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Bacterial Pathogenesis and Antibacterial Control

Edited by Sahra Kırmusaoğlu

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Notice

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Meet the editor

Dr. Ph.D. Kırmusaoğlu is an Assistant Professor of Microbiology at the Department of Molecular Biology and Genetics, T.C. Haliç University, where she had previously held an administrative position as Vice Head of the same department. She specialized in microbiology at Abant Izzet Baysal University (Biology Department), Turkey. Her previous experience includes the position of laboratory manager at microbiology laboratories in several research and private hospitals. Throughout her career, she collaborated with academicians/researchers from AIBU, METU and IU Cerrahpaşa Medical Faculty, and participated in various research projects. She also had worked as a lecturer in the Medical Laboratory Techniques Program held at the Department of Medical Services and Techniques, T.C. Istanbul Kavram Vocational Training School before transitioning to T.C. Haliç University as an Assistant Professor. Dr. Kırmusaoğlu's research interests include pathogenic bacterial biofilms, antibiofilm and antimicrobial agents,the effects and synergistic effects of new alternative agents such as chemical and natural bioactive compounds against pathogenic bacteria, such as antibiotic-resistant and biofilm-producing microorganisms. She is also interested in research on antibiofilm medical devices and food package design for preventing biofilm infections associated with indwelling devices that could lead to systemic infections, food-borne infections, and prolongation of food's shelf life. She has published several international research articles, book chapters, and congress proceedings. More specifically, Dr. Kırmusaoğlu participated as an author in books titled Microbial Biofilms: Importance and Applications and Staphylococcus aureus with her chapters "Staphylococcal Biofilms: Pathogenicity, Mechanism and Regulation of Biofilm Formation by Quorum Sensing System and Antibiotic Resistance Mechanisms of Biofilm Embedded Microorganisms" and "The Mechanism of Methicillin Resistance and the Influence of Methicillin Resistance on Biofilm Phenotype of Staphylococcus aureus." She has been also contributing a book project titled Disinfection as an editor. She is also the author and editor of the book "Genel Biyoloji Laboratuvar Kılavuzu" (General Biology Laboratory Manual) published by Hipokrat Publication.

Contents

Preface

Bacterial virulence factors that initiate bacterial infections lead to the development of symptoms of infection, whereas certain infections are asymptomatic. Pathogenic bacteria reveal their pathogenicity and cause infection by their virulence factors such as peptidoglycan of bacteria, lipopolysaccharides of Gram-negative bacteria, bacterial biofilms, surface proteins that lead bacterial adherence to host cells, toxins that lead bacteria to invade and damage host cells, enzymes, and antiphagocytic factors that cause bacteria to escape from the host immune system. Bacterial pathogens have been becoming the main problem in hospital and community-acquired infections. It is hard to treat the strains that are resistant to antibiotics, due to the causing recurrent and untreatable infections.

To prevent bacterial adherence, invasion and infection, certain new antibacterial chemicals, agents, and natural bioactive substances such as food and plant extracts as well as certain novel technologies have been used and are nowadays vastly researched. Detecting virulence factors, and determining the level of antibacterial activities of antibacterial agents as sole and as combinations, are very important for diagnostic, inhibition and prevention of bacterial infection. In recent years, the combination treatments and the novel technologies have been preferred to overcome the emergence of antibacterial resistance of pathogens.

This book contains 7 chapters from valued contributions from the world leading experts in France, Japan, Romania, Republic of Serbia, Slovakia, Pakistan and India. The goal of this book is to provide an up-to-date review of all topics mentioned. This book can be useful for researchers interested in antibacterials, bioactive compounds and novel technologies.

I would like to thank all the authors who are experts and contributed to this book with their valued chapters. This is very valuable for supporting this book. I would also like to thank Ms. Maja Bozicevic, Mrs. Nina Kalinic and Ms. Dajana Pemac who have helped me in this book project as publishing process managers and IntechOpen Publisher for their concern and encouragement in publishing this book.

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Section 1

Control by Antibiotics and Bioactive Antimicrobials

Chapter 1

Are Herbal Products an Alternative to Antibiotics?

Mihaela Ileana Ionescu

Additional information is available at the end of the chapter

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Abstract

Medicinal plants have been widely used in the management of infectious diseases and by now, many of the ancient remedies have proven their value through scientific methodologies. Although the mechanisms underlying most plant-derived remedies are not well understood, the success of herbal medicine in curing infectious diseases shows that many plants have beneficial effects in various bacterial, fungal, viral or parasitic infections. The modern methodologies in the isolation, purification and characterization of the active compounds, has been a great impact for advancing *in vitro* and *in vivo* research, this step being crucial for further application in clinical trials. Many plant-derived compounds, for example, quinine and artemisinin, have been already successfully used in healing life-threatening infectious disease. The main limitations of plant medicine healing are lack of standardization and reproducibility of plant-derived products. Despite the paucity of clinical trials evaluating their efficacy, phytotherapy, adult plant uses and gemmotherapy, the use of embryonic stem cells should be reconsidered as valuable resources in finding new active compounds with sustained antimicrobial activity.

Keywords: phytotherapy, gemmotherapy, infection, herbal medicine, medicinal plants

1. Introduction

Traditional medicine used for a long time various medicinal plants for infectious diseases healing [1]. Ancient healers often combine medicinal plants with mysterious incantations, recipes being inherited together with the secrets of their employment. On traditional healing practices, the knowledge were orally communicated along with sophisticated protocols about harvesting plants, methods of preparation, applications, dosage, special diets and associations with other secret stories about the nature of illness. This chapter highlights some data on the use of herbal products in the treatment or prevention of infectious diseases. Our knowledge of the role of herbal products in the treatment of infectious diseases has not increased so rapidly

as studies on the discovery of new antibiotics, but the emergence of multi-drug resistant strains requires new therapeutic strategies. Analyzing the evolution of antibiotic resistance has proven challenging due to the countless of factors involved—the genetic adaptation of microorganisms depends not only on exposure to antibiotics [2, 3]. Nowadays, we often find ourselves in a deadlock in choosing effective anti-infective treatment for nosocomial infections [4]. For sure, bacteria are continuously adapted their defense mechanisms, and any new synthetic antibacterial compounds will be sooner or later ineffective. There are some notable exceptions, mentioning only the high activity of penicillin G against *Streptococcus pyogenes.*

Since ancient times, people have faced with infections, ranged from simple urinary tract infections to major epidemics. Obviously, they used their healing the most diverse substances, which included herbs or other natural products found nearby [5]. For instance, a recent paper demonstrates that a well-known natural product—honey has *in vitro* antibacterial activity against *Staphylococcus aureus* and *Streptococcus pyogenes* [6]. Also, many drugs have been discovered from ethnobotanical leads. The most well-known analgesic and anti-inflammatory drug—aspirin—was developed from the plant *Filipendula ulmaria* (queen of the meadow) [7].

