocking proinflammatory cytokines and COX-2 pathways. One of the most important mechanisms that nanoparticles use is the suppression of proinflammatory cytokines, because cytokines enhance immune responses.

The antifungal activity of metallic nanoparticles

In the last few years, fungal contamination and emerging fungal infections have taken center stage in global safety concerns. Using the food industry as an example, fungal contamination may seriously affect public health and food safety, in addition to causing product quality degradation and financial loss.[46] The inappropriate use of antibiotics has worsened this problem, leading to a significant increase in the number of drug-resistant fungi. Therefore, researchers worldwide are working to develop a variety of strategies to stop and manage fungal infections. These strategies include the use of predatory microorganisms, antimicrobial peptides and plant essential oils. Most of these techniques have been shown to function better against fungi in the laboratory, but they also have certain drawbacks, including high costs, unstable ingredients, interference with food components and unpredictable health hazards to humans. However, in the last several years, there has been a significant increase in nanotechnology research. Numerous studies have focused on the reactivity of novel types of nanoparticles with potential uses in food science, including food processing, preservation and nutritional supplementation.[47] Nanoparticles are the most promising alternative to conventional antibiotics for the management of harmful microorganisms.

Silver nanoparticles and graphene materials, as well as single and several walls of carbon nanotubes, are among the many nanoparticles that exhibit strong antibacterial activity. A variety of medical products and therapeutic medications currently use many nano-sized antibacterial agents that contain silver nanoparticles. Silver nanoparticle-based antifungal compounds may not be used in foods because of their high cost, ease of aggregation in tissues and biological adverse effects. Owing to the special characteristics of zinc oxide nanoparticles, they are currently considered to be the most effective antibacterial nanoscale agents. Zinc oxide in nanoscale form is more biocompatible than other nanoparticles. Current scientific data indicate that there is little to no risk to public health when using ZnO nanoparticles.

Antifungal action of zinc oxide nanoparticles

Zinc ions (Zn2+) are present in the medium and nanosized ZnO exhibits improved photocatalytic performance compared to inorganic photocatalysts. Thus, the two main processes by which ZnO oxide nanoparticles exhibit antifungal action are the production of ROS by photons and toxicity caused by the release of Zn2+.

ROS-dependent antifungal activity

Studies by Hirota *et al*. and Xu *et al.* indicated that the antibacterial properties of zinc oxide nanoparticles frequently depend on the presence of hydrogen peroxide or oxygen radicals on their surface via oxygen defect sites.[48] Particularly, when light is present, oxidative stress resulting from the production of ROS may be the main source of physiological effects.

Metal-containing particle-mediated antifungal effect

The exact mechanism of zinc oxide nanoparticles antifungal action in the absence of light is yet unknown. Zinc oxide and other metallic compounds, such as copper and silver oxide, are soluble, which could be the key factor in determining the physiochemical characteristics that affect the effectiveness of metal-containing nanoparticles as antimicrobials.[49] They can dissolve in aqueous solutions to some degree. Therefore, zinc oxide nanoparticles have a restricted source of zinc ions, which could be the cause of the fungitoxic effect of nanoparticles. Researchers have discovered that the toxicity of zinc ions produced by metal oxides causes antibiological action in dark environments.[50]

Anticancer activity of metallic nanoparticles

Cancer is one of the greatest global causes of death and a primary barrier to accelerating anticipation. It is acknowledged on a global scale that it is extremely difficult to handle. Despite

rapid advancements in medicine, certain types of cancer cannot be properly treated with current medications. The adverse effects of traditional treatment methods are one of the main problems associated with malignant growth treatment. One option to consider when investigating novel approaches to cancer treatment is the use of nanomaterials. Nanomaterials have been used as pharmaceutical carriers to increase the *in vivo* anticancer activity of medications for over 30 years. The earliest research, conducted in the 1970s, employed liposomes, which are nanoscale drug carriers that contain anticancer medications. In the clinical setting, metallic nanoparticles have demonstrated innovative uses for diagnosing and treating a range of cancerous growth and other retroviral disorders. Unique and modified bio-based nanoparticles were designed to address hazardous materials without interfering with normal cells. Gold was first utilized for pollution thousands of years in ancient China and India and its application has increased dramatically with the development of nanotechnology. AuNPs are strong candidates for immunotherapy and chemotherapy in the treatment of diseases because of their high surface-to-volume ratio, strength and low cytotoxicity. Nevertheless, many researchers worldwide are engaged in the field of phyto-nanotechnology.

Anticancer activity of zinc oxide nanoparticles

Being a broad bandgap semiconductor, ZnO nanoparticles may easily absorb UV light. This characteristic makes ZnO nanoparticles helpful for a wide range of applications, including biomedical, cosmetic and facial products. ZnO nanoparticles are currently the subject of extensive research because of their potential to prevent cancer. The biocompatibility of ZnO nanoparticles was excellent. Zinc is biocompatible because it is an important co-factor in several cellular processes and maintains cellular homeostasis. The supplied ZnO has the ability to readily biodegrade or participate in the body's active nutritional cycle. Compared to other nanoparticles, ZnO nanoparticles exhibit inherent selective cytotoxicity against malignant cells *in vitro*. One of the distinctive properties of ZnO nanoparticles' cytotoxicity towards cancer cells has been shown to be their capacity to cause oxidative stress in these cells. This characteristic is a result of the semiconducting nature of ZnO. Oxidative stress is induced by the generation of ROS, by ZnO and, when the cell's antioxidant capacity is surpassed, cell death occurs.

Antibacterial activity of metal nanoparticles

The ongoing rise in bacterial resistance has put the scientific community under pressure to develop new antibiotic treatments. Metal nanoparticles are among the most promising of these new antibiotic agents and a wide range of studies have demonstrated their potent antibacterial action. Even when new medications are introduced into the market, antibiotic-resistant bacteria typically emerge within a comparatively short amount of time. However, because nanoparticles target numerous biomolecules simultaneously, preventing the development of resistant strains, it is hypothesized that nanoparticles with antibacterial activity have the potential to minimize or eliminate the creation of increasingly resistant bacteria.

Antibacterial activity of zinc oxide nanoparticles

It has been demonstrated that ZnO nanoparticles change the cell membrane and induce ROS generation. Therefore, when coming into contact with ZnO oxide nanoparticles, bacterial cells absorb Zn+, which then suppresses the function of respiratory enzymes, creates ROS and releases free radicals, thus inducing oxidative stress. Bacterial membranes and DNA are irreversibly damaged by ROS, causing bacterial cells to die.

Mechanism of action of ZnO oxide nanoparticles

ROS are produced in response to ZnO NPs. ROS causes DNA damage, which causes the mitochondrial membrane to release apoptogenic components. Apoptogenic factors cause apoptosomes to develop, which, in turn, causes apoptosis. Primarily bound oxygen atoms on the ZnO surface give them a negative charge. A reduced pH causes protons from the surrounding air to be absorbed by the particle surface, resulting in a positively charged $ZnOH^{2+}$ surface. The particles are absorbed by the cell as a result of the interaction between these positive particles and the negative phospholipids on the outer membrane. Zn+ was released when the ZnO NPs broke down in the acidic lysosomes, preventing respiratory enzymes from carrying out their function and causing the cells to die.

Side effects of ZnO nanoparticles

Because of their high surface area and small size, ZnO nanoparticles have been linked in studies to a number of harmful consequences, including oxidative stress, genotoxicity and cytotoxicity. ZnO nanoparticle inhalation or dermal exposure has been linked to irritation of the respiratory tract, skin irritation and inflammation. Furthermore, the fact that they can cross biological barriers prompts worries about possible systemic effects when they build up in tissues and organs. Furthermore, ZnO nanoparticles have been linked to environmental toxicity, which has an impact on aquatic habitats and creatures. ZnO nanoparticles exhibit intriguing capabilities; nonetheless, due diligence in assessing possible side effects is necessary to guarantee the safe and responsible use of these particles in diverse applications.

Stability and storage of the nanoparticles

The performance and usability of nanoparticles are significantly influenced by their stability and storage. To keep nanoparticles stable and stop agglomeration or degradation over time, proper storage conditions are crucial. These parameters include temperature, humidity and radiation exposure. Nanoparticles are generally kept at regulated temperatures in dry, dark conditions

to reduce chemical reactions and maintain their physical characteristics. To further improve stability during storage, appropriate packing materials may also be used, such as inert gases or specialty coatings. To guarantee the long-term stability and effectiveness of nanoparticles for a variety of applications, from the biomedical to the environmental domains, regular monitoring of storage conditions and the use of suitable handling techniques are essential.

CONCLUSION

This work provides a thorough and concise overview of the anti-inflammatory, anti-cancer and anti-fungal effects of several metallic nanoparticles. The relationship between cells and nanoparticles has also been discussed. Commonly employed tactics for anti-inflammatory effects include scavenging Reactive Oxygen Species (ROS) and inhibiting the NF-B and cyclooxygenase-2 pathways. Inhibiting proinflammatory cytokines is one of the most significant strategies employed by nearly all nanoparticles, as they improve the immune response. Nanomaterials have been utilized as pharmaceutical carriers to increase the *in vivo* anticancer activity of drugs. Gold was first utilized for pollution and is a strong candidate for immunotherapy and chemotherapy in the treatment of diseases because of its strength, high surface-to-volume ratio and low cytotoxicity. Compared with other nanoparticles, ZnO nanoparticles are more biocompatible. Zinc oxide nanoparticles exert antifungal action through two main mechanisms: the formation of ROS by photons and the release of zinc oxide ions, which cause poisoning.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

NPs: Nanoparticles; **GST:** Glutathione S-transferase; **AgNPs:** Silver nanoparticles; **ZnO:** Zinc oxide; **AuNPs:** Gold nanoparticles; **MIC:** Minimum Inhibitory concentration; **FESEM:** Field emission scaning electronic microscope; **HRTEM:** High Resolution transmission electronic microscope; **ROS:** Reactive oxygen species; **DNA:** Deoxy ribose Nucleic acid; **SCC:** Squamous cell melanoma; **BCC:** Basal cell carcinoma; **NMSC:** Non-malignant melanoma.

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Review Article

Marine Sponge Assisted Metallic Nanoparticles and its Biological Activity and its Mechanism

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ABSTRACT

Background: This study presents a systematic analysis of the biogenic synthesis of metallic nanoparticles and their significant biological activities, including anti-inflammatory, antioxidant and anticancer properties. **Aim:** Green synthesis methods, utilizing various biological materials, such as plant extracts and marine sponges, offer environmentally friendly approaches for the production of metallic nanoparticles with specific biological functions. **Methodology:** Synthesized nanoparticles, including silver, gold, zinc oxide and titanium dioxide, exhibit promising therapeutic potential against skin cancer, inflammation and oxidative stress. **Results:** Skin cancer, particularly melanoma and non-melanoma types, is discussed alongside the anatomy of normal skin, diagnostic techniques such as skin biopsy and pathophysiology of skin cancer. Furthermore, this study delves into the mechanisms of inflammation and the roles of metallic nanoparticles in modulating inflammatory responses, emphasizing their potential in targeted therapy. Additionally, the anti-cancer action of metallic nanoparticles, especially in enhancing drug delivery and therapeutic efficacy, has been elucidated. Moreover, the antioxidant properties of metallic nanoparticles and their implications for mitigating oxidative stress-related diseases are discussed. **Conclusion:** Study underscores the importance of metallic nanoparticles in biomedical applications, offering insights into their synthesis, characterization and therapeutic potential across various pathological conditions.

Keywords: Metallic nanoparticles, Green synthesis, Skin cancer, Inflammation, Antioxidant.

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INTRODUCTION

Over the past 20 years, there has been a significant increase in the use of nanotechnology. According to the National Nanotechnology Initiative, structures that fall roughly around the 1-100 nm scale in no less than one direction are considered nanotechnology. Materials are designed and produced using nanotechnology at the atomic and molecular levels.[1] Nanoparticles have emerged as highly promising instruments, with an assortment of applications in medical delivery, diagnosis, skincare products and numerous other biotic and non-biological sectors. Nanoparticles exhibit a range of characteristics based on their dimensions and exterior functions. Nanoparticles are widely used in multiple fields, including electronics, skin care products and both therapeutic and diagnostic medical applications. This is because of the large area they cover and their small stature.[2] Plants are considered bioreactors for the creation of metal nanoparticles. A range of chemical and physical approaches have been used to produce metal nanoparticles with the required properties. Conifers possess particular qualities for the creation of metal nanoparticles. A biological synthesis method that is effective, trouble-free, affordable and environmentally benign is called "Green synthesis" for metallic nanoparticles. Metal oxides and pure metals are both included in metallic nanoparticles, which have a variety of benefits.[3] In the field of medical science and illness therapy, the creation of biologically inspired green production of AgNPs has garnered significant attention globally. The environmentally friendly production of AgNPs is used to treat cancer and germs resistant to antibiotics.[4] Among the several metallic nanoparticles employed for biomedical applications, one of the most significant and fascinating nanomaterials is AgNPs. AgNPs are crucial in nanoscience and nanotechnology, especially in nanomedicine. Although several valuable metals have been used for diverse purposes, silver nanoparticles are drawing attention because of their potential use in the detection and therapy of cancer. AgNPs have become increasingly prevalent in a range of industries, including the food, pharmaceutical, medical, consumer and industrial sectors, owing to their distinctive chemical and physical characteristics. Multiple biological and therapeutic applications of AgNPs include anti-bacterial, anti-viral, anti-fungal, anti-inflammatory, anti-cancer and anti-angiogenic properties.[5]

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Marine sponges are porous protozoans of the phylum Porifera that are multicellular and filter-feeders. The sessile creatures were sponges. Marine sponges yield more than 5,000 naturally occurring chemicals.[6] Currently, marine sponges are the richest source of potent anticancer substance is marine sponges.[7] Marine sponges are among the best sources of naturally occurring bioactive substances with potential applications in medicine.[8] Certain medications made from marine sponges have been shown to affect Hodgkin's disease, malignant lymphoma and metastatic breast cancer.[9] There are numerous applications for metabolites from bacteria, fungi and algae that live on sponges in cosmetics, medicine and environmental preservation. Microorganisms found in sponges produce substances with anti-viral, anti-oxidant, anti-inflammatory, anti-cancer and anti-bacterial properties. Moreover, alkaloids with pharmacological properties have been obtained from marine sponges.[10]

METHODOLOGY

In this study, we first performed a systematic analysis of the biogenic synthesis of metallic nanoparticles.[11] In addition, they exhibit anti-inflammatory, anti-oxidant and anticancer activities. Green synthesized nanoparticles are now employed to inhibit the expression of proinflammatory cytokines and ROS scavenging methods by suppressing the NF-B and COX-2 pathways.[12] Various metal and metal oxide nanoparticles, such as platinum,[13] silver,[14] titanium dioxide,[15] gold,[16] and zinc oxides,[17] have certain biological activities. Silver-fortified Sponge (AFS) spheres were successfully synthesized after method optimization using various extracts of marine sponges. Gram-positive and gram-negative bacteria are effectively inhibited by growth by AFS, which are precisely spherical, micro-sized and effective. 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl Tetrazolium bromide (MTT) and morphological alterations were used to evaluate the cytotoxic response of AFS. The patent database, conference abstracts, publications, news releases and current pipeline reviews were analyzed to provide a wealth of knowledge on the environmentally friendly synthesis of metallic

Skin Cancer

Skin cancer is the most prevalent type of cancer worldwide. Most skin cancers are not melanomatous. Melanoma arises when the pigment-producing cells of the skin exhibit malignant tendencies. Prolonged exposure to Ultraviolet (UV) radiation is the primary risk factor. When an older, fairy skinned person develops scaly, indurated lesions on sun-exposed areas, especially the head and neck, diagnosis is typically assumed. With a dermatoscope, proper lighting and magnification, clinical diagnosis accuracy can be improved. For a conclusive diagnosis, biopsy along with histopathological confirmation is required. Because the depth of the lesions determines how the cancer will be treated and how long it will last, a full-thickness biopsy is necessary for melanoma evaluation. Globally, there has been an increase in the number of instances of skin cancer among all types.[19]

Anatomy of normal skin

Clinicians need to have a fundamental understanding of the skin to properly comprehend skin cancer. The layers of the epidermis, reticular and papillary dermis and subcutaneous fat comprise normal skin. Four major cell types and four sublayers constitute the epidermis. Over the course of 30 days, actively dividing cells or keratinocytes undergo several stages of maturation, which are represented by these sublayers.[20]

Skin biopsy

Skin biopsy is an important diagnostic technique for cutaneous malignancies. It can also be helpful for the detection of infectious and inflammatory diseases when used in conjunction with clinicopathological correlations. Biopsy is performed to indicate suspected neoplastic lesions, bullous disorders (a rare skin condition that causes large, fluid-filled blisters) and if the

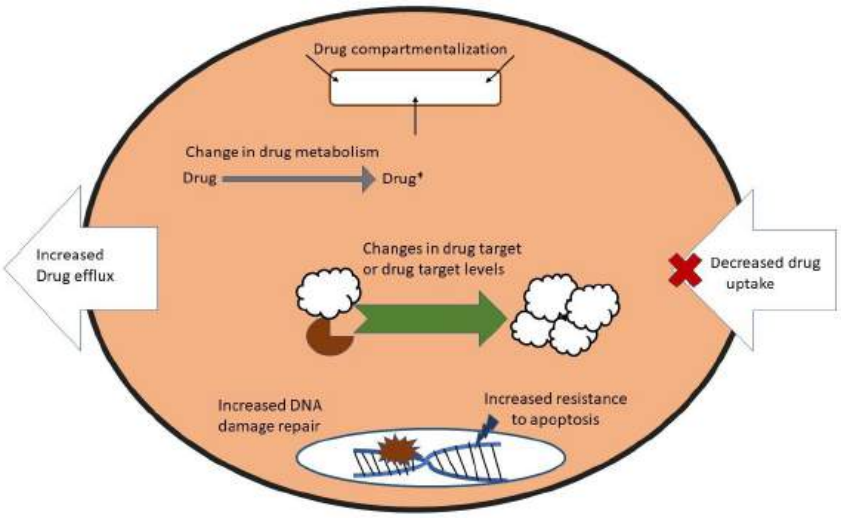

Figure 1: Drug resistance possible mechanisms in cancer cells.

diagnosis is not cleared and/or for therapeutic treatment. The site selected for biopsy has the following properties: thickest, most pigmented area, area with the most inflammatory changes and newly formed vesicles should be chosen for blistering lesions. There are three types of skin biopsy is present, they are punch biopsy, shave biopsy and excision biopsy.

Skin cancer pathophysiology

Skin cancer has a multifactorial etiology. The primary causative factor for the formation of NMSC and malignant melanoma is UVR from sunshine. UVA and UVB are the two primary subtypes of Ultraviolet (UV) radiation. Compared with UVB rays, UVA rays can cause deeper skin damage (Figure 1), such as elastosis, because they can penetrate the skin more deeply. Sunburn and erythema are the main effects of UVB irradiation. Inflammatory reactions, oxidative stress, immuno suppression, DNA damage and gene alterations are all induced by UVR.[20]

Types of cancers

Malignant melanoma and Non-Malignant Squamous Cell carcinoma (NMSC), of which Basal Cell carcinoma (BCC) and Squamous Cell Carcinoma (SCC) are the two most common types of skin cancers. Because BCC and SCC cases are exempt from reporting requirements to national registries, it is challenging to estimate the true number of NMSC.[19]

Basal cell carcinoma

BCC cancer initiates growth in the epidermal basal layers and auxiliary cells. This cancer growth will be present in almost all the places in the human body and exclusively, frequently visible open skin areas such as the nose, ears and cheeks, where most of the sun's UV light will fall. This type of cancer is inactive and rarely spreads slowly throughout the body.

Squamous cell carcinoma

Cutaneous SCC is the second most common type of skin cancer worldwide. Epidermal keratinocytes proliferate in a malignant and controllable manner, leading to cutaneous SCC. Similar to BCC, UV exposure is the primary cause of SCC. An estimated 20% of the occurrences of nonmelanoma skin cancer in the US are thought to be SCC instances.

Melanoma

Melanoma is an aggressive malignant tumor that originates in melanocytes. Epidermal melanocytes are present in the basal layer. A buildup of genetic alterations that activate oncogenes inactivates tumor suppressor genes and impedes DNA repair when exposed to ultraviolet radiation. Melanoma may result from these pathways, as melanocytes proliferate uncontrollably.[19]

Enzyme involved in cancer treatment

A few specific enzymes play a role in identifying cancer cells and in eliminating and killing these cells. Enzymes degrade the environment surrounding cancer cells.[21] The enzymes glutaminase, phenylalanine ammonia lyase, lysin oxidase and methionase are utilized in cancer treatment.[22] Dihydropyramidine Dehydrogenase (DPD) is one of the enzymes used in chemotherapy to break down the medications fluorouracil and capacitabin. 5,6-dihtdrouracil and 5,6-dihydrothymine are produced when uracil and thymine are reduced by the DPD enzyme.[23] Drug-metabolizing enzymes play a role in cancer and cancer therapy by metabolizing medications that can boost their effectiveness. Consequently, drugs are processed in tumors and metabolic organs with the aid of drug-metabolizing enzymes.[24]

Skin cancer is prevented by avoiding sun exposure, use of full-length clothing that covers exposed skin, use of hats and sunglasses and use of broad-spectrum (UVA/UVB) sunscreens and unblocks with frequent reapplications.[19]

Inflammation

Infection or other types of damage can cause inflammation in any part of the body, leading to redness, pain, swelling and other symptoms. It is thought to be a pathological cornerstone. Because of the favorable host reaction, cellular homeostasis, tissue form and function are restored. Infections, wounds and tissue damage cannot heal without an inflammatory response (Figure 2). Innate and adaptive immune responses are the two main mechanisms of host defence that control this reaction. Granulocytes, phagocytes and other cells are involved in the adaptive immune response, whereas the innate immune response is the initial reaction of the host to any foreign substance. The hallmark of adaptive immunity, which aids in the early stages of infection, is specific. There are situations in which the inflammatory response can persist longer than necessary and cause more harm. As more blood must be delivered to the injured area, the small artery branches that supply it swell. Redness and heat are produced by increased blood flow through dilated arteries, which are transported by an increase in the erythrocyte count. Increased phagocytic cell infiltration in this region causes swelling and discomfort. When a host with a functioning innate immune system interacts with external stimuli, inflammation usually begins within minutes.[25] As innate immunity is the primary cause of inflammation, both immune and non-immune cells, including fibroblasts and endothelial cells, contribute to the inflammatory process. Immune cells include neutrophils, mast cells, macrophages, dendritic cells and Endothelial Cells (ECs). Depending on the type of stimulation, inflammatory pathways and target tissues are activated. Inflammation has a set duration that varies according to the extent of damage caused by inflammation. Extended inflammation also has a systemic effect: elevated levels of cytokines and coagulation factors cause hepatocytes to produce

prostaglandins and acute-phase proteins such as C-reactive protein, which affect the central nervous system and cause pain, exhaustion and fever. Acute inflammation is more localized and less severe than chronic inflammation, which arises when pathogens causing acute inflammation are not eliminated.[26]

Methods for synthesis of metallic nanoparticles

Metallic nanoparticles have unique properties that make them useful for a variety of applications.[27] Metallic nanoparticles can be synthesized using a variety of physical and chemical approaches. Principal methodologies include green synthesis, top-down and bottom-up techniques.

Green synthesis

Green synthesis is one of the most effective methods for creating metallic nanoparticles is green synthesis. Plant extracts, fungi, bacteria and other biological materials can be processed using green synthesis. The availability of phytoconstituents in specified species, such as ascorbic acid, carboxylic acid, phenols, aldehydes, ketones, flavones, amides and terpenoids, must be considered in order to synthesize metallic nanoparticles.[28] Metal salts can be reduced to metal nanoparticles. This technique is safer for the environment, uses safe solvents, lowers pollution and prevents the formation of harmful byproducts.

Mechanism for metals and their oxides

Through various processes, distinct microorganisms can be used to form different nanoparticles. Through the influence of electrostatic interactions between silver ions and negatively charged enzymes on the cell wall, silver ions are introduced onto the surface of fungal cells. Silver ions are subsequently reduced to the corresponding nuclei and grow as separate silver nanoparticles.[29]

Top-down technique

Metallic nanoparticles are produced from the bulk of materials, which involves the use of physical methods and employs mechanical energy, which is used in grinding, milling and crushing, as well as electrical energy used in the electrical arc discharge and thermal energy. The nanoparticles obtained using this technique have sizes in the range of 10-100 nm.[30] The nanoparticles were of high purity and had a uniform size distribution.

Bottom-up technique

This involves the formation of complex clusters and the nucleation and growth processes. They commonly use chemical and biological syntheses.[31]

Characterization

Numerous nanoparticles created using environmentally friendly methods have been characterized using a range of techniques such as Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), X-ray Diffraction (XRD) and spectroscopic techniques such as UV-vis and FT-IR.[32]

Silver nanoparticles

They have a wide variety of applications in various fields, such as industry, food and biomedicine. AgNPs have multiple biological and therapeutic applications, including antifungal, antibacterial and anti-inflammatory activities.[33]

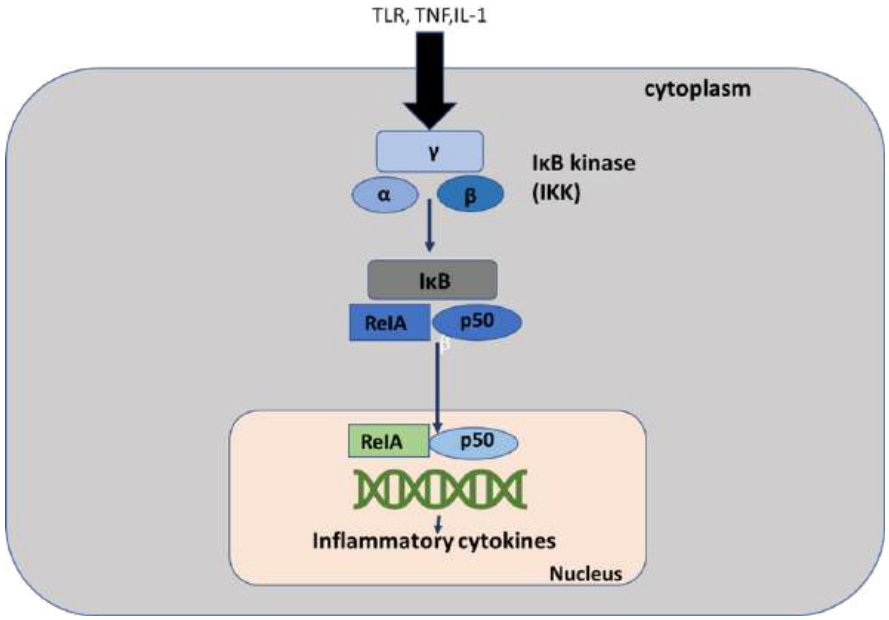

Figure 2: **Mechanism of Inflammation.**

Zinc oxide nanoparticles

They are widely used in medical applications and possess good biocompatibility, making them suitable for anti-fungal, anti-bacterial, anti-viral and anti-cancer activities. They show selective toxicity towards tumor cells.[34]

Gold nanoparticles

They have a wide variety of applications owing to their highly efficient drug-delivery ability. They exhibit excellent properties such as stability, biocompatibility and physical and chemical properties. Nanogold particles can easily bind to enzymes, antibodies and cytokines. They also exhibit anti-oxidant and anti-inflammatory properties. Cytotoxic drugs can also be directly targeted to cancerous cells.[35,36]

Titanium nanoparticles

They exhibit excellent antimicrobial properties and can be used in various biomedical applications. They also exhibit immunomodulatory and immune-toxic effects.[37,38]

Selenium nanoparticles

They have a wide range of applications in the medical field. They can target drugs in cytotoxic and normal cells. The main principle is the generation of Reactive Oxygen Species (ROS). They play an important role in protection against oxidative stress and inflammatory reactions.[39,40]

The anti-inflammatory action of metallic nanoparticles

A localized physical condition known as inflammation occurs when an infection or injury causes a portion of the body to become swollen, red, or painful. This is recognized as the fundamental basis of pathology. When pathogenic germs injure or infect tissues, the body produces an inflammatory response. This response attracts macrophages, killer cells and stem cells, all of which help regulate the reaction. Large, heterogeneous, mononucleated, phagocytic cells called macrophages are produced in the bone marrow and flow in the bloodstream as monocytes or mobile White Blood Cells (WBCs). These leukocytes travel from macrophages to infection sites in different tissues. There are two types of macrophages: M1 and M2. M2 macrophages, which are activated in response to anti-inflammatory reactions, cause the remodeling of struck tissues and organs and are pro-inflammatory. M1 macrophages, whose production induces inflammation, During an inflammatory response, macrophages produce activation signals to further trigger inflammation, in addition to phagocytosing cellular and tissue debris and causing macrophage activation. Activation signals involved in this process include extracellular matrix proteins, Lipopolysaccharide (LPS) and cytokines such as tumor necrosis growth factor, chemokines, lymphokines, interleukins and interferons.

The improved ability of nanoparticles to penetrate inflammatory and epithelial cells results in improved therapeutic efficacy and stability. Additionally, their ability to target specific areas, such as inflammatory cells or tissues, is enhanced. Anti-inflammatory activities have been shown for a number of metals and metal oxide nanoparticles, such as nickel, zinc oxide, zinc peroxide, magnesium oxide, titanium dioxide,[41] silver,[42] gold,[43] selenium,[44] copper,[45] iron oxide,[46] and cerium oxide.[47]

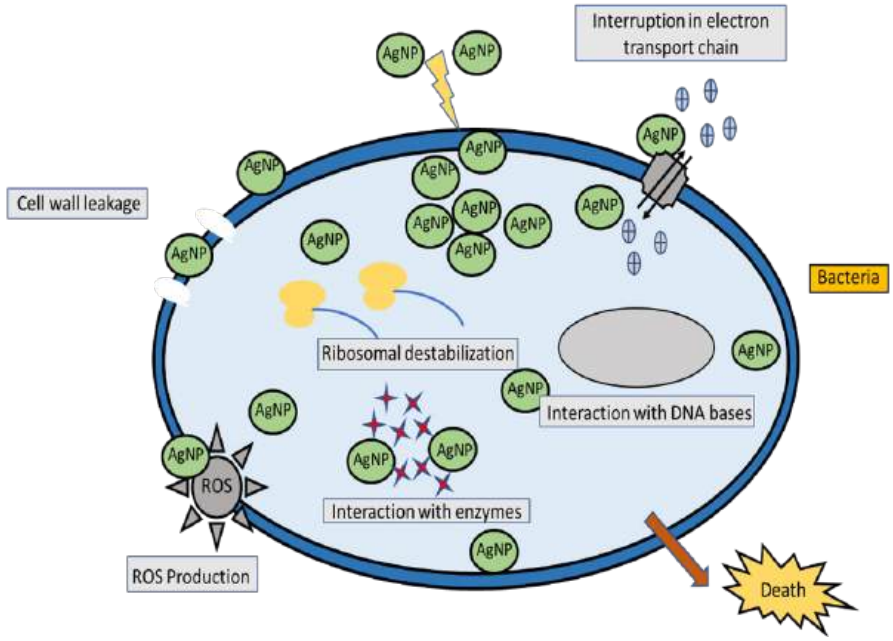

Figure 3: Anti-Cancer action of metallic nanoparticles.

The anti-cancer action of metallic nanoparticles

A malignant tumor mass is more likely to occur in the body when cells continue to proliferate uncontrollably owing to genome instability, deficiencies in the DNA repair mechanism and harmful mutations in tumor suppressor genes. By targeting the pathophysiology of tumor cells, metallic nanoparticles enable efficient transport of anticancer medications into tumors, thereby improving therapeutic outcomes (Figure 3). Applications of metallic nanoparticles in cancer detection and treatment include Magnetic Resonance Imaging (MRI) and colloidal mediators for the magnetic hyperthermia effect with Photothermal Ablation (PTA) therapy using magnetic nanoparticles. When the gold nanoparticles were coupled with other medications, such as TMZ and Cisplatin, something newly developed. In particular, they have the ability to attach to a wide range of chemical and biological molecules, a strong absorption range and minimal toxicity. Silver nanoparticles have generated much interest because of their potential use as antitumor agents in cancer therapy. It serves as a delivery system for medications containing antisense oligonucleotides and other small molecular structures. Drug transporters, better stability of surface-bound nucleic acids, a variety of surface ligand attachments, transmembrane delivery, protection of the attached therapies from degradation and the possibility for improved regulated intracellular drug delivery are just a few of the advantages that AgNPs provide. Nanoparticles have been used in many advanced tests to treat a range of illnesses.[48] The anti-cancer potential of the ZnO Nanoparticles was assessed after they were conjugated with unique hydrophobic peptides. Better cytotoxicity was observed for the nanoparticle-peptide complex than for the peptide or nanoparticle alone. Therefore, the ZnO nanoparticles tested in our study demonstrated anti-cancer activity against colon cancer cells. Moreover, it can be conjugated with peptides to more effectively target cancer cells.[49]

Anti-oxidant Action of Metallic Nanoparticles

Oxidative stress is a condition that occurs when an excess of oxidants, such as Reactive Oxygen Species (ROS), Nitrogen Species (RNS) and organic molecules, upsets the balance between the cell's antioxidative defense and oxidants. Contains sulfur and produces alkyl sulfinyl radicals.[50] One class of factors linked to a number of illnesses and aging is oxidants. Electrical stimulation or natural oxidative processes, which are produced by reduction-oxidation activities, are the usual sources of Reactive Oxygen Species (ROS). Research has revealed a possible relationship between aging and ROS damage. Recently, there has been an increase in interest in the free radical theory of aging, which focuses on mitochondria as both a source and target of ROS. The majority of current research has focused on identifying ways to reduce the effects of these oxidants. Although some metallic and metallic oxide nanoparticles have been shown to behave well as antioxidants, others are considered to have the potential to cause oxidative stress and result in unfavorable health outcomes. The unique properties of AuNPs have led to their application in many different industries.[51] The Antioxidant properties of AgNPs may be used to treat degenerative Alzheimer's disease and cancer. For instance, human lung cancer cells were treated with well-characterized PVP-coated AgNPs to examine the reactive oxygen species that lead to programmed cell death.[50] To discover novel compounds derived from organic compounds, anti-oxidant capabilities are currently being thoroughly investigated for a wide range of materials, especially organic compounds.[52]

CONCLUSION

In conclusion, silver nanoparticles were consistently produced using a marine sponge to decrease the silver precursor concentration. Silver nanoparticles are one of the most important nanoparticles that exhibit cytotoxic activity towards cancer cells. Silver nanoparticles produce an autophagy effect on cancerous cells and lead to the death of cancer cells. AgNPs are effective against inflammation without any toxic effects. *In vitro* and *in vivo* experiments showed that AgNPs have better anti-inflammatory activity. Nanoparticles up to 100 nm in size are useful for anti-cancer, anti-inflammatory and anti-oxidant activities because of their nanoscale size, which is highly permeable and has the strongest structure and provides better pharmacological actions.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Review Article

Unraveling the Molecular Mechanisms of Antimicrobial Resistance: A Comprehensive Exploration

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ABSTRACT

Background: The worldwide health community faces a serious threat of Antimicrobial Resistance (AMR), which occurs when bacteria develop resistance to drugs, thereby reducing the effectiveness of therapy. **Aim:** Unraveling the Molecular Mechanisms of Antimicrobial Resistance: A Comprehensive Exploration. **Materials and Methods:** Conducting a comprehensive literature search using various databases (PubMed, Google scholar, Research gate etc.,) to identify relevant studies on antimicrobial resistance mechanisms. Selected articles were critically evaluated, synthesized and organized to present a cohesive overview of the topic. **Results:** The key mechanisms underlying AMR include efflux pumps, target site changes, gene mutations, biofilm formation, quorum sensing and enzyme inactivation. A multimodal strategy involving cautious antimicrobial usage, strong infection control protocols, research on novel medicines and international collaboration is required to tackle this epidemic. **Conclusion:** The threat of AMR is jeopardizing global healthcare systems and calling for immediate action to protect public health and maintain the efficacy of antibiotic treatment.