The purpose of the paper is not to develop the history of empirical antimicrobial therapy, but only to recover the valuable results of ancient healing prescriptions that have led to current remedies. The origin of the particular history of healing with plants is lost in the dawn of mankind's history. People have always tried to understand and heal the diseases, just as they have tried to understand the human-nature relationship. We could say that they approached illness in a philosophical manner, seeking the causes and remedies in everything that meant life. In the past, people were more attentive, more patient and time was redeemed differently. They were ardent observers of the environments and were able to make amazing connections between the smallest details. How ancient people, without any medical device or epidemiological resources, masterminded the therapy strategies is beyond our understanding. A first observation of the ancient way of life is that they traveled far less than today—with some notable exceptions of ocean voyagers—and the commodity exchanges were not so extended, therefore they had had to use the local products to prepare their healing recipes. Undoubtedly plants, almost always wild plants, and animal products were widely used with more or less efficiency. For many of them, outstanding studies demonstrated their scientific value.

Many health care professionals draw a distinct border between allopathic medicine and complementary and alternative medicine, claiming the ascendance of reductionist approaches or, contrary, the naturalistic approaches. For some time, there is a more balanced point of views in identifying favorable aspects of both ways and taking advantages in health care provision [8]. Accurate studies about the efficacy complementary and alternative medicine methods of are scarce until now, but the positive perception of patients toward this way of healing, even for pediatric patients, will, inevitably, force us to analyze more carefully this field [9].

This chapter does not discuss the ascendancy of the various branches of herbal medicines against allopathic medicinal products, it only highlights herbal remedies with proven antimicrobial efficacy [10]. The chapter is not intended to be encyclopedic, but a synthesis of commercial plant products proven by substantial evidence as having biological activity in human infectious diseases. Further, research articles on plant extracts with antimicrobial activity have

been reviewed to find evidence that supports plants value in infectious diseases. Since this chapter is dedicated to health care professionals, in the following sections, the plant products are systematized according to their clinical conditions, without intending to cover all herbs and all infectious diseases. Phytotherapy and chemotherapy applications in infectious diseases will be described separately as there are differences in the healing process underlying these methods.

2. Phytotherapy

2.1. Overview

It can be said that phytotherapy is one, if not the oldest method, used in the treatment of infectious diseases. Many countries have a long tradition in herbal medicine [11–14]. People in countries where traditional medicine is widespread are more prone to continuing these practices [15, 16]. Nowadays, traditional healers are trying to treat contemporary infectious diseases (e.g., HIV) with herbs without any evidence of their efficacy or toxicity [17]. Moreover, certain associations of antibiotic plants are antagonistic, therefore the failure of antibiotic treatment may be the result of the addition of an apparently harmless plant product such as rose and pomegranate flowers [15]. Thorough out the world, there are many lost legends about traditional medicine, people lifestyle being intertwined with the plants cycle of life, but the subject is far more complex to be developed in the present chapter [7].

Phytotherapy uses the entire plant or parts of plants prepared by various methods. Presently, there are a wide range of plant products—oils, tinctures, plant extracts, mother tincture (TM), essential oils, powders, suppositories, syrups, inhalants and all sort of mixtures. There are studies that not only demonstrate the antibacterial effect of some plants, but also identify the possible target of action. *Juglans regia* (walnut) in combination with *Camellia sinensis* (tea plant, tea shrub) acts synergistically to inhibit multiple-resistance bacteria (MDR), most likely targeting the bacterial cell wall [18].

2.2. Phytotherapy in bacterial infections

Oral hygiene. Today there are thousands of commercial herbal mouthwash and dentifrices. People prefer natural products for oral hygiene, and dentists also agree to use effective alternatives to prophylactic products containing chlorhexidine or sodium fluoride [19]. Due to the interest of dental professionals, many clinical trials have been performed with promising results. Whereas washing with water and neutral soap proved to be the most efficient protocol against *Candida albicans* and *Staphylococcus aureus* biofilms on the maxillofacial silicone polymer, *Cymbopogon nardus* (citronella grass) and *Hydrastis canadensis* (goldenseal) are also efficaces [20]. *Ribes nigrum* (blackcurrant) and *Hippophae rhamnoides* (sea buckthorns) juices may reduce the risks for both tooth decay and gingivitis, by inhibition of bacterial grow [21] and by their anti-inflammatory effect [22]. The assessment of a paste containing *Myrtus communis* (myrtle) in the treatment of recurrent aphthous stomatitis, an idiopathic oral ailment, gave positive results [23].

Respiratory tract infections. Essential oils are widely used in respiratory illnesses. Although there are attempts to accurately characterize active principles, the results of specific identification of a single antibacterial compound are not promising. Among well-known plants with therapeutic potential in healing or prevention bacterial respiratory infections are *Urena lobata* (Caesarweed) [24], *Rosmarinus officinalis* (rosemary), *Thymus vulgaris* (thyme) or *Primula veris* (primula root) [10]. The essential oils mixtures—*Cinnamomum zeylanicum* (cinnamon), *Daucus carota* (wild carrot), *Eucalyptus globulus* (eucalyptus) and *Rosmarinus officinalis* (rosemary)—are also effective in treating influenza infection and bacterial complication of influenza [25].

The **urinary tract infections** are frequently encountered especially in woman, and many plants formulations have been used by centuries with both antibacterial and anti-inflammatory potential—*Astragalus membranaceus* (Mongolian milkvetch) root, *Sophora flavescens* (shrubby sophora), *Lindera aggregata* (lindera) root, *Oldenlandia diffusa* (snake-needle grass), *Phellodendron amurense* (Amur cork tree), *Desmodium styracifolium* (Guang Jing Qian Cao) [26]. Aqueous extract of *Calluna vulgaris* (common heather) and *Vaccinium vitis-idaea* (lingonberry, cowberry or bearberry) extract directly inhibit most uropathogenic strains or prevents biofilm formation [27, 28].

Cutaneous infections. Cosmetology widely uses herbs in various forms to treat other skin infections difficult to treat [29]. Treatment for acne—a chronic condition which deteriorates the quality of life—is discouraging, relapses and duration of treatment often lead both patients and doctors to use herbal medicines. Topical treatment with various plants or plant-drugs formulations is extensively used. Their antimicrobial efficacy is supported by numerous *in vitro* and *in vivo* studies showing that certain plant and essential oil extracts inhibit bacterial species encountered in cutaneous infections [30].

Digestive infections. Undoubtedly, the main plant species recommended in digestive infections is the *Vaccinium myrtillus* (bilberry) [31]. Gastritis due to *Helicobacter pylor*i infections could be treated with *Punica granatum* (pomegranate) [32]. A clinical trial shows the efficacy of traditional herbs used in Chinese medicine, Jiechang mixture, in the treatment of infantile mycosis enteritis [33].

2.3. Phytotherapy in viral infections

Most herbal formulations with viral infection benefits are dedicated to respiratory diseases. Traditional Chinese herbal medicine (TCHM) formulas are by far the most used and play an important role in virus infectious diseases like **respiratory syncytial virus infections** [34]. TCHM formulas contain a long list of plants and its underlying mechanism is not yet elucidated. Do not forget that traditional Chinese medicine, due to its philosophical approach, is more profound and more sophisticated than the simple use of some curative formulations [35]. *Echinacea angustifolia* (narrow leaf echinacea), a versatile immunomodulator, is probably the best choice for **common cold** and prevention of **influenza** complications [36]. Essential oils, referred to bacterial infections, administered separately or in different mixtures, are also beneficial in preventing influenza complications. Infectious herpes simplex is other recurrent viral infections and the emergence of acyclovir-resistant clinical isolates

has made the treatment more difficult. Birch bark efficacy has been shown to inhibit the acyclovir-sensitive and acyclovir-resistant clinical isolates of **herpes simplex virus type 1** (HSV-1) in the early phase of infection [37].

2.4. Phytotherapy in parasitosis

Many parasitic infections are of major public health concerns, but the helminth infections are one of the most common infections [38]. The effectiveness of human parasitosis treatment is diminished by the resistance to parasite medication. Also, some parasitic diseases require high doses for long terms of drugs with substantial adverse effects. Therefore, it is not surprising that researchers are interested in healing parasitic infections with plant remedies with proven effectiveness [39]. Traditional herbal healing of parasitosis rely on well know plants like garlic, ramson or pumpkin seeds, but interesting studies show that innovative technologies can bring new insights into treating parasitic diseases [40, 41].

Allium ursinum TM (ramsons) stimulate the digestion and help at eliminating the **intestinal worms**. Alternatively, in pediatrics, suppositories may be recommended. *Nigella sativa L.* seeds (black cumin, fennel-flower and negrilla) has been used in the treatment of quite different clinical conditions—helminths infections, epilepsy [42] or preventing oral malodor [43]. Promising results were obtained in helminths infections—a new formula of highly antiparasitic compound silver doped titanium dioxide nanoparticles (TiAgNps) and *Nigella sativa* L. essential oil is very efficient on **cutaneous Leishmaniasis** [40]. A recent *in vitro* study shows that a nano emulsion of *Zataria multiflora* (Shirazi thyme) essential oil determines the decrease in the size, number and weight of **hydatid** (*Echinococcus granulosus*) cysts [41]. This species of thyme is also effective against the protozoan *Trichomonas vaginalis* [44]. *Artemisia annua* L. (sweet wormwood) may prolong survival of animals experimentally infected with *Acanthamoeba* sp., therefore can be used for general and local treatment of **acanthamoebiasis**, or in combined therapy with antibiotics [45]. **Malaria** has always been a challenge for health practitioners, even today, when attempts to develop a commercially available vaccine are already under way in clinical trials [46]. Nowadays, the main antimalarials originated from plants (quinin, artemisinin and newly lupane triterpenes) [47–49]. Since phytotherapy has a holistic approach of illnesses, the combination of antimalarials with curcurma oil has positive effects [50].

Many producers have developed several formulations, more simple to administrate especially, for children or elderly persons. In Romania, there are an antiparasitic mixture *Vermicin* (www.fares.ro) which consists of *Olea europaea* L. folium (olive leaf), *Thymus vulgaris* (thyme, thymi herba), dry extract of rhizome of *Hydrastis canadensis* (goldenseal), berberine sulfate (alkaloid derived from *Berberis aristata*) [51], *Inula helenium* L (Inulae radix) [52] and flower bud of *Eugenia caryophyllata* (Caryophylli flos). It is not recommended for pregnant women due to possible effects of stimulation of uterine contractions or women who are breastfeeding because active substances pass into breast milk and can alter the taste of milk. A formulation recommended by Ovidiu Bojor (www.plantextrakt.ro) is *Giardinophyt* and contains extracts of *Thymus vulgaris* (thyme, thymi herba), *Chrysanthemum vulgare* (tansy), seeds of *Anethum graveolens* (dill), *Eugenia caryophyllata* (cloves) and propolis cera. The producer claims that parasite removal is favored, along with other beneficial effects on the digestive system. Seven-day treatments are recommended to prevent relapses.

3. Gemmotherapy

3.1. Overview

Gemmotherapy is part of complementary and alternative medicine, being a form of herbal therapy which uses embryonic stem cell tissues of plants—inflorescences, buds, bark of the stalk, young branches of the spring plants, young roots, acorns, sap, seeds or bark of roots. Gemmotherapy, the youngest branch of herbal therapy, was developed by a group of homeopaths—Henry Pol, Max Tétau and O.A. Julian—and is characterized by a different approach to illness, health and life in general. Although this chapter is not dedicated to gemmotherapy, we can not ignore its fundamental principles. The most important intriguing concept is that then prior to any treatment, it is essential to understand that symptoms are considered an expression of energetic status of a certain organ. Therefore, the efforts are directed to restoring the dynamic balance of the affected organs rather than simply treating the pathological symptoms; in other words—a holistic approach to human being and disease.

Principle of preparation of the extracts used in gemmotherapy. The procedure involves two steps—the fresh harvested plants are first treated with alcohol, then a hydro-glycerin solution is added. In the first step, there is inhibition of enzymatic activity of the plant followed by further extraction and stabilization of active principles. The extracts are stored as concentrated glycerin macerate 1DH (dilution 1:10) in glass brown bottles at room temperature. The warranty period is 5 years [53].