Keywords: AMR, Superbugs, Efflux pump, Gene mutation, Biofilm.

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INTRODUCTION

AMR occurs when bacteria, viruses, fungi and parasites evolve, rendering medications ineffective. This phenomenon makes infections more challenging to treat and increases the likelihood of diseases. AMR jeopardizes the efficacy of treatment for a variety of infections caused by these microorganisms.[1] Antimicrobials encompass a broad category of medications, including antibiotics, antivirals and antifungals, all designed to combat infections. These powerful drugs play a crucial role in preventing and treating a wide range of microbial infections, from bacterial to viral and fungal diseases. By targeting and inhibiting the growth or spread of microorganisms, antimicrobials play a vital role in safeguarding public health and promoting well-being. Their use is pivotal in both clinical settings and broader public health initiatives aimed at controlling the spread of infectious diseases.[2]

Superbugs

Superbugs, colloquial terms for microorganisms that have developed resistance to antimicrobial treatments, pose a significant threat to public health. Several factors contribute to the emergence and spread of Antimicrobial Resistance (AMR). One critical mechanism is the active efflux of drugs, where microorganisms expel antimicrobial agents from their cells, thereby reducing their effectiveness.[3] Additionally, microorganisms can modify their target sites, making them less susceptible to antimicrobial drugs. Gene mutations play a pivotal role in AMR by altering the genetic makeup of microorganisms and allowing them to withstand the effects of medications (Figure 1). Biofilm formation and quorum sensing enable microorganisms to thrive in protected environments and coordinate their resistance mechanisms.[4] Physicochemical factors, such as variations in pH and temperature, can influence the efficacy of antimicrobial treatments. Furthermore, microorganisms may employ enzyme inactivation strategies to neutralize antimicrobial agents. Understanding these multifaceted factors is crucial for developing effective strategies to combat the increasing threat of antimicrobial resistance.[5]

Mechanism of antibacterial resistance by efflux pump

In eukaryotic cells, efflux pumps have been known since the discovery of P-glycoprotein in 1976 by Julian and Ling. Efflux, as a mechanism of antibiotic resistance, was first described in 1980. Genetic elements encoding efflux pumps may be encoded on chromosomes and/or plasmids, thus contributing to both intrinsic and acquired resistance. Efflux pumps are transport channels that transport antibiotics from the intracellular to the external environment. The expression of several efflux pumps

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may lead to broad-spectrum resistance. Primary efflux pumps obtain energy from ATP hydrolysis, whereas secondary efflux pumps obtain energy from the chemical gradient mechanism of the protons (Figure 2).

- MATE: Aminoglycosides, Cationic drugs, etc.,
- RND: Multiple drugs.
- MFS: Chlorhexidine, cetrimide etc.,
- SMR: Benzalkonium.
- ABC: Multiple drugs.

Target site modification

The enzyme or protein essential for microorganism survival undergoes genetic modification that renders the antimicrobials ineffective.

Alterations in target protein structure.

Preventing antimicrobial agents from binding effectively.

Modifying enzymatic activity to bypass the inhibitory effect of antimicrobial agents.

This alteration renders the antimicrobial agent ineffective at inhibiting or killing microorganisms. In bacteria, this often involves mutations in genes encoding target proteins, such as enzymes or cell membrane receptors, which are usually essential for microorganism survival. mutations can lead to changes in the structure and shape of proteins (Figure 3).[6]

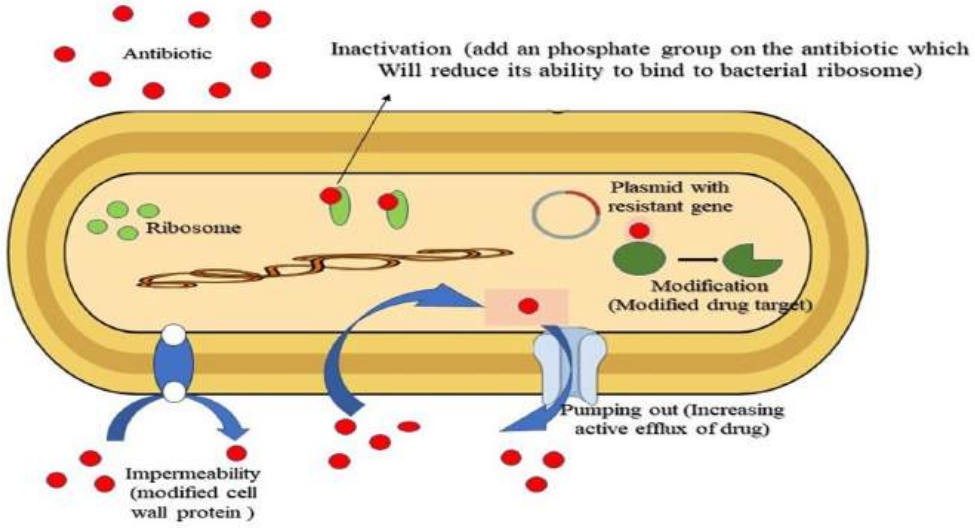

Figure 1: Mechanism of antibacterial resistance.

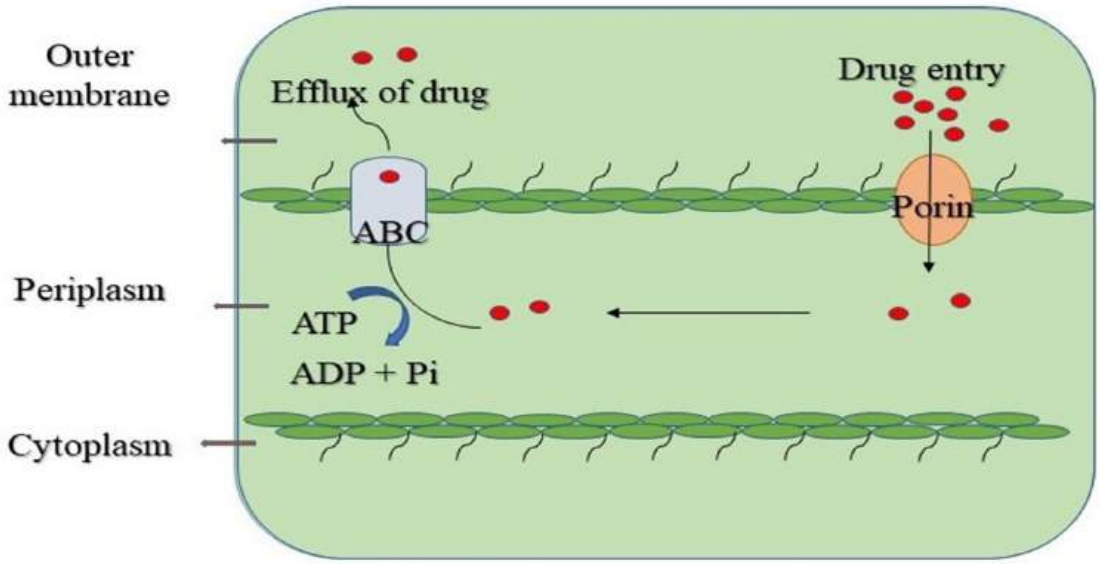

Figure 2: Antibacterial resistance mechanism by efflux pump.

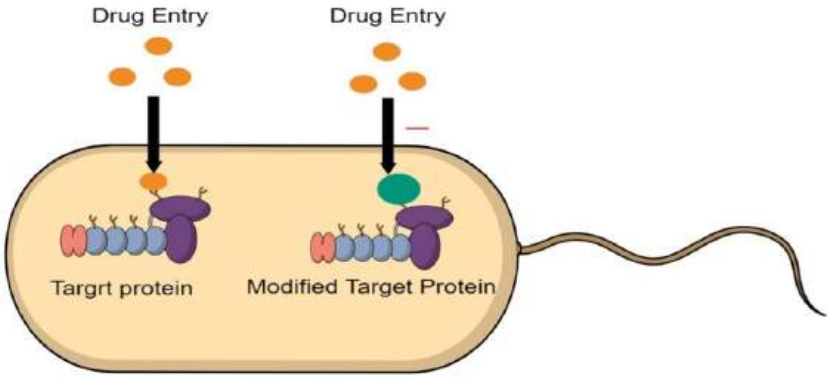

Figure 3: Antibacterial resistance by receptor target site alteration.

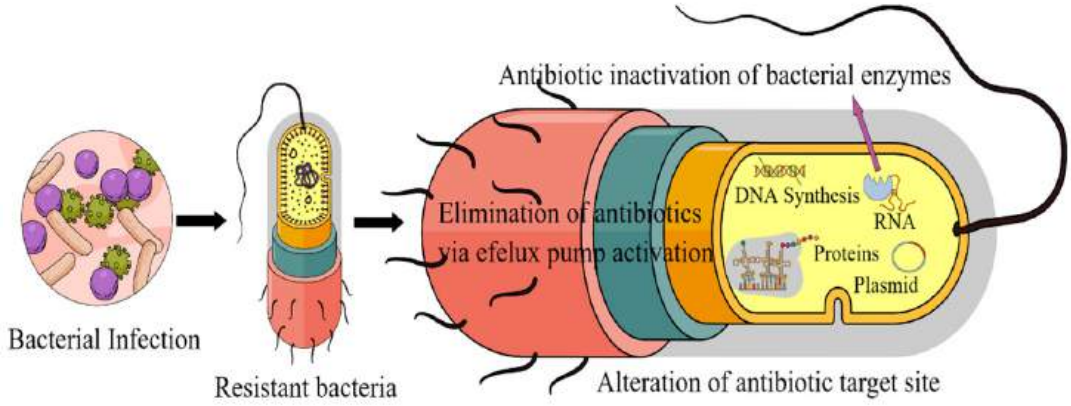

Figure 4: Antibacterial resistance mechanism target changes by gene mutation.

Gene mutation

Gene mutations are an important mechanism by which microorganisms develop antimicrobial resistance. This works through spontaneous mutation: Bacteria have a natural tendency to acquire genetic mutations during replication and occur randomly in bacterial DNA, leading to changes in the genetic code. Conjugation: This is a process whereby a donor bacterium conjugates or makes physical contact with a recipient bacterium through a sex pilus and transfers genetic elements to it. Resistance Transfer Factor (RTF)-This plasmid is of great importance as it leads to the spread of multiple drug resistance among bacteria. Selection pressure: Exposure to antimicrobial agents creates selective pressure that favors the survival of bacteria with mutations that confer resistance (Figure 4). Target modification: Mutations occur in genes encoding target sites, such as enzymes or cell wall components. Efflux pumps: Mutations that increase efflux pump activity can reduce intracellular antibiotic concentrations and promote resistance. Enzyme production: Mutation can also lead to overproduction or modification of enzymes involved in antibiotic inactivation and degradation. Horizontal gene transfer Mutations can occur in genes involved in horizontal gene transfer mechanisms, such as plasmids or transposons, which enhance the transfer of resistance genes between bacteria and accelerate the spread of resistance within the bacterial population. Gene mutations are dynamic processes that enhance the evolution of antimicrobial resistance.[7,8]

Biofilm formation and quorum sensing

Biofilms are a complex community of bacteria that adhere to surfaces and are encased within a self-produced extracellular polymeric substance, which serves as a physical barrier that protects bacteria from antimicrobial agents. Bacteria within biofilms can enter a dormant or slow-growing state, known as persister cells. Bacteria hide exopolymeric substances in biofilms and provide stability to inhabiting cells. Quorum Sensing (QS) plays an important role in biofilm formation-once the quorum is reached, bacteria initiate the expression of genes involved in biofilm formation, leading to the secretion of extracellular matrix components and assembly of structured community of bacteria (Figure 5).[9]

Enzyme inactivation

The most common mechanism of resistance in pathogenic bacteria to aminoglycosides, beta-lactams (penicillin and cephalosporin) and chloramphenicol involves the enzymatic

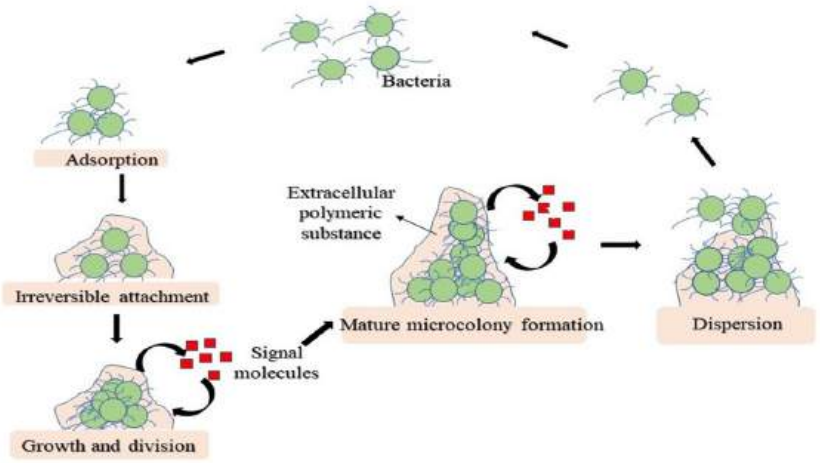

Figure 5: Antibiotic resistance by biofilm formation.

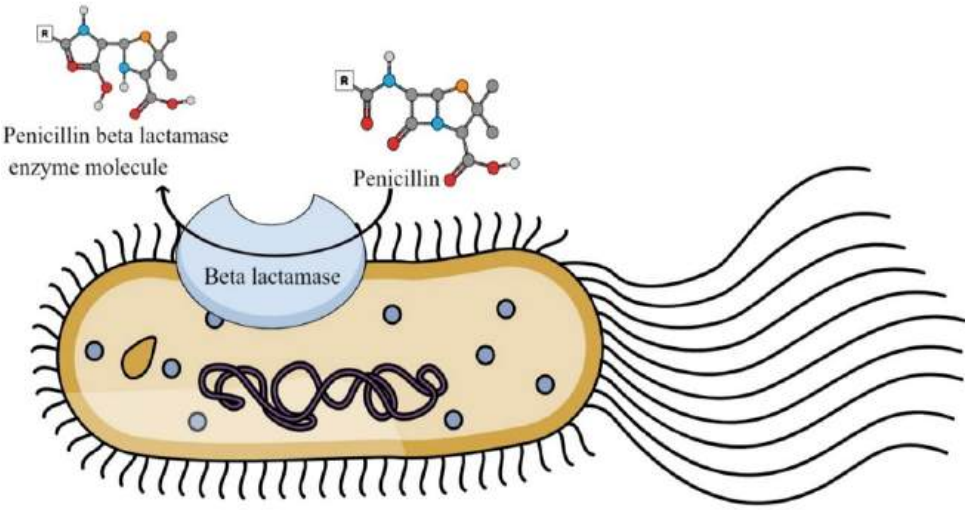

Figure 6: Antibiotic resistance by enzyme inactivation.

inactivation of antibiotics by hydrolysis or formation of inactive derivatives (Figure 6).[10]

CONCLUSION

AMR poses a serious and complex threat to public health. The efficacy of medicinal interventions against infections is threatened by the evolution of bacteria that resist antimicrobial therapies through a variety of methods. Comprehensive and cooperative efforts, including stakeholders from the healthcare, research, policy and international sectors, are needed to tackle Antimicrobial Resistance (AMR). Coordinated efforts are needed to address Antimicrobial Resistance (AMR) by promoting the responsible use of antibiotics, improving infection prevention and control methods, advancing research into novel treatment alternatives and fostering worldwide cooperation. Inaction against AMR compromises global public health systems and puts current medical procedures at risk of failure. Prioritising and maintaining efforts to lessen the effects of AMR and guarantee the ongoing effectiveness of antimicrobial medicines for future generations is essential.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

AMR: Antimicrobial resistance; **ATP:** Adenosine triphosphate; **MATE:** Multidrug and toxic extrusion; **RND:** Resistance-nodulation-division; **MFS:** Major facilitator superfamily; **SMR:** Small multidrug resistance; **ABC:** ATP-binding

cassette; **ADP:** Adenosine diphosphate; **Pi:** Phosphate group; **RTF:** Resistance transfer factor; **DNA:** Deoxyribonucleic acid; **RNA:** Ribonucleic acid; **QS:** Quorum sensing.