Mechanism of action of the extracts used in gemmotherapy. The principles of gemmotherapy derived from the drainage concepts from homeopathy; for the very first time it was named in France as "drainage therapy". The main action of the extracts used in gemmotherapy is the rebalancing of cellular and tissue homeostasis, targeting the reticulohistiocytosis tissue. The most important characteristic of these products is their holistic activity, separated molecule do not have the same activity like the whole extract. Until now, we could not separate a certain molecule in order to accurately characterize its activity due to coordinated action of the active principles. The comprehensive studies about precise mechanism of action of extracts used in gemmotherapy are very rare, therefore, in my opinion, at this moment clinical observation is the most relevant way of validate gemmotherapy.

Treatment regimen in gemmotherapy. There is not a standardized therapeutic regimen in gemmotherapy, but the glycerine macerate 1DH are usually administrated 40–50 drops/1–2 times/day diluted in 100 ml water. Fernando Piterà explained that this water dilution not only releases active principles, but triggers crucial electronic exchanges between the molecules. If a complementary regimen is recommended, 50–70 drops/once a day are recommended. The association of different glycerine macerate 1DH are often proposed. Usually the treatment lasts for a month and can be repeated after at least 2 weeks. Some authors do not recommend the mixtures and administration of more than one extracts at once, although therapeutic regimens often includes more then one extract. The main argument for avoiding mixtures is that of avoiding reactions between different active compounds that can cause inactivation or even the formation of toxic compounds.

Since the scientific literature dedicated to gemmotherapy is so scant, recommendations on the application of gemmotherapy products to infectious diseases—bacterial infections, viral infections or parasitosis—are selected from the Fernando Piterà's *Compendio di gemmoterapia clinica (Meristemoterapia) con indice clinico,* 3rd edition, Genova [53]. Inflammatory response is a well-known feature of many infectious diseases, so most of the gemmotherapy regimens include *Ribes nigrum* (black currant) that reduces inflammation and stimulates and supports the adrenal glands. Also, *Juglans regia* (walnut) is considered to be the major stem cell plant-derived "antibiotic". Note that embryonic plant cell stem tissues are unique in structure and composition therefore their properties and clinical applications differ from the adult plant.

3.2. Gemmotherapy in bacterial infections

Oral hygiene. The aphthous stomatitis and gingivitis—a common and mild form of periodontal disease—are diseases commonly encountered characterized by frequent recurrences and the lack of specific treatment. Gemmotherapy may have a real benefit in preventing relapses of the oral ulcers or in gingivitis by admiration of *Alnus glutinosa* (alder) associated with *Vaccinium myrtillus* (bilberry), *Juglans regia* (walnut), *Ligustrum vulgare* (privet) and *Ulmus campestris* (elm). In paradontosis, *Abies pectinata* (silver fir), *Betula pubescens* (downy birch) or *Quercus pedunculata* (oak) could be prescribed.

Respiratory tract infections. Fernando Piterà claims that in recurrent tonsillitis and group A streptococcal pharyngitis *Vitis vinifera* (common grape vine) associated with *Juglans regia* (walnut), *Ampelopsis veitichii* (peppervine), *Ribes nigrum* (black currant) and *Rosa canina* (dog rose) can reduce the level of streptolysins and prevent the complications of rheumatic fever. In tuberculosis the dynamics of immune response greatly influence the clearance and dissemination of *Mycobacterium tuberculosis*, the heterogeneity of the disease being closely related to underlying immune dysfunctions [54]. Tuberculosis convalescence is a critical period for healing the illnesses, and gemmotherapy may help in this critical period by *Prunus spinosa* (blackthorn)—an excellent tonic which greatly stimulate the immune response—in combination with *Juglans regia* (walnut) and *Rosa canina* (dog rose).

In **urinary tract infections**, the most important gemmotherapy products according to Fernando Piterà are *Calluna vulgaris* (common heather) and *Vaccinium vitis-idaea* (lingonberry, cowberry or bearberry). *Calluna vulgaris* has diuretic and disinfectant action on the urinary tract. The young branches of the spring plant of *Vaccinium vitis-idaea* have antiseptic, antimicrobial and anti-inflammatory effect on urinary tract. In acute cystitis, Fernando Piterà recommends *Calluna vulgaris* (common heather), *Vaccinium vitis-idaea* (lingonberry, cowberry or bearberry) and *Vaccinium myrtillus* (bilberry); in recurrent cystitis—*Calluna vulgaris, Vaccinium vitis-idaea* and *Juniperus*.

Genital infections. *Buxus sempervirens* (common box) is a small, always green and toxic shrub because of cyclobuxine. The glycerine macerate 1DH of young branches of *Buxus sempervirens* (common boxwood), harvested in vegetative stage, even have not a specific prescription, it appears to cure the syphilis sequelae. Vaginosis has several causes, but very often is associated to vaginal microbiota imbalance [55]. *Rubus idaeus* (raspberry) was already identified as

targeting the hypothalamic-pituitary-gonadal axis and along with *Calluna vulgaris* (common heather) and *Juglans regia* (walnut) have a beneficial impact on reestablishing the equilibrium of vaginal microbiota [56].

Digestive infections. *Vaccinium myrtillus* (bilberry) is strongly recommended in enteritis, diarrhea, dysentery and membranous colitis. *Juglans regia* walnut) and *Vaccinium vitis-idaea* (lingonberry, cowberry or bearberry) could be associated.

Cutaneous-mucosal infections. *Juglans regia* (walnut) is recommended in many cutaneous illnesses like acne, dermatoses, mycosis, tinea, blepharitis. There are several notable associations of *Juglans regia* in complicated infections—in pustular acne, furunculosis complicated with staphylococcal and streptococcal infection with *Ulmus campestris* (elm); in infected wounds with *Buxus sempervirens* L. (boxwood). *Juglans regia* is helpful in the treatment of superficial forms of panaritium along with *Ficus carica* (common fig). In oral candidiasis, the combination of the following three product—*Buxus sempervirens* L. (boxwood), *Ligustrum vulgare* (common privet) and *Rubus fructicosus* (blackberry)—is the best choice.

3.3. Gemmotherapy in viral infections

Ribes nigrum (black currant) is indispensable for the treatment of **viral respiratory infections** and may be associated with *Abies pectinata* (silver fir) and *Rosa canina* (dog rose) in the common cold and with *Alnus glutinosa* (alder), *Betula pubescens* (downy birch), *Lonicera nigra* (blackberried honeysuckle) or *Salix alba* (white willow) in the flu, the last one if hyperpyrexia and arthralgia accompanies the illness.

Juglans regia (walnut) with *Acer camestre* (field maple), *Ulmus campestris* (elm) and *Rosa canina* (dog rose) prevent relapses of **herpes simplex**. Also, *Acer camestre* may be combined with *Prunus spinosa* (blackthorn) in recurrent **herpes simplex keratitis**.

In Epstein-Barr virus infectious **mononucleosis**, one of the common viral infectious of young people around the world, gemmotherapy offers quite a long list of products: *Alnus glutinosa* (alder), *Betula pubescens* (downy birch), *Juglans regia* (walnut), *Juniperus communis* (common juniper), *Ribes nigrum* (black currant), *Tamarix gallica* (French tamarisk) and *Vitis vinifera* (common grape vine).

The *Radical Secale* (rye) root has a selective tropism for hepatic parenchyma, being recognized for its hepatocyte regenerative capacity. It is used in **acute viral hepatites** in combination with *Rosmarinus officinalis* (rosemary), *Berberis vulgaris* (barberry), *Corylus avellana* (hazel), *Lonicera nigra* (blackberried honeysuckle) or *Zea mays* (corn).

Radical Secale (rye) root, *Rosmarinus officinalis* (rosemary), *Berberis vulgaris* (barberry), *Corylus avellana* (hazel), *Juniperus communis* (common juniper), *Ribes nigrum* (black currant) and *Rosmarinus officinalis* (rosemary) are used in **chronic hepatites**. Note that *Rosmarinus officinalis* has a specific tropism for the liver, cholecyst, biliary tract, adrenal glands and gonads, which contributes to the liver regeneration.

The treatment of **warts**, common viral infections caused by the human papilloma virus, is discouraging for patients and physicians too, but gemmotherapy offers some alternatives:

Buxus sempervirens L. (boxwood), *Ficus carica* (fig), *Juglans regia* (walnut), *Rosa canina* (dog rose), *Taxus baccata* (yew), *Thuja orientalis* (Chinese thuja) and *Vitis vinifera* (common grape vine).

3.4. Gemmotherapy in parasitosis

The *Buxus sempervirens* (common boxwood), also used phytotherapy, in combination with *Alnus incana* (gray alder) and *Juglans regia* (walnut) may be an effective therapy in parasitoses.

4. Active compounds from medicinal plants

The attempts to understand the antimicrobial effect of medicinal plants and precise mechanisms of action are delayed by two major obstacles. First, the antimicrobial effect is the result of the combined action of several factors, some of which do not have direct antimicrobial action but plays an essential role in achieving the therapeutic effect. Second, for the vast majority of medicinal plants, aside of antimicrobial effect, the active compounds have additional effects—for example, anti-inflammatory effect. In particular, gemmotherapy has stated that all active principles in embryonic cell stem tissues act in a coordinated manner, so attempting to divide the whole effect is useless. Even so, identifying of therapeutically active ingredients in order to obtain a pure compound is mandatory for further experimental tests and clinical trials [57].

Tracing connections between the active compounds and their degradation products is an issue more challenging than isolating a pure compound and to determine the structure of the pure substance. Nowadays, there exist extraction methodologies and accurate protocols for analyzing the physico-chemical features of pure substances, but considering all possible interconnections is much more complicated than that. Healing is the result of an entire network of substances, therefore very often the mechanism of action of plant products is not well-understood. What is really necessary is the feasible protocols to bioassay the reaction chain between the active compounds. The advance in recognizing the therapeutic effect of plant products begins with the precise characterization of the active compounds. Progresses made in nanotechnologies are encouraging in the direction of developing more effective, more stable and manageable plant-derived formulations. In the near future, the nanoparticles may find an immediate application in controlling side-effects, for example, toxicity, of some drugs used for the treatment of infectious diseases [40, 50, 58–60].

Herein some of plant active compounds are quoted.

1,8-cineole (ethyl-dimethyl-(3-sulfopropyl)azanium) ($C_7H_{18}NO_3S^+$) (PubChem CID 448830) is found in many essential oils, for example, *Eucalyptus globulus* (eucalyptus) oil, *Melaleuca alternifolia* (tea tree) or *Rosmarinus officinalis* (rosemary). It is one of the main active compound, which explains the antimicrobial effectiveness of essential oils [61].

Anthocyanins (2-phenylchromenylium) (Cyanidin: $C_{15}H_{11}O_6Cl$; Peonidin: $C_{16}H_{13}O_6Cl$; Malvidin: $C_{17}H_{15}O_7Cl$; Delphinidin: $C_{15}H_{11}O_7Cl$; Petunidin: $C_{16}H_{13}O_7Cl$; Pelargonidin: $C_{15}H_{11}O_5Cl$ or $C_{15}H_{11}O^+$) (PubChem CID 448830) are found in *Vaccinium myrtillus* (bilberry). A recent study about intestinal accessibility and bioavailability show that the colon is a significant site for anthocyanins and their degradation products [62].

Arbutin ((2R,3S,4S,5R,6S)-2-(hydroxymethyl)-6-(4-hydroxyphenoxy)oxane-3,4,5-triol) ($C_{12}H_{16}O_7$) (PubChem CID 440936) is found in plants from the families *Ericaceae, Asteraceae* and *Rosaceae*. It explains the antiseptic properties, but its relation with microorganisms are not enough studied, notably the mutagenic effect of its metabolites hydroquinone [63].

Allicin (3-prop-2-enylsulfinylsulfanylprop-1-ene) ($C_6H_{10}OS_2$) (PubChem CID 65036) found in family *Alliaceae*, has a broad spectrum antimicrobial and immunomodulatory activity, along with many other beneficial effects for human health [64, 65].

Artemisinin ($C_{15}H_{22}O_5$) (PubChem CID 68826) is one of the most celebre semi-synthetic plant-derived compound used in infectious diseases. The discovery of artemisinin, the active compound of *Artemisia annua* (sweet wormwood) has brought new hope for medical community [47, 48, 66]. The emergence of artemisinin-resistant *Plasmodium falciparum* explains the critical need to identify new antimalarials [67]. Hope comes again from herbal compounds—a recent paper describe **lupane triterpenes**, originated from *Buxus sempervirens* (common boxwood), as being the next antimalarials [49].