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Research Article

Retrosynthesis: An Approach to Explore the Functional Group Interconversions of an Antiviral Drug Pyrrolo[2,1-f][triazin-4-amino] Adenine C-Nucleoside (GS-5734)

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ABSTRACT

Planning chemical synthesis is a key feature of many aspects of chemistry, especially drug discovery and development. Retrosynthesis is the process of deconstructing a target molecule into readily available starting materials by the imaginary breaking of bonds or the conversion of one functional group into another. The identification of possible functional group interconversions with stable intermediate compounds is considered to be a successful approach in organic chemistry, which helps in identifying suitable synthetic routes and throws light on the possible synthons. The synthetic plan generated from retrosynthetic analysis will be a roadmap for guiding the synthesis of the target molecule. Herein, we present a multiscale approach for functional group interconversion and bond formation to facilitate an easier sense of the suitable retrosynthetic pathway of pyrrolo (2,1-f)(triazin-4-amino) Adenine C-Nucleoside (GS-5734).

Keywords: COVID-19, Remdesivir, Retrosynthesis, Functional group Interconversion, Synthons.

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INTRODUCTION

Remdesivir, a nucleoside analog antiviral drug, was developed by Gilead Science. This drug was mainly used to treat Ebola virus disease in 2010. It has also shown activity against the Middle East respiratory syndrome-related coronavirus (MERS-coV), the hemorrhagic fever Marburg virus and the severe acute respiratory syndrome coronavirus (SARS-CoV).[1] Recently researchers began extensive studies to evaluate the ability of remdesivir to treat the COVID-19 viral disease. The potential biological activity of remdesivir inspired us to explore the synthetic route of the drug using retrosynthetic analysis.

The task of making complex structures proficiently and in fewer advances than in recently revealed combinations is a progressing challenge in numerous research facilities around the globe.[2] Recent computational tools offer potential new ways to augment retrosynthetic analysis.[3] Among which the disconnection approach is significant for the development of algorithms to promote the identification of chemical bond disconnections.[4-9]

This retrosynthetic investigation was first utilized by Robinson in tropinone synthesis and eventually formalized by Corey *et al*. It is a fundamental technique that organic chemists use to understand the synthetic routes of the target molecule.[10,11] A retrosynthetic disconnection of a chemical structure or Targeted Molecule (TM) leads to two or more fragments, which are recognized as synthons with their corresponding reagents or functional groups. In another way, it is also known as Functional Group Interconversion (FGI).

In this work, we illustrated important reactions that can support a possible synthetic route, along with the substrate scope and functional group interconversions. The article is organized by the functional group interconversions and bond formations to

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facilitate an easier sense of the suitable retrosynthetic pathway of pyrrolo (2,1-f)(triazine-4-amino) Adenine C-Nucleoside (GS-5734) (Figure 1).

Functional group-based strategies

Conversion of functional groups in synthetic organic chemistry is an art that requires careful analysis in order to reduce or avoid side or unwanted reactions. The chemo-, region-, stereospecific- and stereoselective aspects can guide researchers in designing more direct routes to the precise target, consequently decreasing the number of functional group manipulations or alterations. Functional group interconversion is generally used to generate retrons from the target molecule. Antithetical disconnections are thought to divide the target molecule into a negatively and positively charged species made as units in synthesis are called donor synthons (d) and acceptor synthons (a). They are derived from reagents that contain various functional groups.

Formation or cleavage of P-O bond

Phosphate esters are scaffolds that are present in a variety of biologically active molecules and are also widely used in agrochemicals, pharmaceuticals, plasticizers, flame retardants, etc.[12-17] Similarly, phosphate esters are an essential part of various naturally occurring biological molecules, such as proteins, nucleic acids, steroids, carbohydrates and coenzymes and are also used as pro-drugs.[18] Phosphorylation of alcohols leads to the formation of phosphate esters, which have a great diversity of applications. As shown in Figure 2, the intention to install the phosphate ester functional group compound 1 can be achieved by the reaction of an alcohol (compound 2) with halo phosphoramidate (compound 3) in the presence of a polar solvent (step A). The mechanism involves the nucleophilic substitution of alcohol as an alkoxide ion (donor synthons) on a phosphoramidate ion (acceptor synthons) to obtain phosphate esters. The presence of an electron-withdrawing group or a good leaving group favors the attack of alkoxide ions on the electrophilic phosphoramidate ions. The disconnection of phosphate esters (P-O) by acidic hydrolysis provides reagents with practically acceptable donor (alkoxide ion) and acceptor synthons (phosphoramidate ion) information (Table 1). Existing modern and conventional methods support the FGI of Phosphate esters from alcohols via phosphorylation.[19-23]

General Mechanism of Phosphorylation

Formation and cleavage of C-O bonds

The C-O bond is quite common in structural motifs of numerous synthetic molecules and natural products with various important functional groups. In this study, we restricted our focus from alcohol-to-ether and ether-to-alcohol conversion.

Ethers are important functional groups in organic chemistry that contain an oxygen atom bonded to two alkyl or aryl groups. Depending on the nature of the alkyl or aryl groups bonded to the ether side of oxygen, they have different structures. Organic ethers are one of the most important classes of chemicals that have significant applications as herbicides, disinfectants, pharmaceuticals, plasticizers, solvents, drug intermediates and solvents in organic synthesis.[24-29] Generally, ethers are prepared from Brønsted acids and Lewis acid catalysts.[30] Among which a few procedures for the conversion of alcohols into ethers are Mitsunobu-type reactions,[31] hydroalkonylation of alkynes,[32] reductive condensation of esters and ketones,[33] alcoholysis of epoxides[34,35] and oxidative C-H alkoxylation of arenes.[36]

The dehydration of alcohols is the most important organic transformation that is being extensively used for the preparation of ether compounds.[37] The reaction of an alkyl halide with an alkoxy anion under basic conditions (Williamson synthesis) and the acid-promoted dehydrogenate condensation of alcohols gives ether functional group compounds. The dehydration of alcohols using many homogeneous catalysts, such as Lewis acids or Brønsted acids, has been reported for the etherification of alcohol functional groups.[38,39]

The reactions (E, G, I, J, K and L) with the alcohol group undergo dehydration, followed by nucleophilic substitution to give ethers, as depicted in Figure 3. Alcohols are converted to alkoxide ions using an inorganic base, which follows a nucleophilic substitution reaction with primary or secondary alkyl halides (S_N2 displacement mechanism, Williamson ether synthesis) in the presence of an aprotic polar solvent to give ethers. The reaction often competes with the base-catalyzed elimination of the alkylating agent, the nature of the leaving group and the reaction conditions (temperature and solvent), which can have a strong effect on the type of product formation (substitution or elimination). In reaction E, alcohol 13 undergoes intramolecular Williamson ether synthesis to give the cyclic ether compound 12. The disconnection of alcohol (O-H bond) by dehydrogenation condensation gives the donor synthon (alkoxide ion). The generated alkoxide ion attacks the carbon atom attached directly to the halide atom of the alkyl halide by a displacement reaction (halide atom). Here, the carbon atom attached to the halide atom acts as an acceptor synthon in a transition state, with both attacking and leaving groups. The possible synthetic information is presented in Table 2.

The cleavage of carbon-oxygen bonds in ethers can be achieved by nucleophilic substitution, which is a useful synthetic transformation in organic as well as medicinal chemistry.[40-46] The activation energy is significantly higher for bond cleavage in ethers.[47] This may be due to the reluctance of the C-O bond towards the oxidative addition reactions, the lower tendency of the alkoxy group to act as a good leaving group and the nucleophile employed. To promote this ionization, bond cleavage usually requires assistance from a protic or Lewis acid. The reaction may occur either via the S_N1 or S_N2 mechanism, depending on

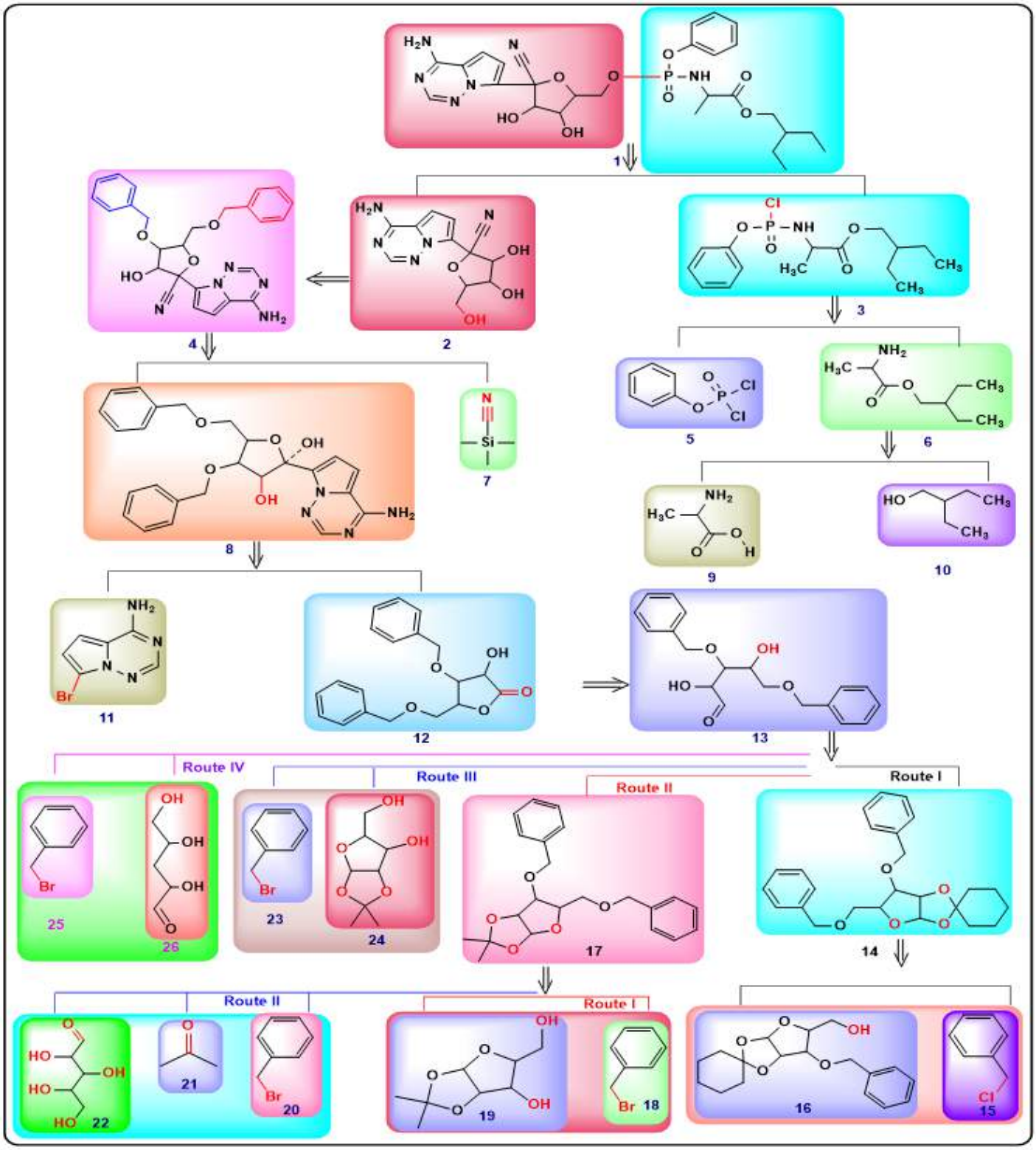
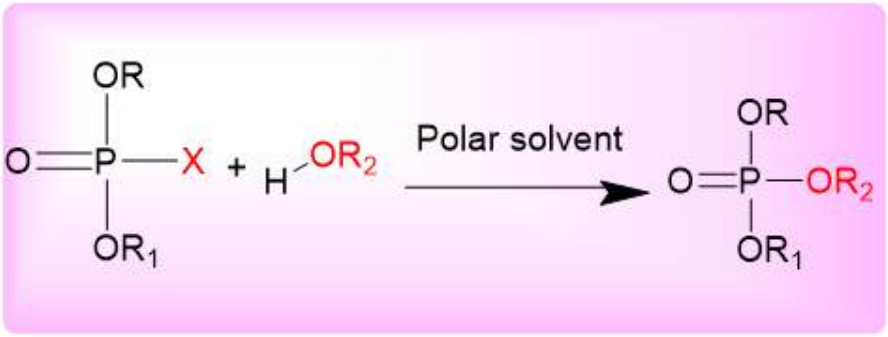


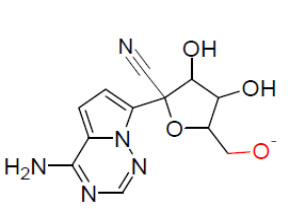
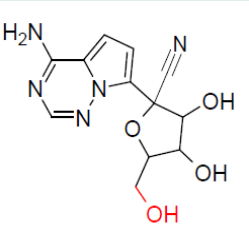
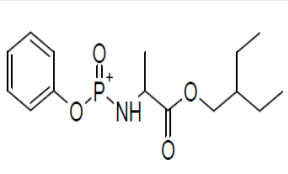
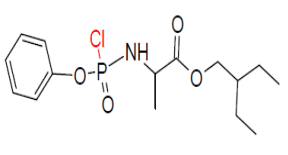
Figure 1: Complete schematic representation of retrosynthetic analysis of Pyrrolo(2,1-f)(triazin-4-amino) Adenine C-Nucleoside (GS-5734).



Retrosynthetic reaction for possible FGI

Figure 2: **General mechanism of phosphorylation, Disconnection of compound 1 (Phosphate esters) to compound 2 (alcohol) and compound 3 (Halo phosphoramidate).**

Table 1: **Types of synthons with respect to their available reagents.**

Type of synthons	Structure of synthons	Reagent	Functional group
Donor	Alkoxide ion 		Alcohol
Acceptor	Phosphoramidate 		Halo phosphoramidate

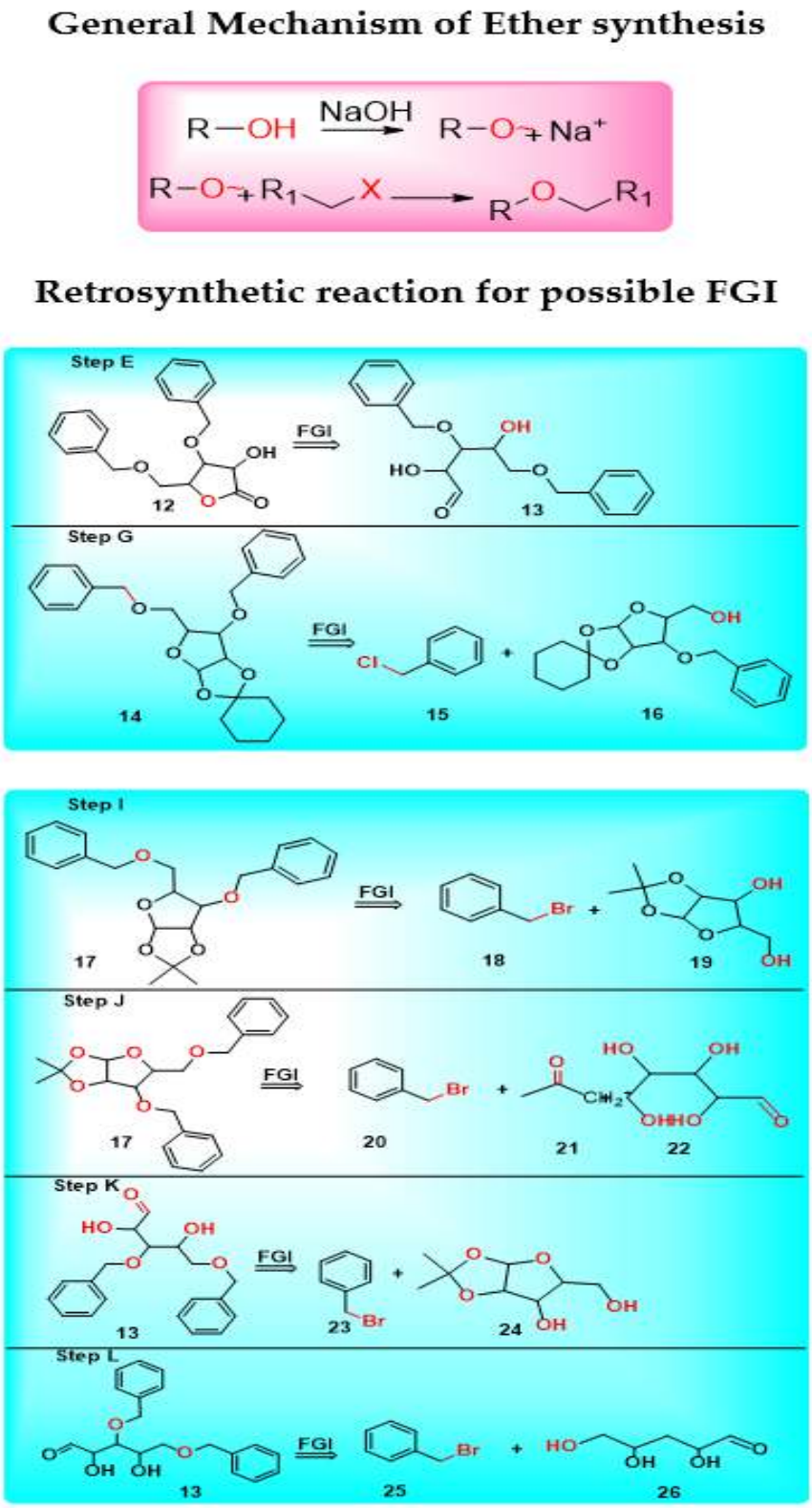


Figure 3: General mechanism of Ether synthesis, Disconnection of compounds 12, 13, 14 and 17 (ether functional group) to compound 13, 15, 16, 18, 19, 20, 21, 22, 23, 24, 25 and 26 (alcohol and alkyl halides).