Benzoic acid ($C_7H_6O_2$) (PubChem CID 243), found in the fruits of *Vaccinium vitis-idaea* (lingonberry, cowberry or bearberry), has antiseptic effect which explains the preservation of jam.

Curcumin ((1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione) ($C_{21}H_{20}O_6$) (PubChem CID 969516), an active compound extracted from aromatic *Curcuma aromatica* (wild turmeric), is not only a powerful antioxidant, anti-inflammatory and anticancer ingredient but also has antiparasitic activity, *in vitro* studies showed antimalarial, antileishmanial and antitrypanosomal activity [68, 69].

Essential oils are ethereal oils consisting of complex mixture of many volatile compounds. Even if research on their mechanism of action is lacking, their antimicrobial efficacy is demonstrated by the results of their use in infectious diseases [70].

Quinine ((R)-[(2S,4S,5R)-5-ethenyl-1-azabicyclo[2.2.2]octan-2-yl]-(6-methoxyquinolin-4-yl) methanol)($C_{20}H_{24}O_2N_2$ or $C_{20}H_{24}N_2O_2$) (PubChem CID 3034034) originates from *Cinchona*. It was for decades the main cure in malaria, other active compounds of plant origin demonstrated their value.

Resveratrol (5-[(E)-2-(4-hydroxyphenyl)ethenyl]benzene-1,3-diol)($C_{14}H_{12}O_3$)(PubChem CID 445154) is best known for its antioxidant properties, but also has antimicrobial activity [71].

Thymoquinone (2-methyl-5-propan-2-ylcyclohexa-2,5-diene-1,4-dione)($C_{10}H_{12}O_2$) (PubChem CID 1028) is the main active compound of *Nigella sativa L.* seeds essential oil. It has not only an anti-inflammatory effect [72], but it is a versatile immunomodulator, novel mechanisms being recently discovered [73]. The role in activation cellular immunity, by stimulating CD4+ T lymphocytes and production of γ-interferon, explain the positive results of *Nigella sativa L.* seeds essential oil in viral infections [74].

5. Limitations of phytotherapy and gemmotherapy

The main limitation of phytotherapy and gemmotherapy, as well for any traditional healing treatment, is the lack of *standardization* of the treatment. This is one of the reasons for

the low credibility regarding the efficacy of medicinal plants, so most doctors do not even consider herbs as an alternative to antibiotics. Basically, they are right, does anyone have the courage to treat their own severe pneumonia only with thyme essential oil? On the other hand, the antibiotics overuse is a notable factor in emergence of MDR bacterial isolates. Very often patients claimed antibiotics even for uncomplicated common cold. Luckily, empirically antibiotics use is restricted by general health laws. Recently many studies have focused on the identification and isolation biologically active compounds, and this is the first step in the process of developing new drugs with clear mechanism of action and a standardize employment. Finding more about the pharmacology of plant-derived active compounds will surely lead to the standardization of the therapeutic regimens [33]. Health care professionals are accustomed to precise therapeutic protocols, and are more confident in prescribing certified medicines than to use a plant extract of uncertain composition. We must recognize that in the field of phytotherapy or gemmotherapy, prescribe a concise therapeutic regimen for a specific infection, adapted to the pathology of each patient, requires a well-trained professionals.

Another important limitation is the *reproducibility* of the composition of plant products—the same product may have different properties depending on the suppliers. Researchers are interested in analytical characterization and authentication of active compounds in order to establish quality control procedures. Once again, the step is a real challenge because of the various factors that need to be taken into account. DNA barcoding and metabarcoding are proposed for authentication single plant ingredient, and mixed plants products, respectively [75]. Poor characterization of plant products is the main reason for different clinical trial results. A plant product credibility started with a certified manufacturing method—International Food Standard, Community herbal monograph, German Homeopathic Pharmacopeia or other international recognized quality standards [76]. The major obstacle in the manufacturing plant products is the natural genetic variation of plant species. A product from the same supplier, obtained by the certified manufacturing method, could be different because of the origin of the raw materials. Plant quality depends very much on environmental conditions, pollution being an important variable.

Third, the *availability* of quality plant species, in particular exotic plants, is limited to a particular geographical area. Only ancient people relied solely on wild plants for healing, hence the interest in massive cultivation of medicinal plants. Concomitantly, there are other variables ranging from plant species to environmental conditions, that significantly influence the availability of quality medicinal plants [4, 77, 78]. At present, environmental pollution is a problem that seems to grow continuously, and it also seems hopeless to find a viable solution. Even if we can not change environmental conditions, we need to be aware that the place where plants grow is the first thing that affects the quality of a final product.

Finally, there are no extensive clinical trials evaluating the *effectiveness* of phytotherapy and gemmotherapy for infectious diseases and the determination of *adverse effects* [79]. Assessing the antimicrobial efficacy of plant products requires standardized tests and guidelines to compare experimental data. As for antibiotic and antifungals, we have standards provided by the Clinical and Laboratory Standards Institute (CLSI) [80] and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (www.eucast.org), we lack consistent procedures to test plant products. Pure active compounds could easily be tested for minimal inhibitory concentration (MIC), but different methods are needed to test plant extracts or essential oils [81].

6. Discussions

To answer at the question of effectiveness of herbal products in infectious diseases, clear evidence should be stated before any other debate. Undeniably, some plant species contain antimicrobial substances, elaborated for their own defense against infections. Certainly, plant pathogens and human pathogens are not the same, and plant defense mechanisms are quite different from human defense mechanisms. But these differences should not be a reason to deny the efficacy of plants in certain infectious diseases. Conversely, the demonstrated antimicrobial effects of certain plants should not overestimate their capability and discontinue the therapeutic regimens of conventional drugs. However, one of the latest scientific certification of plant usefulness in infectious diseases is the discovery of artemisinin, which saved millions of lives, by Youyou Tu, a pharmacologist at the Chinese Academy of Chinese Medical Sciences in Beijing, who received the Nobel Prize in 2015 [47, 48, 66].

With the abundance of all range of over-the-counter herbal products, more or less expensive, it looks like it is not difficult to break into natural products market. People are more prone to try something accessible, cheaper and presented as without side effects. By adding a professional advertise, the suppliers' success is guaranteed. Where does this confidence in herbal products come from? We can only guess that the first reason is we use plants in daily life in the form of tea, spices or simply as soft drinks—lemonade, mint water and so on. Hot tea with lemon and honey is almost always indispensable in the treatment of the flu, so we can think that there is nothing wrong with the administration of herbal products such as, for example, essential thyme oil. Nothing wrong at first sight, but should not forget the main problem of the therapy with herbal products—standardization. We are talking not only about dose standardization but also especially about the standardization of the therapeutic regimen when two or more herbal products are recommended. It is noteworthy that mixed herbal products are presented as a simpler alternative when several plants are included in therapeutical regimen. Any reasonable person should admit that we cannot blend any kind of substances without alter the effect of each component. An herbal product is already a mixture and its effect is the result of the subtle interactions of its molecules, which is why random mixing of several plants can even lead to the cancelation of the therapeutic effect. Due to the lack of accurate studies on plant interactions, at this time we cannot understand the complexity of the network formed in a recipient which contain more than one species of plant. Most plant products has not been evaluated by the Food and Drug Administration (FDA) and the lack of clear-cut studies on the safety of certain herbs, for example, echinacea and elderberry, suggest that certain risk groups, such as pregnant women, should not be treated with them [82]. Very often, natural products are considered superpower remedies, but recent studies have shown that they should be used with caution in the absence of accurate evidence of their effectiveness [83]. However, there are certain clinical situations when plant-derived products can have a real benefit—in infections characterized by frequent recurrences such as simplex hepatic infections or when immune modulation is important for complete recovery, for example, flu or tuberculosis. In pediatrics or in geriatric, parasitic infections are one of the most common infections, especially in nurseries or day care centers. Considering that relapses are quite frequency in such care centers, plants could be of real benefits in cure and prevention. We are now able to study herbal products by analytical methods that separate into individual components, but in my opinion the physico-chemical analysis of all plant components cannot answer the main question: we can

ever synthesize antimicrobial substances that have similar effects to their natural counterparts. It is like trying to listen to a symphony by isolating the sounds of each instrument. Fernando Piterà argues that this approach neglects that the effects of each natural component depend fundamentally on the entire plant. He describes a plant as a "phytocomplex"—a biological unit with internal dynamics. In fact, this is the doctrine of gemmotherapy. One may say that conventional research methods are not suitable in clinical trials of phytotherapy or gemmotherapy. Experienced doctors in complementary medicine argue that it is not impossible to find research methods that meet the rigor of conventional medicine [84].

It is not exaggerate to remember here the title of a famous etching of Goya "El sueño de la razón produce monstruos" ("The sleep of reason produces monsters"). When it comes to the success or failure of treating a disease, reason is all the more important. Health care providers are aware of the consequences of a wrong therapeutic regimen for the treatment of infectious diseases. If the immediate consequences are therapeutic failure, the long-term consequences are resistance to antibiotics and the spread not only in clinical wards but also in the environment of these antibiotic resistance genes.

7. Conclusions

The ancient herbal therapeutic schemes differ from one geographic region to another, influenced directly by the flora existing in those regions. The folklore is the foundation of medicinal herbs, and the references to relevant bibliographies are rare. In countries with a strong tradition of plant healing, people are more confident in using plant products. Even so, the current trend in antimicrobial resistance has convinced many research teams to orient their resources toward selecting and standardizing plant treatments beneficial to healing or preventing infectious diseases. Embryonic stem cells used in gemmotherapy are often multiple organotropic, the products recommended for infectious diseases are more immunomodulatory than genuine antimicrobial agents. Also, embryonic stem cells have different characteristics from adult plants. The positive outcomes of many plant-based products or plant-derived compounds in healing of infectious diseases justify the reconsideration of their therapeutic potential. The emergence of bacterial, viral or parasitic resistant to various drugs and the toxicity of synthetic drugs are only two reasons for designing precise *in vitro* and *in vivo* studies targeting the antimicrobial efficacy of plants. There are so many unexplored features of the plant's therapeutic potential, that somewhere behind these unknown features is some hidden evidence of the mechanisms of action but evidence of new plant uses.

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Chapter 2

Synergistic Activity of Antibiotics and Bioactive Plant Extracts: A Study Against Gram-Positive and Gram-Negative Bacteria

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Additional information is available at the end of the chapter

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Abstract

The alarming growth of the number of antibiotic resistant bacteria and difficulties in treatment of infections have initiated a search for new antibacterial compounds and develop new alternative strategies in combating bacterial infections. Plant-derived compounds could exhibit a direct antibacterial activity and/or an indirect activity as antibiotic resistance modifying compounds, which, combined with antibiotics, increase their effectiveness. This ability of plant active substances reflects in modification or blocking of resistance mechanism so that bacterium becomes sensitive to antibiotic or the antibiotic acts when in lower concentrations. The systematic screening of plant-derived bioactive compounds, including those which can synergistically act with antibiotics, as resistance modifying agents represents a potential approach to overcome bacterial resistance. Therefore, the goals of this chapter are (i) an update of literature review on synergism between plant extracts and antibiotics, (ii) presentation of experimental results of synergistic activity of selected plant extracts and antibiotics and (iii) concluding remarks.

Keywords: antibacterial activity, synergism, antibiotic, plant extract, mode of action

1. Introduction

From the beginning of the antibiotic era, it was noticed that bacteria had the potential to develop resistance to antibiotics. Those early treatment failures with antibiotics did not represent a significant clinical problem because other classes of agents, with different cellular targets, were available [1]. But, in time, the number of antibiotic resistant bacteria has increased and antibiotic resistance has become a global public health threat [2].

The remarkable ability of bacteria to adapt to adverse environmental conditions makes them capable of surviving at clinically relevant concentrations of existing antibiotics resulting in the selection of resistant strains. The misuse and overuse of antibiotics are accelerating this process. An antibiotic, as a selective agent, induces genetic changes of bacteria, contributing to development, selection and spreading of resistant strains [3]. This process of acquired resistance is supported by rapid mutation and horizontal transfer of resistance genes. Resistance genes (via plasmids, transposons) may be transferred between individuals of the same or related bacterial species, between members of commensal or pathogenic microbiota and between different environmental habitats, thus spreading the resistance. Even more, there is evidence that some clinically relevant resistance genes have environmental origin [4]. The final score is the list of multi-drug, health-threatening resistant bacteria: methicillin-resistant *Staphylococcus aureus* (MRSA) and coagulase-negative staphylococci, vancomycin-resistant *Enterococcus* species (VRE), extended-spectrum β-lactamases producing Enterobacteriaceae, multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, at present.