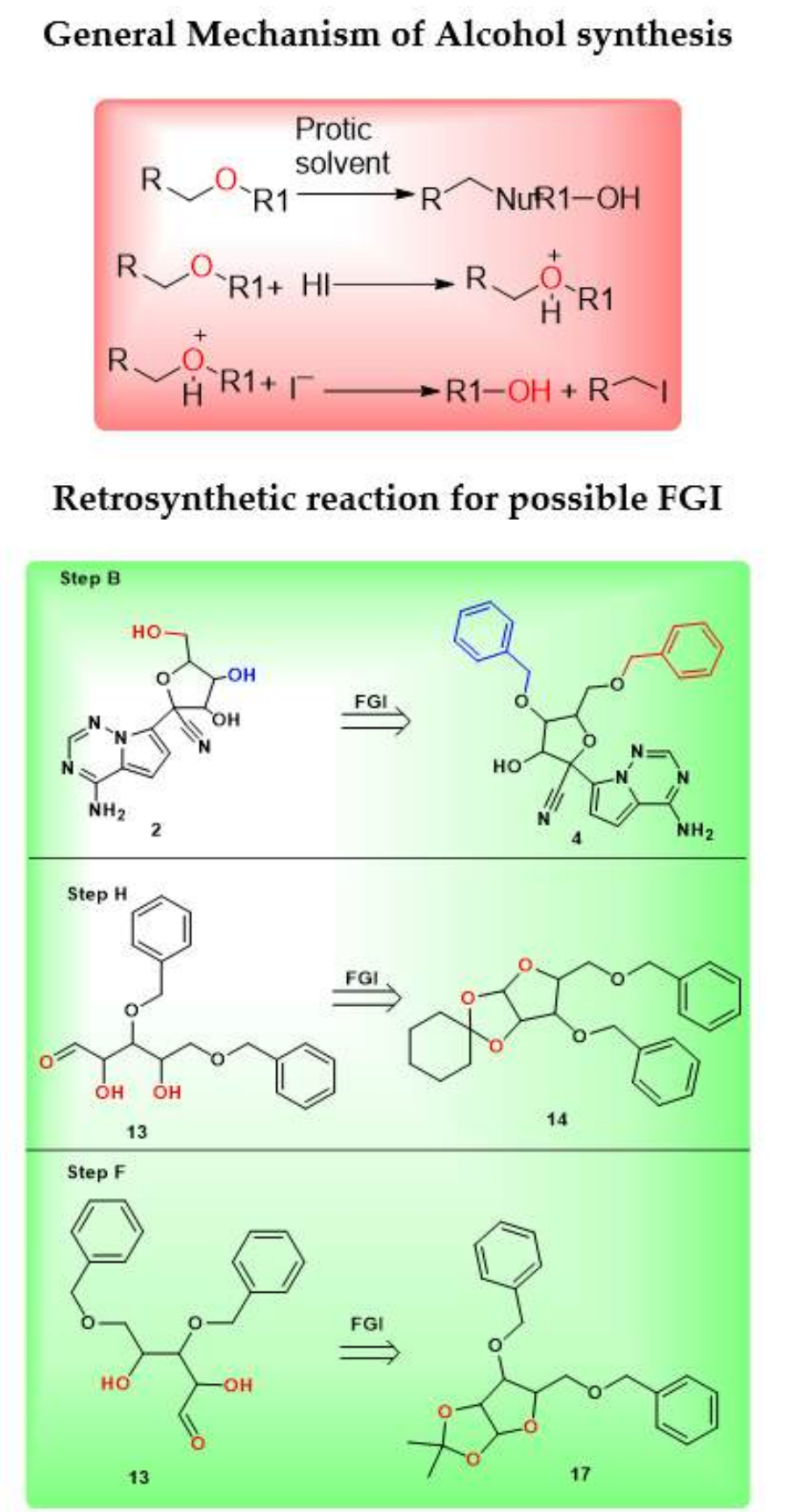
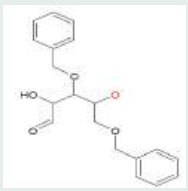
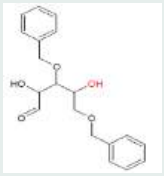
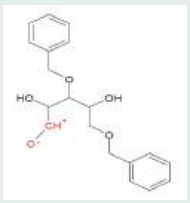
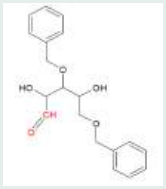
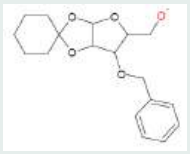
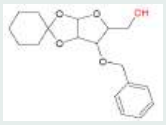
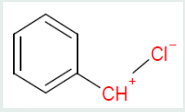
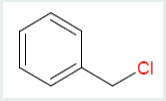
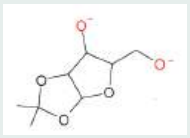
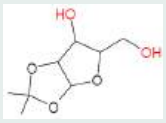
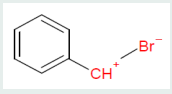
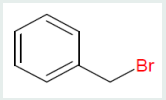
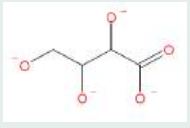
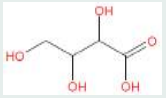
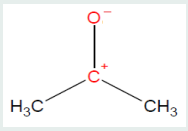
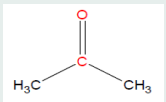
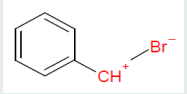
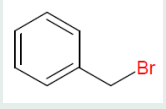
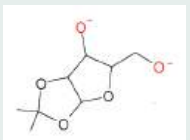
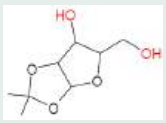
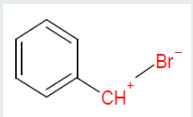
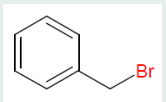
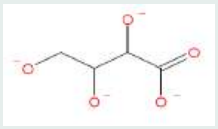
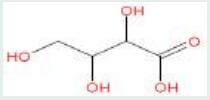
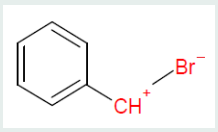
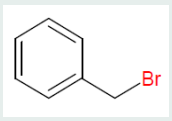


Figure 4: **General mechanism of Alcohol synthesis, conversion of compound 2 and 13 alcohol group to compounds 4, 14 and 17 ether group.**

Table 2: Types of synthons with respect to the available reagents.

Step	Type of synthons	Structure of synthons	Reagent	Functional group
Step E	Donor	alkoxide ion 		Alcohol
Step E	Acceptor			Aldehyde
Step G	Donor			Alcohol
Step G	Acceptor			Alkyl halide
Step I	Donor			Alcohol
Step I	Acceptor			Alkyl halide
Step J	Donor			Alcohol
Step J	Acceptor			Ketone
Step J	Acceptor			Alkyl halide
Step K	Donor			Alcohol
Step K	Acceptor			Alkyl halide

Step	Type of synthons	Structure of synthons	Reagent	Functional group
Step L	Donor			Alcohol
Step L	Acceptor			Alkyl halide

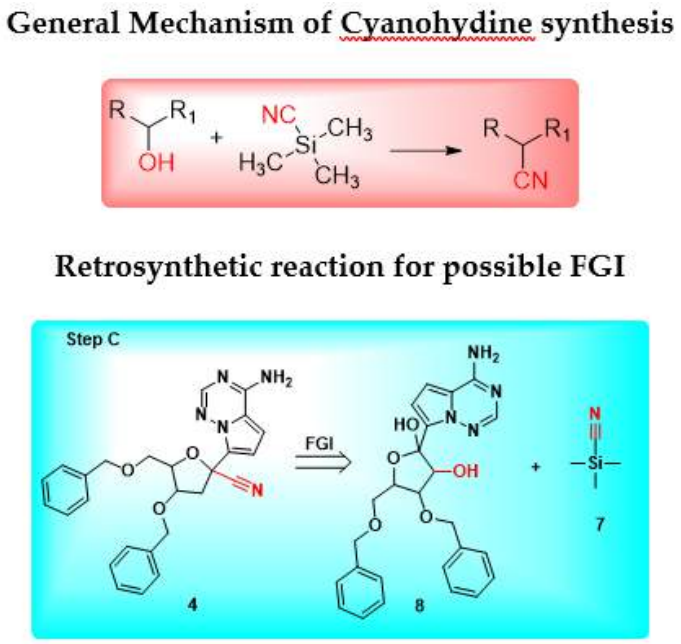


Figure 5: General mechanism of Nitriles synthesis, Disconnection of compound 4(Cyanohydines) to compound 8 (alcohol) and compound 7 (Nitrile).

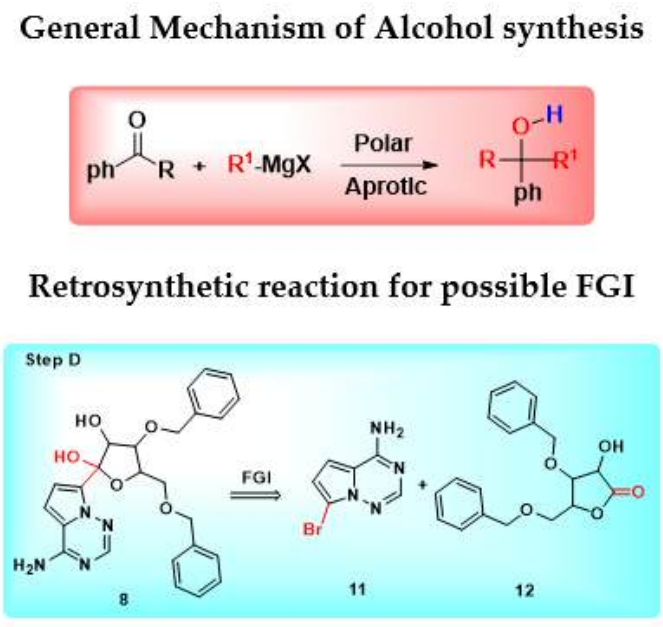


Figure 6: General mechanism of Alcohol synthesis, Disconnection of compound 8 (alcohol) to compound 11 (aryl halide) and compound 12 (ketone).

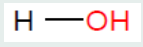
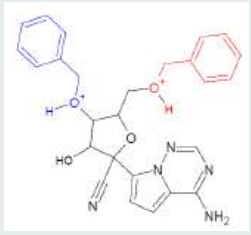
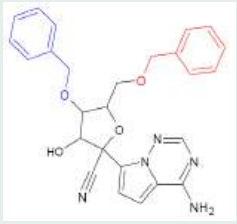
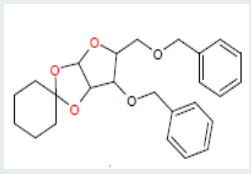
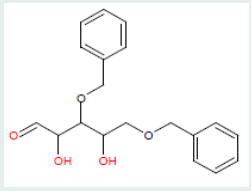
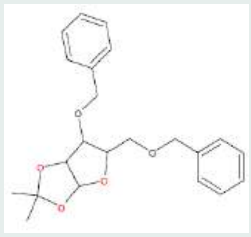
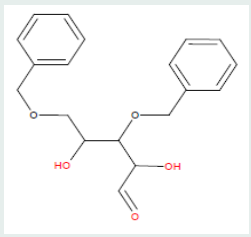
the alkyl group attached to the oxygen atom. Fortunately, recent advances in this area have led to dramatic progress in the use of innovative catalytic techniques that employ the cleavage of aryl alkyl ethers.

In reaction B, the aliphatic ether group undergoes hydrolysis to give alcohol and reactions F and H with cyclic ether undergo hydrogenolysis to give alcohol and carbonyl functional groups. Compound 4 underwent a nucleophilic addition reaction to give compound 2 via the S_N2 mechanism with a transition state in the presence of an acidic solvent, as depicted in Figure 4. Compounds 14 and 17 underwent catalytic hydrogenation to give 13 bearing hydroxyl and carbonyl groups. Possible synthons are listed in Table 3.

Formation of C-C bond by activation of C-O bond

The substitution reactions of alcohols with nucleophiles are generally more difficult than those of the corresponding halides. This was mainly due to the poor ability of the hydroxyl group to act as a good leaving unit. However, substitution is still a fascinating convention in synthetic organic chemistry, mainly due to the greater availability and cost effectiveness of alcohols over other functional groups. Thus, much attention has been paid to the direct substitution of alcohols with various nucleophiles, such as indoles,[48,49] amines,[50] amides,[51] linear thiols,[52] trimethoxybenzene,[52] enolsilane,[53] allylsilanes,[53] 1,3- dicarbonyls[54-58] and silyl ketene acetal.[59] The substitution of a hydroxyl functional group in alcohols with cyanide has attained great interest.[60-69] Nitriles are important precursors for the synthesis of drugs such as naproxen, cicloprofen and indoprofen.[70-72]

Table 3: Types of synthons with respect to the available reagents.

Step	Type of synthons	Structure of synthons	Reagent	Functional group
Step B	Donor			Hydroxy
Step B	Acceptor			Ether
Step F	Intra-molecular conversion		Stereoselective conversion	Ether
Step F	Intra-molecular conversion		Stereoselective conversion	Alcohol
Step H	Intra-molecular conversion		Stereoselective conversion	Ether
Step H	Intra-molecular conversion		Stereoselective conversion	Alcohol

Alcohols can be converted into nitriles by the Mitsunobu reaction[73] in the presence of a condensing agent (dicyclohexyl carbodiimide),[74] Michael reactions,[75] Strecker synthesis.[76] Even homogeneous Lewis acids (indium halides) and $B(C_6F_5)_3$[77,78] were found to be efficient for the transformation of alcohols with trimethylsilyl cyanide into nitriles. To troubleshoot the problems in the previous methods nowadays a heterogeneous along with moisture-tolerant catalyst were using instead of a homogeneous system.[79-92] In reaction C the transformation of alcohol (compound 4) to cyanohydines (compound 8) is achieved by nucleophilic substitution of nitrile group with alcohols (Figure 5). The disconnection of compound 4 gives possible synthons: compound 7 with a nitrile group and compound 8 with an alcohol functional group. Compound 7 acts as a donor synthon and compound 8 acts as an acceptor synthon (Table 4).

Formation of C-C and O-H bond by activation of C-O bond

The carbonyl ketone group undergoes a nucleophilic addition reaction with a Grignard reagent to give alcohol and this is the most versatile method for the synthesis of alcohols.[93-97] Recently, several modifications have been developed to enhance the yield of the product by suppressing side reactions such as changing the solvent,[98-103] addition of an excess amount of organic bases,[104-107]

Table 4: Types of synthons with respect to the available reagents.

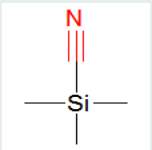
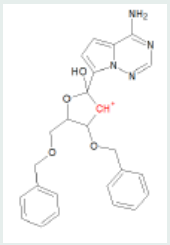
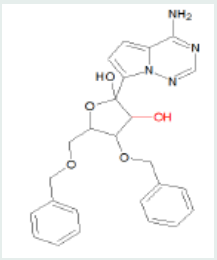
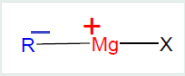
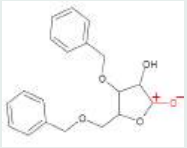
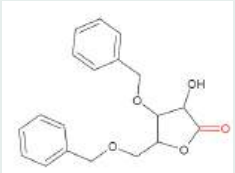
Step	Type of synthons	Structure of synthons	Reagent	Functional group
Step C	Donor			Nitrile
Step C	Acceptor			Alcohol

Table 5: Types of synthons with respect to the available reagents.

Step	Type of synthons	Structure of synthons	Reagent	Functional group
Step D	Donor			Aryl halide
Step D	Acceptor			Ketone

inorganic salts,[108-118] and tetrabutyl ammonium bromide.[119] In reaction D, compound 11 undergoes a nucleophilic addition reaction with a Grignard reagent to give compound 8 in the presence of moderately polar aprotic solvents such as dry ether and tetrahydrofuran (Figure 6). The disconnection of compound 8 gives possible synthons, compound 12 with the ketone group and compound 11 with the aryl halide group. The ketone carbonyl group undergoes ionization and the carbonyl carbon atom gets a positive charge due to the electronegativity of an oxygen atom, which acts as a positive synthon. The aryl halide acts as a donor synthon and attacks the positive carbon atom of the carbonyl carbon atom (Table 5). Simultaneously, the hydride ion or hydrogen atom attacked the carbonyl oxygen atom to facilitate the reaction.

CONCLUSION

In this study, we have proposed a retrosynthetic model for a targeted molecule, Pyrrolo(2,1-f)(triazin-4-amino) Adenine C-Nucleoside (GS-5734), to understand the various transformations of the intermediate scaffolds with possible synthetic routes. The resulting donor and acceptor synthons support the formation of stable intermediate compounds with various functional groups. The disconnection and functional group interconversion were carried out by exploring the inverse route from the target molecule to a pair of reactants in the given synthetic route, where all possible combinations of purchasable reagents spanned the feasible solution space. The identified diverse routes may help stimulate the ideas of organic researchers to narrow down the possibility of the synthetic route for the remdesivir drug. Even inter conversion of the functional group may provide an outlook for adopting alternative green synthetic techniques.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

TM: Targeted Molecule; **FGI:** Functional Group Interconversion.

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Research Article

Isolation and Identification of *Penicillium herquei*: Antibacterial, Antioxidant and Cytotoxic Properties of *Penicillium herquei* Ethyl Acetate Extract

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ABSTRACT

Background: Fungi are organisms with wide diversity and are an essential source of bioactive compounds. **Objectives:** Genotypic characterization and analysis of antibacterial and antioxidant activities of *Penicillium herquei* ethyl acetate extract. **Materials and Methods:** In the present study, *Penicillium herquei* Bainier and Sartory-A2 type strains were isolated and characterized from environmental sources (*Phyllanthus niruri* plant leaf). *Penicillium herquei* was selected for genomic identification by sequencing the ITS regions. Fungal DNA was isolated and ITS regions were amplified using primers ITS5 and ITS4. Phylogenetic analysis was performed using the NCBI server and the fungal sequence submitted on the NCBI website (NCBI accession number: MG909554.1). Fungal cultures were grown in a solid medium using potato dextrose agar. Fungal mats were extracted using ethyl acetate and evaluated for antibacterial activity, antioxidant activity (DPPH) and cytotoxicity (SK-MEL-3 cell**)** evaluations. **Results:** Fungal DNA sequenced NCBI BLAST analysis showed 99% similarity with *Penicillium herquei. Penicillium herquei* mat ethyl acetate extract showed antibacterial activity against *E. coli* (13 mm), *B. cereus* (5 mm), *S. aureus* (3 mm) and *K. pneumoniae* (12 mm). Ethyl acetate extract reduced by giving hydrogen atoms (DPPH) exhibited high scavenging activity compared to ascorbic acid and it also showed significant cytotoxicity (16 µg/mL) against human skin cell lines (SK-MEL-3 cells). **Conclusion:** This study concluded that *Penicillium herquei* with bioactive secondary metabolites has good antibacterial, antioxidant and cytotoxic properties.

Keywords: *Penicillium herquei*, ITS5 and ITS4, NCBI, Antibacterial, Antioxidant, Cytotoxicity.

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INTRODUCTION

Filamentous fungi of the genus Penicillium, classified under Ascomycetes, are ubiquitous in the environment and have played a pivotal role in the history of medicine since the discovery of penicillin. Penicillin, a β-lactam antibiotic produced by *Penicillium chrysogenum*, marked the beginning of recognition of the therapeutic potential of antibiotics.[1,2] Beyond penicillin, the genus Penicillium boasts a rich diversity of over 354 acknowledged species, many of which are known to produce bioactive compounds with pharmacological activities.[3,4] Endophytic fungi, specifically filamentous members of the Penicillium genus, have emerged as valuable sources of low molecular weight bioactive substances. For instance, Chrysogenum is known to produce essential bioactive secondary metabolites. The genus has significantly contributed to the development of various bioactive molecules, with some species producing antibiotics such as Penicillium brevicompactum, which yields mycophenolic acid used in immunosuppressive treatments.[5] Exploration of Penicillium species has revealed a plethora of bioactive compounds, showing their diverse pharmacological potential. These compounds exhibit antimicrobial, anticancer and antifungal activities.[6,7] As a result, an increasing number of Penicillium species have been subjected to bioactive chemical evaluation from various environmental sources. Among Penicillium species, *Penicillium herquei* strains have been identified and secondary metabolites extracted from these strains have been evaluated for their biological activity. This study focused on assessing the biological potential of *Penicillium herquei* and exploring its secondary metabolites for potential applications. This research not only contributes to our understanding of the bioactive potential within the Penicillium genus, but also highlights the significance of these fungi in drug discovery and pharmaceutical development. The

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diverse ecological compounds identified within this genus exhibit antiviral, anti-inflammatory and cytotoxic activities.[8,9,10]

MATERIALS AND METHODS

Sampling site and environmental data

Penicillium herquei Bainier and Sartory A2 was isolated from *Phyllanthus niruri* leaves collected from Palakkad, Kerala. The leaves were rinsed with tap water to obtain a fine powder. Adequate powder was transferred to PDA medium and incubated at room temperature for three days. Fungal cultures were separated and purified using the streaking method.

Isolation and Identification of fungi

Targeting specific sections within ribosomal RNA gene clusters via universal primers over PCR amplification is a different selection method for identifying fungi in nearby species.[11,12] This molecular study identified fungi using universal primers ITS4 and ITS5 in the rDNA ITS region (NCBI Accession number MG909553). Fungal cultures were deposited in the National Fungal Culture Collection of India (NFCCI accession no: 4233).

Microscopic study of *Penicillium herquei*

Fungal morphology was observed using a microscope and morphotaxonomy was observed in the conidial heads, colonies, sterigmata, chlamydospores, conidiophores and vesicles.[13]

Subculturing of the *Penicillium* spp.

The *Penicillium* spp. subcultured onto PDA plates prepared by dissolving 39 g of PDA in 1000 mL of distilled water and sterilizing at 121°C for 15 min. After inoculation to the plate, this incubated at 27°C for 7-10 days the well-grown sample used for further study.

Preparation of production media

Two different media were used to optimize the compounds (Media 1 and 2). Medium 1 contain K_2HPO_4 1.0 g, KCl 0.5 g, $NaNO_3$ 3.0 g, $FeSO_4$ 0.01 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, yeast extract 5.0 g, sucrose 30.0 g and NaCl 20.0 g, dissolved in distilled water 1,000 mL. Medium 2 contain $NaNO_3$ 3.0 g, beef extract 5.0 g, KH_2PO_4 1.0 g, $FeSO_4$ 0.01 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, glucose 30.0 g and NaCl 15.0 g in 1000 mL of distilled water. After preparing the media, sterilization was allowed and a 5mm thick plugged fungal culture using a cork borer and incubated at 30°C for seven days.

Extraction and purification of compounds

media were prepared separately and incubated at 29°C for 7- 10 days. The mycelial mat was partially grown within 7days and after obtaining the mycelial mat, the compounds were extracted. First, the broth medium was filtered and the mat was crushed with ethyl acetate at a ratio of 1:3 (w/v). After mixing, the mixture was incubated for 3 hr with intermediate mixing to obtain an extract. Finally, the aqueous phase was separated and stored to obtain a purified sample, which was used for further studies.[14]

Identification of secondary metabolites

The purified solution was used for the identification of the compounds and TLC was performed. A 4 cm wide and 8 cm high TLC plate was marked from the bottom of 1 cm to load the sample loaded with nearly 50 µL and allowed to dry at room temperature. The dried leaf was allowed to run in a solvent system of chloroform: methanol: 25% of ammonium hydroxide (89.95: 9.95: 0.1), the plate was allowed to dry and the R_f value calculated using the formula.

Antibacterial activity of *Penicillium herquei* ethyl acetate extracts

The purified fractions showed antibacterial activity by well diffusion method against *E. coli, B. cereus, S.* aureus and *K. pneumoniae*. Mueller-Hinton agar was prepared by dissolving 39 g in 100 mL of distilled water and was used after sterilization. The solidified medium 60 µL of µL culture was swabbed with a cotton swab and made using a cork borer. To the wells, 20 µL of the sample added (TLC plate mg/mL in DMSO) and Chloramphenicol (C-30mcg) standard antibiotic and incubated at 37°C for 24 hr. After incubation, the zone of inhibition was measured.[15,16]

Antioxidant activity of *Penicillium herquei* ethyl acetate extracts

Stock solutions were prepared for both the ascorbic acid and ethyl acetate extracts at a concentration of 1.0 mg/mL. Different doses of ascorbic acid (5, 10, 15, 20 and 25 µg/mL) were combined with the ethyl acetate extract in a methanol solution mL of 0.5 mM DPPH in methanol and ethyl acetate extract (0.5 mL) with the respective ascorbic acid solution was prepared. Absorbance at 517 nm was measured using a Stat Fax 4200 ELISA reader (USA) after 30-minute incubation at room temperature in the dark. Each experiment was conducted twice and the inhibition percentage (I%) was calculated accordingly.[17] % inhibitory=(Mean absorbance of the control-Mean absorbance of the sample)/Mean absorbance of the control×100.

Cytotoxicity activity of *Penicillium herquei* ethyl acetate extracts

The cytotoxicity of the ethyl acetate extract of *Penicillium herquei* on SK-MEL-3 cells was evaluated as described by Mosmann in 1983. SK-MEL-3 cells were harvested, counted using a diluted hemocytometer in DMEM (1×104 cells/mL) and seeded into individual wells of 96-well plates. After 24 hr of incubation for cell attachment, the wells were treated with either control or various concentrations of the *Penicillium herquei* ethyl acetate extract. Following treatment, SK-MEL-3 cells were cultured for an additional 24 h at 37°C in a humidified 95% air and 5%

CO_2 incubator. After washing with fresh culture medium, the drug-exposed cells were further incubated for 4 hr at 37°C with MTT (5 mg/mL in PBS). The purple-precipitated formazan was then diluted in 100 µL concentrated DMSO and cell viability was determined by measuring the absorbance at 540 nm using a multi-well plate reader. To express the results, the percentage of viable cells relative to the control was calculated using the following formula: % cell proliferation inhibitory=(mean absorbance of the control - mean absorbance of the sample)/mean absorbance of the control×100. The IC50 values, representing the concentration of the extract inhibiting 50% of SK-MEL-3 cells compared to the control, were computed from the dose-response curve generated by the *Penicillium herquei* ethyl acetate extract. Each experiment was conducted in duplicate and repeated at least thrice.

Study of apoptotic induction properties of *Penicillium herquei* ethyl acetate extract

The investigation of apoptotic cell death using microscopic fluorescence adhered to the methodology outlined by Baskic *et al*. (2006). SK-MEL-3 cells were initially seeded at a density of 5×104 cells/well in a six-well plate and allowed to incubate for a complete day. Following 24 hr incubation with *Penicillium herquei* ethyl acetate extract, the cells were extracted, washed with cold PBS and stained for five minutes at room temperature using a mixture of Acridine Orange (AO) and Ethidium Bromide (EB) in a 1:1 ratio, each at a concentration of 100 µg/mL. The stained cells were observed under a fluorescence microscope at 40x magnification. After completion of treatment, the cells were detached and washed three times with PBS. Finally, the plates were stained with Acridine Orange/Ethidium Bromide (AO/EB) at a 1:1 ratio at a concentration of 100 µg/mL for 5 min and examined under a fluorescence microscope at 40x magnification. The identification and quantification of apoptotic cells was based on the total cell count within the field of view.

Statistical analysis

Values are presented as mean±Standard Deviation (SD). Statistical analyses were conducted using SPSS version 12.0 for Windows (SPSS Inc., Chicago; http://www.spss.com), employing one-way Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). Statistically significant differences were considered when the *p*-value was <0.05.

RESULTS AND DISCUSSION

Morphological and Genotypic analysis

Two Penicillium strains were collected from plant leaves and enriched with Potato Dextrose Agar (PDA). Isolates purified by a single spore isolation technique are characterized by microscopic observation and measurement, including penicillin. *Penicillium herquei* Bainier and sartory SCOPS-A2 colonies on CYA fast-growing, velutinous, reverse pale yellow or yellow-brown, 16×18 mm in 7 d. Penicilli strictly verticillate, rarely terverticillate, terminally produced. Metulae 3-5 in number, smooth, hyaline, 9.63x2.88 µm. Phialides ampulliform, cellular short 2-5 per matulae, hyaline, smooth-walled, up to 10.15x2.5 µm. Conidia globose to oval light oluvaceous, 1.95-2.67x2.67-2.94 µm (Figure 1a, 1b and 1C).

Molecular identification of *Penicillium herquei*

The isolates were identified using a standard protocol. The raw sequence obtained from the ABI 3100 automated DNA sequencer was manually edited for inconsistency. The quality of the sequence was subjected to BLAST analysis using the NCBI server. The tested fungal strain showed a 99 % sequence similarity to *Penicillium herquei*. Sequence analyses with NCBI accession number MG9095543 and *Penicillium herquei* isolate P14910 resulted in the following alignment statistics. Alignment statistics: Query Length, 523; score, 935 bits (1036); expect, 0.0; identities, 521/523 (99 %); gaps, 0/523 (0%) Strand; Strand-Plus/Plus (Figure 2).

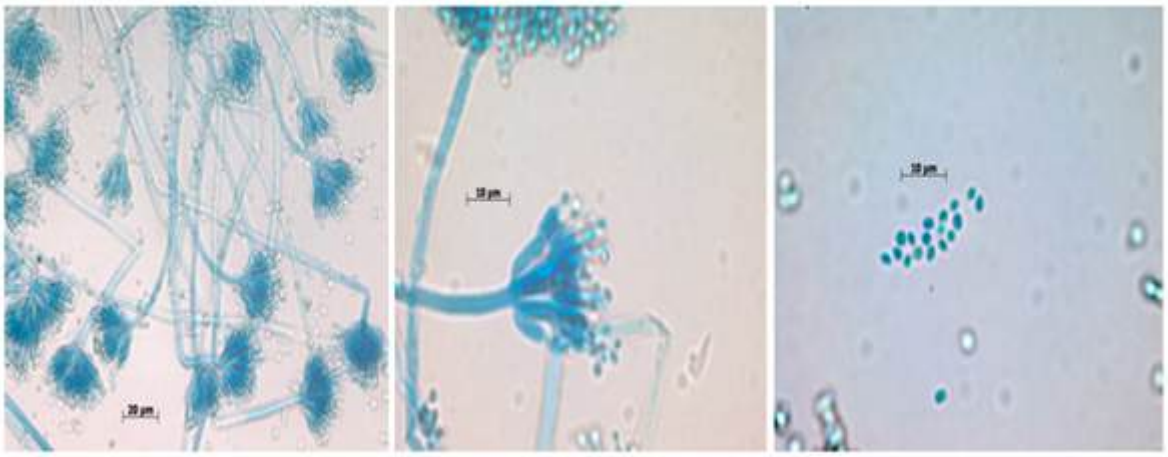

Figure 1: Microscopic observation of *Penicillium herquei*.

Large scale cultivation

The fungus *Penicillium herquei* was cultivated in solid-enriched media. The mycelial mat was isolated and homogenized with ethyl acetate/aqueous in a ratio of 1:3 (w/v) and from the extraction of bioactive secondary metabolites isolated using chromatography techniques (see the experimental methods).

Antibacterial activity

Investigation of the antibacterial activity of *Penicillium herquei* ethyl acetate extract, assessed by the disc diffusion method against various clinical bacteria, yielded noteworthy results. The extract demonstrated significant zones of inhibition, particularly against *E. coli* (13 mm) and *K. pneumoniae* (12 mm), suggesting its potent antibacterial effects. While *B. cereus* showed a moderate response (5 mm), *S. aureus* exhibited comparatively weaker inhibition (3 mm). In contrast, the standard antibiotic Cefixime-30 displayed varied inhibitory zones. These findings underscore the potential therapeutic applications of the extract, especially against E. coli and K. pneumoniae, warranting further exploration through additional studies, including Minimum Inhibitory Concentration (MIC) determination, to elucidate its specific applications and mechanisms of action in combating bacterial infections (Table 1 and Figure 3).

Determination of antioxidant activity *Penicillium herquei* ethyl acetate extract by using DPPH method

Determination of antioxidant activity of *Penicillium herquei* ethyl acetate extract using the DPPH method yielded significant insights into its potential as a radical scavenger. The antioxidant capabilities of the extract were compared to those of ascorbic acid standard, a well-known antioxidant. The percentage of inhibition by *Penicillium herquei* ethyl acetate extract was measured at various doses. The observed color change from purple to yellow during the inhibition process was indicative of the reduction of DPPH radicals by the extract. This alteration suggests the addition of hydrogen atoms to the DPPH radicals, resulting in a visible color shift. This phenomenon signifies strong scavenging activity, as the extract effectively reduced the concentration of free radicals. These results imply that *Penicillium herquei* ethyl acetate extract of *P. herquei* possesses antioxidant properties, as evidenced by its ability to neutralize DPPH radicals. The comparison with ascorbic acid served as a reference point, highlighting the efficacy of the extract in scavenging free radicals. This antioxidant potential is crucial for mitigating oxidative stress-related damage in biological systems (Figure 4).

Cytotoxic activity (MTT assay)

Examination of *Penicillium herquei* ethyl acetate extract for its cytotoxic effect against SK-MEL-3 cancer cells at various concentrations, ranging from 10 µg/mL to 35 µg/mL, yielded noteworthy results after a 48 hr incubation period. The *in vitro* cytotoxicity assessment revealed a dose-dependent response, as demonstrated by the modified shape of the SK-MEL-3 cells, as illustrated in Figure 5. In particular, at a concentration of 20 µg/mL, *Penicillium herquei* ethyl acetate extract exhibited a significant inhibitory effect on the growth of SK-MEL-3 cells in comparison to the control cell viability. This concentration markedly reduced cell proliferation, indicating the potential cytotoxic effect of the extract on the SK-MEL-3 cancer cell line. The observed

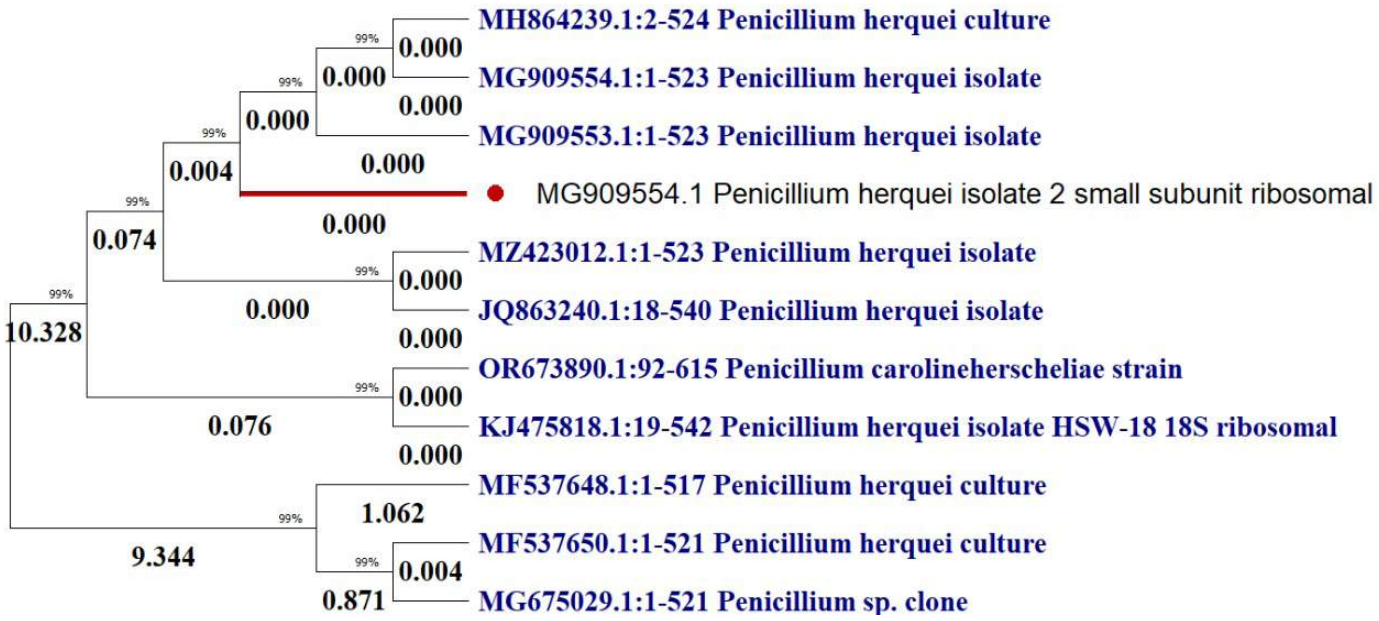

Figure 2: **Phylogenetic tree displays the relation of strain *penicillium herquei* to other relevant fungal species recovered from NCBI GenBank (18S ribosomal ribonucleic acid).**

Table 1: Antibacterial activity of *Penicillium herquei* ethyl acetate extract against clinical bacteria by disc diffusion method.

Sl. No.	Test organism	Zone of Inhibition (mm) (*n*=2)	
		Ethyl acetate extract	Standard (Cefixime-30)
1	*E. coli*	13	Nil
2	*B. cereus*	5	7
3	*S. aureus*	3	8
4	*K. pneumoniae*	12	5

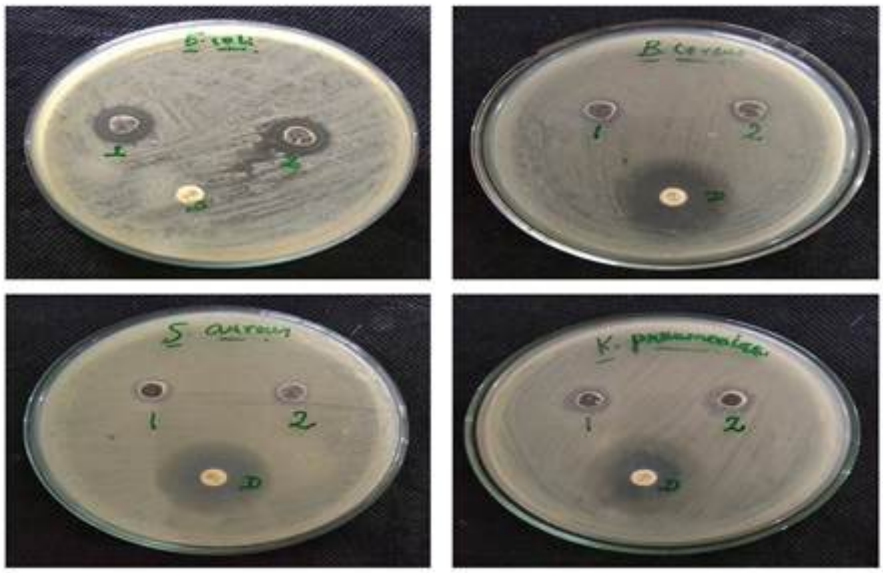

Figure 3: Antibacterial activity of *Penicillium herquei* ethyl acetate extract against clinical bacteria by disc diffusion method.

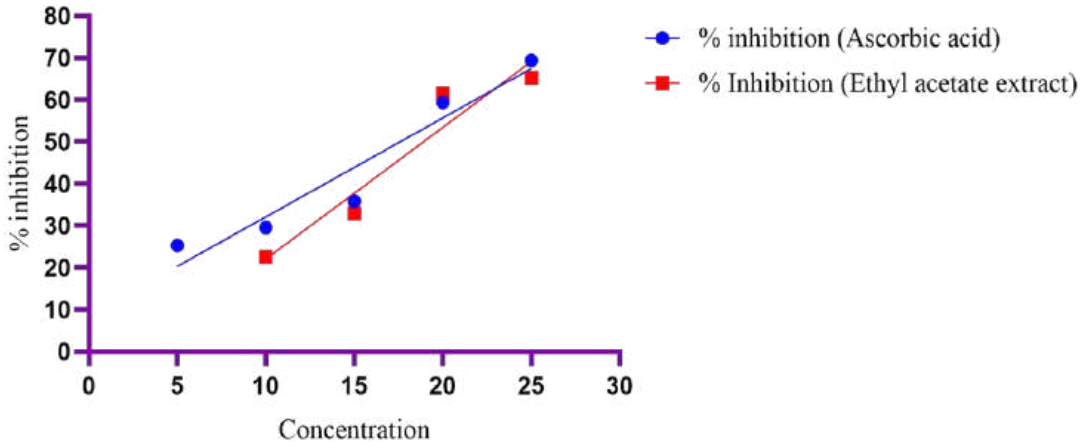

Figure 4: Antioxidant activity *Penicillium herquei* ethyl acetate extract.

dose-dependent cytotoxicity suggests that the *Penicillium herquei* ethyl acetate extract may contain bioactive compounds with anti-cancer properties. A concentration of 20 µg/mL emerges as a pivotal point, demonstrating marked inhibition and emphasizing the potential of the extract as a candidate for further investigation in cancer therapeutics. These results underscore the importance of exploring natural sources, such as fungal extracts, for potential cytotoxic compounds that could contribute to the development of novel anti-cancer agents. Further studies are warranted to elucidate the specific mechanisms of action and to identify the bioactive components responsible for the observed cytotoxic effects.

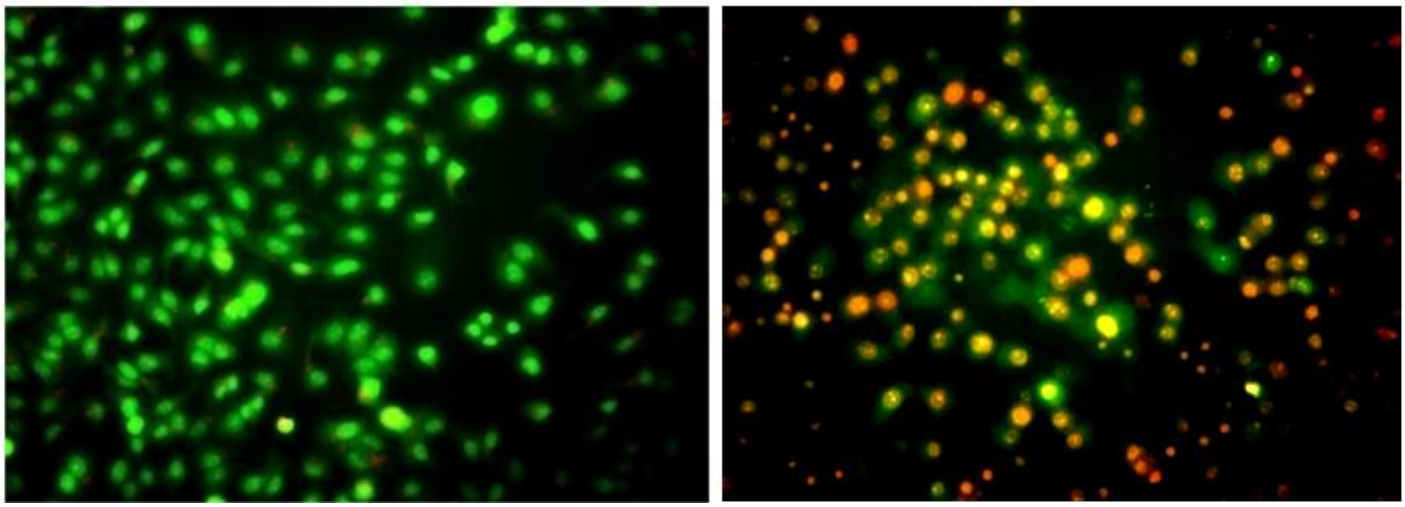

Figure 5: Fluorescence microscopy was used to investigate the dual dye AO/EB-stained *Penicillium herquei* ethyl acetate extract (20 µg) and regulated SK-MEL-3 cells after 24 hr. The standard green nucleus first emerged in living cells; later, necrotic cells and chromatin condensation or fragmentation of orange nuclei and yellow nucleus with chromatin occurred.

CONCLUSION

A comprehensive investigation of *Penicillium herquei* involved morphological and genotypic analyses, molecular identification, large-scale cultivation and the evaluation of its bioactive potential. Morphological characterization revealed distinctive features, such as fast-growing colonies with specific Metulae and Phialides characteristics. Genotypic analysis confirmed the identity of *Penicillium herquei*, exhibiting a 99% sequence similarity with the reference strain. The large-scale cultivation and extraction of bioactive secondary metabolites from *Penicillium herquei* demonstrated a robust approach for potential pharmaceutical applications. The ethyl acetate extract exhibited notable antibacterial activity, particularly against *E. coli* and *K. pneumoniae*, demonstrating its potential therapeutic significance. Comparison with the standard antibiotic, Cefixime-30, provided a valuable reference for the efficacy of the extract. Further exploration through Minimum Inhibitory Concentration (MIC) determination is crucial to understand its specific applications and mechanisms of action against bacterial infections. Additionally, the antioxidant activity of *Penicillium herquei* ethyl acetate extract was evaluated using the DPPH method, showing its potential as a free radical scavenger. Cytotoxicity assessment of SK-MEL-3 cancer cells demonstrated a dose-dependent inhibition of cell growth, suggesting the potential of the extract in cancer therapeutics. Fluorescence microscopy provided visual insights into the effects of the ethyl acetate extract on SK-MEL-3 cells. The multifaceted approach, integrating morphological, genotypic and functional analyses, provides a comprehensive understanding of its potential applications in medicine and contributes to the ongoing exploration of microbial resources for therapeutic purposes. Further studies are warranted to explore the specific bioactive compounds responsible for these observed effects and validate the potential of the extract in various biomedical applications.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Research Article

Clone Based Sequence for the Identification and Phylogenetic Study of Lichenized Fungi: A Case Study from *Usnea*

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ABSTRACT

Background: *Usnea* is a large genus in the family Parmeliaceae, with more than 350 species widely distributed in polar, temperate and tropical regions. **Aim:** The *Usnea* genus is recognized based on the fruticose thallus, branches with a cartilaginous central axis and usnic acid in the cortex. **Materials and Methods:** The phylogenetic relationships and the morphological variation among *Usnea* species have studied. The morphological characters traditionally used for species recognition of several European *Usnea* species analysed regarding their reliability. The evolution and distribution of the morphological characters looked to a phylogeny based on sequence data. It is easiest to obtain sequences from fresh *Usnea* material (not older than five years). DNA from the central axis has extracted to minimize the risk of contamination with lichen parasites. Since lichens are a combination of multiple organisms (fungi and algae), obtaining a single organism's DNA is difficult. These lichenized organisms cannot quickly be grown in axenic culture or manually teased apart from their associated microbial communities. It is a common phenomenon to observe multiple DNA bands following PCR caused by DNA from various organisms. **Results:** There is a greater chance of failure in the sequencing process. The cloning approach is the best one to check the sequence. To do the cloning, consider purifying at least five bands separately from the gel electrophoresis. Thus, get five clones to get an accurate picture of which sequences contained in the DNA. That way, even if the sequencing fails, you will still have the cloned products as a backup. Thus, clone-based sequencing is more efficient than that traditional sequencing methods. **Conclusion:** In the present study, we use this method for the phylogenetic interpretation of the genus *Usnea* and the results compared with the global data sets.

Keywords: Lichen, ITS, PCR, Phylogenetic, RAPD, Sequence.

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INTRODUCTION

Lichens are biologically distinct entities composed of fungus (mycobiont) with photosynthetic partners (photobiont), usually green algae or cyanobacterium. The algal partner synthesizes the food by photosynthesis and shares it with a fungal partner; in turn, the fungus protects the algae. They grow in diverse climatic conditions and on various substrates. The ability to quickly absorb and retain water from many sources makes it possible for lichens to live in harsh environments like deserts, Polar Regions and exposed surfaces like bare rocks, walls, roofs and tree branches. The photobiont is not known to reproduce sexually in the lichen state. However, the fungal partner is specific to the lichen taxon, so that the classification of lichens based on the sexual characteristic of the fungal partner.

Characteristic features and diversity of *Usnea*

The genus *Usnea* (Parmeliaceae, Ascomycota) recognized by fruticose (hair-like) thallus with a radial organization (Figure 1). An axis consisting of a cartilaginous strand of longitudinally arranged hyphae gives rise to many branches and the presence of usnic acid in the cortex. The variation in morphological characters such as the colour of the thallus, the thickness of the main branch and the thallus' length makes it difficult to distinguish one species of *Usnea* from another. According to Clerc,[1] morphological feature of *Usnea* that are constant and that do not change with the changing environmental conditions of the geographical area should be used to distinguish species of *Usnea*. Such characters include pigmentation of the basal part of the thallus, cortex and medulla; density of fibrils; the shape of the branches, branching type; and the ratio of Cortex, Medulla and Axis (C/M/A) in longitudinal section.

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Lichen genus *Usnea* is represented by ca. 350 species globally,[2] and India represent 60 species.[3] Motyka published the first world monograph of genus *Usnea* and divided *Usnea* into six subgenera *viz*., *Protousnea* Motyka, *Neuropogon* (Nees and Flot.) Motyka, *Lethariella* Motyka, *Chlorea* (Nyl.) Motyka, *Eumitria* (Stirt.) Motyka and *Euusnea* Jatta. All these subgenera later became independent genera except *Eumitria* (Stirt).[4] Motyka and *Euusnea* Jatta are the two genera combined and considered the genus *Usnea*. Different authors have discussed the delimitation of Usnea and the generic concept changed from time to time.[5-7] Many taxonomic uncertainties remain at the species level. Species identification of *Usnea* thought to be exceptionally difficult by most lichenologists because they are incredibly variable on morphology. The eco-phenotypes of the same species often look radically different.

General remarks on taxonomy and molecular work

The identification and taxonomic positioning of fungi, bacteria, viruses and parasites are essential for biologists interested in their diversity or conservation and others to help distinguish the organism of their interest (*e.g.*, plant pathologists, manufacturers of pharmaceutics). The conventional taxonomic approaches usually based on floral characteristics alone. Traditional methods like environmental sample culturing, laboratory identification by morphology and biochemical tests are still fundamental. Traditional microscopic techniques fail to identify the vegetative hyphae.[8] Such casual observations cause ambiguity in the classification of closely related species and populations. Molecular techniques were used to investigate genetic diversity and relationships among species. These data provide helpful tools for taxon delimitation, especially in plant groups in which the number of diagnostic morphological characters is limited.[9] Most of the biological identifications still carried out using traditional paper-based expertise, which has to be followed manually, although molecular methods are becoming more common.[10] While using this taxonomic key, the user makes a series of choices from a successive species group. This identification is only as good as the expert's observations who have compiled the key and its correct interpretation by the user.

Lichens are complex organisms formed by many fungi and algae species. To determine which one is more dominant or which one is most significant in the taxonomic perspective is a puzzling question generally we the lichenologist ask. Although most of the lichens are identified based upon the lichenized fungi, secondary fungi presence in the form of lichenicolous or as an endolichenic form is not rare, creating confusion performing molecular identification.

Gene sequences in Phylogeny

To infer phylogenetic and taxonomic relationships among lichens, both morphological and molecular data often considered. In recent years advances in technology and knowledge of gene sequences have significantly contributed to angiosperm phylogeny.[11] Biologists have utilized chloroplast, nuclear, mitochondrial genes to elucidate relationships at all levels of taxonomic rank. Molecular approaches for analyzing phylogeny have become increasingly valuable, especially where morphological characters have been insufficient to distinguish taxa at different levels.[12,13]

The application of molecular markers has been the most active research area in animal and plant systematic analysis. The analysis of the genetic changeability within and among populations of the species is crucial for understanding their future maintenance and developing their improvement strategies and conservation programs. Based on polymorphism found in proteins or DNA, molecular markers development has dramatically facilitated research in various disciplines such as taxonomy, phylogeny, ecology, genetics and plant breeding.[14]

There are several DNA-based marker systems for studying phylogeny, each with its pros and cons. These markers are phenotypically stable and are not prone to environmental

Figure 1: A and B Saxicolous and Corticolous *Usnea* species.

change.[15] In recent days, there has been constant recognition that the higher-order structure is fundamental for establishing meaningful structure-function relationships among biological macromolecules.[14-16]

One of the extensively used regions in a different organism's phylogenetic studies is the nuclear ribosomal DNA cistron.[17] The nuclear gene encoding the cytoplasmic ribosomal RNAs (rDNA) are in most eukaryotes organised into transcriptional units with a Small (18S/SSU), 5.8S and a Large (28S/LSU) subunit rDNA region, separated by internal transcribed spacer regions ITS1 and ITS2. The DNA sequences for LSU and SSU rDNA are under stabilising solid selection due to their critical role in ribosome synthesis. The non-coding region, like the ITS rDNA regions, is not under similar functional constraints. Several general features of the ITS region promotes its use for phylogenetic analysis of angiosperms. First, along with the other components of the nrDNA multigene family, the ITS region is highly repeated in the plant nuclear genome. The entire rDNA repeat unit is present in many thousands of copies arranged in tandem repeats at a chromosomal locus or multiple loci. This high copy number promotes detection, amplification, cloning and sequencing of nrDNA. Second and most importantly, this gene family undergoes rapid concerted evolution via unequal crossing over and gene conversion from phylogeny reconstruction. This property promotes Intra genomic uniformity of repeat units, even between nrDNA loci on non-homologous chromosomes. And in general, it also supports the accurate reconstruction of species relationships from these sequences.[17]

Internal Transcribed Spacer (ITS) region is the primary choice for molecular identification of fungi. It is two highly variable spaces ITS1 and ITS2 are usually species-specific, whereas the intercalary 5.8s gene is highly conserved. White *et al.*[18] designed several primers to sequence the ITS region widely used by phylogeneticists. Along with the development and application of molecular techniques, the Internal Transcribed Spacer (ITS) of rRNA has frequently been used to investigate phylogenetic relationships of *Usnea*. Due to the high evolution rate existing as size variation and sequence divergences in the ITS region, it became somewhat difficult to reliably construct alignments reflecting speciation relationships. Hence, some other primers should also be sequenced to get a clear picture.

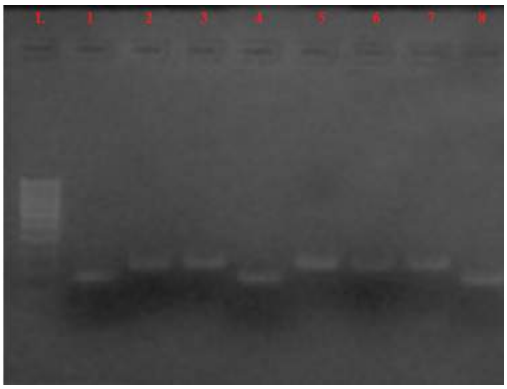

Figure 2: Gel Image Showing the DNA fragments obtained after the purification. L: 100 bp LADDER, Sample1-8.

The quantitative comparison among nucleotide sequences has recently become a useful tool to facilitate species identification.[19,20] However, several factors, such as variable evolution rates, divergent pseudogene copies, highly complex evolution and the ways to construct a phylogenetic tree may confirm the construction of evolutionary relationships. Like other phenotypic characters, rRNAs secondary structure has proposed a molecular marker for identification, classification and phylogeny of fungi.[21,22]

Previous molecular studies of *Usnea* mainly focused on the taxonomic status of some subgenera. The phylogenetic relationships among different species explored. The nucleotide sequence comparison used as the primary analysis method in these studies.[23,24] Here, our work concentrated on the application of morpho-molecular features to delineating phylogenetic groups and species determination. Species with close relationship share similar secondary structures, which indicate that the secondary structure of ITS2 is a helpful character in taxonomy and phylogeny of *Usnea*. The differences of the secondary structure appear relatively easy to be identified in contrast to nucleotide base comparison. Similar to morphological characters, the molecular structure is classified further to explore lichenological systematics.

We used ITS universal primers (ITS4 and ITS5) for the species delimitation, which is ideal for identifying species in lower taxonomic levels. Since lichens are also difficult to culture, the thallus' DNA is used directly for the PCR and sequencing studies. In the case of lichens, due to many organisms within the species, we got multiple bands of uncultured natural isolates in the PCR product. Although we purify the genetic materials using professional DNA purification Kits like Quegen Plant Mini-Kit, complete purification is not assured. In the gel electrophoresis, we got multiple bands indicating impurities or a mixture of the genetic materials. If we use these impure genetic materials for sequencing, there is a greater chance of failure of sequencing or mismatch of sequence. To overcome the sequence difficulties the clone-based sequencing was used. If we get more than one band and want to sequence them to check which one is the correct band, the best approach would be to clone-based sequencing technique.

MATERIALS AND METHODS

Sample collection and DNA isolation

For DNA isolation to identify the taxa and phylogenetic studies, the fruticose lichens were collected with more caution. Samples are collected from different areas in five replicates each. They were kept in plastic zip-lock covers and put in the icebox until bringing it to the lab. After returning to the laboratory, freshly

collected materials are washed in distilled water and stored in 1.5 mL Eppendorf tubes in a -20ºC deep freezer until using for DNA extraction. Qiagen DNeasy® Plant Mini Kit isolates the DNA from the foliicolous lichens following the standard protocol (Qiagen-2012). Purification of the DNA obtained is tested by the OD260:OD280 ratios (the OD ratio for pure DNA is 1.8±1). The OD260 value measures DNA concentration (approx. 1.0 reading at OD260 is equivalent to 50 µg/mL).

Gel Electrophoresis

Agarose gel (0.8 g) powder is boiled in 100 mL of 0.5X TBE buffer for 1-2 min. 0.5 µg/mL of ethidium is added to this agarose solution and allowed to cool. Coombes is adjusted to about 0.5-1.0 mm above the plate with a stand in a gel tray to form wells for loading the DNA samples. Agarose solution poured onto the plate and the gels tray is allowed to cool to about a temperature of 40º-50ºC and allowed to polymerise for about 10-15 min (when ready, turns whitish and opaque). Coombes was carefully removed once the gel polymerised. Then the gel is placed in the electrophoresis tank containing 0.5X TBE buffer. About 6 µL of the genomic DNA mixed with 6 µL sterilised water and 2 µL of 10X loading dye and loaded in the wells, against 10 µL of the DNA marker Lamda DNA HindIII-EcoR1 double-digit as a standard to compare the band intensities. The setup is then covered with a lid and connected to a power supply. The DNA is allowed to diffuse with gel and runs for 1-2 hr at a constant voltage (80 V). After the electrophoresis completed power supply is turned off, the gel is removed and visualised and archived using Gel Documentation System. The presence of highly resolved high molecular weight bands confirms the excellent quality of DNA.

PCR Amplification

The ~700 bp Internal Transcribed Spacer (ITS) region amplified using a high-fidelity Polymerase Chain Reaction (PCR) polymerase. PCR amplification was performed by using universal primers (nuITSrDNA: ITS4-5'-GAAACCTTGTT AYGMCTD-3'and ITS5-5'-GGAAGTAAAAGTCGTNASAAGR-3'). For the samples in which full length (~650 bp) PCR does not work, internal ITS primers spanning ~300 bp are used. The PCR amplicon was column purified and cloned into a cloning vector (T-vector). TA cloning is one of the simplest and most efficient methods for the cloning of PCR products. The procedure exploits the terminal transferase activity of specific thermophilic DNA polymerases, including *Thermusaquaticus* (Taq) polymerase. Taq polymerase c has non-template dependent activity, which preferentially adds single adenosine to the 3'-ends of a double-stranded DNA molecule. Thus, most of the molecule's PCR amplified by Taq. Polymerase possesses single 3'-A overhangs. Using a linearised "T-vector" with single 3'-T overhangs on both ends allows direct, high-efficiency cloning of PCR products, facilitated by the complementarity between the PCR product 3'-A overhangs and vector 3'-T overhangs. Eco-RI and Hind-III sites used as identifiable restriction sites.

Master-mix for PCR amplification: DNA: 1 µL (100 ng), forward primer: 400 ng, reverse primer: 400 ng, dNTPs (2.5 mM each) 4 µL, ×10 Taq DNA polymerase assay buffer 10 µL, Taq DNA polymerase Enzyme (3 U/µL) 1 µL, water X µL to make up the total reaction volume: 100 µL. All reagents were of chromous make.

The following PCR cycle times set for the different processes: Initial denaturation at 94ºC for 5 min, followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at 55ºC for 30 sec, extension at 72ºC for 45 sec and a final extension at 72ºC for 5 min. The PCR amplified product was subjected to 1.2% agarose gel (with ethidium bromide) electrophoresis for base-pair size analysis.

DNA Cloning

To clone the genetic material, we used a PCR product cloning kit. In this method, we add our PCR product to the vector and ligate it in (the exact procedure varies, but it's straightforward). Effectively we will have cloned all the different products produced by our PCR. Then we perform colony PCR of >5 colonies and identify those who have the correct size product. We can then grow up the plasmid, miniprep it and sequence. Using a host cell to carry the vector allows for easy amplification and retrieval of specific clones from the library for analysis. Cloning the amplicons on T-vectors or Gateway plasmids is easy. Then take five clones and extract plasmid DNA.

Sequencing

Cleaned DNA products sequenced in Chromous Biotech Pvt Ltd., Bengaluru. The same set of primers as for PCR (nu ITS rDNA: ITS4-5'-GAAACCTTGTTAYGMCTD-3'and ITS5-5'-GGAAGTAAAAGTCGTNASAAGR-3') were used for Sequencing. Sequencing the PCR amplified product was performed on ABI 3500×L Genetic Analyzer of Applied Biosystem Micro Amp, USA, using cycle sequencing kit and Big Dye Terminator Version 3.1. 10 µL of the sequencing analysis mixture contained 4 µL of Big Dye Terminator Ready Reaction Mix, 1 µL of PCR amplified product (100 ng/µL), 2 µL primer (10 pmol/λ) and 3 µLMilli-Q Water.

Analysis conditions for sequencing were programmed to-denaturation at 96ºC for 1 min, followed by 25 cycles of denaturation at 96ºC for 10 sec, hybridisation at 50ºC for 5 sec and elongation at 60ºC for 4 min. The resultant nucleotide sequence was analysed using the software Seq Scape version 5.2, which follows an analysis protocol of BDTv3-KB-Denovo_v 5.2.

Jukes-Cantor corrected distance model was used to generate a distance matrix. A minimum comparable position of 200 ignoring alignments inserts used. The phylogenetic tree was created

using Weighbor with alphabet size 4 and length 1000 using the sequences aligned with a system software aligner SeqScape_v 5.2.

Sequence fragments assembled in Sequencher 5.2 (Gene Codes Corp., MD, USA) software. Further, the final phylogenetic tree obtained by performing a heuristic search using maximum likelihood and Bayesian analysis. Strength of the tree branching analysed by 1000 bootstrap repeats. For the statistical analysis and tree building of the sequencing data, Mr Base v 3.1.2, BEAST v 1.7, Fig Tree v 1.3.1 and tree view v 1.6.

RESULTS AND DISCUSSION

Analysing sequence for identification of species

The DNA sequence analysis using universal lichen (fungal) primers, species-specific primers and comparative Gene Bank searches provide specific identification of a more significant number of *Fusarium* and *Aspergillus* species. However, this would require the use of appropriate DNA target regions that display sufficient interspecies sequence variation without it being excessive. The DNA target regions examined for such purposes. In addition, the variable parts at the 5' of the 28S rRNA gene (D1-D2 region) and the internal transcribed spacer regions 1 and 2 (ITS1 and ITS2) of rDNA have examined by other sequencing targets.[25-27] Once a sequence database is established, it has to be analysed further. As the first step, sequences compared with existing databanks (*e.g.*, Gene Bank, RDP and EMBL) perform a Basic Local Alignment Search Tool (BLAST) search in most cases. BLAST did online via the internet. As a result, it is possible to construe quickly whether the determined sequences need to align or not with other sequences. The public alignment package ClustalW produces good alignments from scratch, but other programs can be used as well.

A case study from *Usnea*

In this study, eight species of *Usnea* subjected to phylogenetic analysed. The DNA sequenced by both the conventional method and the clone-based method. Figure 2, we can see the photography of the gel image taken after running the purified DNA before sequencing. Thus, generated sequence data aligned. The aligned sequences used to create phylogenetic trees.

The cladograms for the eight species of *Usnea* created using conventional DNA sequencing data and clone-based sequence data. In both cladograms, the evolutionary history inferred using the Maximum Parsimony method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa.

Uses of Clone based sequencing

- They are rapid and require a tiny sample.
- Can be performed for environmental samples, i.e., without culturing.
- Meagre rate of sequence failure.
- Assurance of good quality sequence.
- It can be repeated easily.
- All the symbionts in the system (lichen) sequenced without any confusion or contamination.
- Detection of species is accurate and can get good BLAST result.

CONCLUSION

Although direct sequencing is much cheaper and more comfortable to carry out, comparatively, clone-based sequencing is the most efficient sequencing method. Next-gene sequencing also comes into the limelight, where the organisms' complete genomes are sequenced in the present days. In India, the next-gene sequencing is not yet completely into practice in all places and it is much costlier. Hence, clone-based sequencing is better than the blindfolded direct sequencing of organisms for the time being.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

DNA: Deoxyribonucleic acid; **rDNA:** Recombinant DNA; **LSU:** Large subunit; **SSU:** Small subunit; **ITS:** Internal Transcribed Spacer; **PCR:** Polymerase chain reaction; **RDP:** Ribosomal Database Project; **EMBL:** European Molecular Biology Laboratory; **BLAST:** Basic Local Alignment Search Tool.

SUMMARY

This study focuses on the Usnea genus, a large group within the Parmeliaceae family, known for its fruticose thallus and usnic acid presence. It examines the phylogenetic relationships and morphological variation across various Usnea species, particularly in Europe, assessing the reliability of traditional morphological characteristics used for species identification. The research highlights the challenges in extracting and sequencing DNA from these lichenized fungi due to their symbiotic nature with algae and susceptibility to contamination. A cloning approach

to sequencing was adopted to improve accuracy, involving the purification of multiple DNA bands from gel electrophoresis and subsequent cloning. This method, compared against traditional sequencing techniques, proves more effective in managing the complexities of lichen DNA and provides a robust framework for understanding the evolutionary and phylogenetic context of the Usnea genus, with findings aligned with global datasets.

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