The alarming growth of the number of antibiotic resistant bacteria and difficulties in treatment of infections have initiated a search for new antibacterial compounds and develop new alternative strategies in combating bacterial infections. Medicinal plants, with their long history of use in folk medicine for the treatment of infectious diseases, have become a promising new source of antibacterial agents. Plant-derived compounds could exhibit a direct antibacterial activity and/or an indirect activity as antibiotic resistance modifying compounds, which, combined with antibiotics, increase their effectiveness [5]. The systematic screening of plant-derived bioactive compounds, including those which can synergistically act with antibiotics, as resistance modifying agents represents a potential approach to overcome bacterial resistance. Therefore, the goals of this chapter are: (i) an update of literature review on synergism between plant extracts and antibiotics, (ii) presentation of experimental results of synergistic activity of selected plant extracts and antibiotics and (iii) concluding remarks.

2. Plant-derived antibacterial compounds

Plants produce a whole series of different compounds, which are not of particular significance for primary metabolism, but represent an adaptive ability of a plant to adverse abiotic and biotic environmental conditions. They have a remarkable effect to other plants, microorganisms and animals from their immediate or wider environment. All these organic compounds are defined as biologically active substances and generally represent secondary metabolites, given the fact that they occur as an intermediate or end products of secondary plant metabolism. Apart from determining unique plant characteristics (color, scent, flavor), these compounds also complete the functioning of plant organism, showing both biological and pharmacological activities of a plant [6]. They represent a structurally diverse group of compounds, classified in three major groups: phenolic compounds (simple phenols, phenolic acids, flavonoids, quinones, tannins and coumarins), terpenes and alkaloids. These compounds can be isolated from plant material as a solvent extract, an essential oil or a supercritical extract. Crude extracts represent complex mixtures of compounds (of both secondary

and primary metabolites), belonging to different biosynthetic and chemical classes that share some general mutual characteristics, such as polarity and/or volatility [7]. Plant extracts have long been known to possess broad antimicrobial activity and were frequently studied and reviewed [7–13]. Their marked antibacterial activity, classification as GRAS (generally recognized as safe) substances and low risk of bacterial resistance development have made them as suitable source for development of novel antibacterial agents.

2.1. Mechanisms of action of plant-derived antibacterial compounds

The antibacterial efficiency of plant compounds depends on several factors: (i) characteristics of target microorganism (the type, genus, species, strain), (ii) characteristics of plant material (botanical source, composition of the bioactive compounds as well as time of harvesting, stage of development or method of extraction) and (iii) chemical properties (hydrophilicity, lipophilicity, concentration, pH value). It is widely accepted that plant extracts, because of complex nature, possess multiple mechanisms of action. Plant extracts and their main components may exhibit activity by: (i) inhibiting bacterial growth or viability, (ii) targeting bacterial virulence factors or (iii) potentiating effectiveness of antibiotics as resistance modifying agents.

The inhibition of bacterial growth occurs through several mechanisms: disruption of membrane function and structure (including the efflux system), interruption of DNA/RNA synthesis and function, interference with intermediary metabolism and induction of coagulation of cytoplasmic constituents [7, 8, 14, 15].

Phenolic compounds, initially, affect cell membrane, as high correlation between toxicity and hydrophobicity of different phenolic compounds, changing the permeability and causing the leakage of cellular content or interfere with membrane proteins resulting in structure disrupting [7, 8, 14–16]. Besides the effect on cellular membrane, flavonoids, also, inhibit nucleic acid synthesis (caused by topoisomerase inhibition) and energy metabolism (caused by NADH-cytochrome c reductase or ATP synthase inhibition) as well as interrupt cell wall and cell membrane synthesis [17]. Quinones have a potential to form irreversible complex with nucleophilic amino acids in proteins. Probable targets in the microbial cell are surface-exposed adhesins, cell wall polypeptides and membrane-bound enzymes [8]. Tannins are subdivided into two groups: hydrolysable (gallotannins and ellagitannins) and condensed (proanthocyanidins) tannins. Proanthocyanidins possess several mode of action such as destabilization of cell membrane, inhibition of extracellular microbial enzymes, direct actions on microbial metabolism or deprivation of the substrates required for microbial growth [14]. The activity of gallotannins is attributable to their strong affinity for iron, and it is also related to the inactivation of membrane-bound proteins [18]. Coumarins cause a reduction in cell respiration [8]. Terpenes, compounds built up from isoprene subunits, because of lipophilic nature cause cell membrane disruption [19]. Alkaloids, one of the earliest isolated bioactive compounds from plants, possess ability to intercalate with DNA, interrupt activity of enzymes (esterase, DNA-, RNA-polymerase) or cell respiration [19].

Above that plant compounds have impact on growth and viability of bacteria, several research papers have discussed the effects of these compounds in modulating various aspects of bacterial virulence. It was observed that plant extracts inhibit bacterial biofilm formation, motility,

attachment and cell communication [20–23]. Biofilm formation is additional virulence factor that helps in the persistence of pathogens, and bacteria within a biofilm are more resistant to host defense and to antibiotics what make difficulties in eradication of infections. Moreover, plant extracts and their main compounds are able to suppress bacterial toxin production by reducing the expression of major virulence genes. Upadhyay et al. [24] have summarized the published results and showed that selected plant extracts inhibit the production of cholera toxin by *Vibrio cholerae*, reduce the production of *Staphylococcus aureus* α-hemolysin, enterotoxins and toxic shock syndrome toxin 1, reduce the production of verotoxin and inactivate Shiga toxins.

Finally, numerous investigations have shown that plant extracts in combination with antibiotics increase their activity and decrease the doses of antibiotics and their side effects. These positive interactions are considered as a potential strategy to combat bacterial resistance. The following sections will focus on synergistic activities of plant extracts and antibiotics.

2.2. Synergistic antibacterial activity of plant extracts and antibiotics

Synergistic interaction between two agents, in which one agent enhances the effect of the other and together they act more efficiently than as individual agents, motivated many scientists to examine and assess the significance of synergistic acting of plant-derived compounds and traditional antibiotics [25, 26]. It is well known that plant extracts possess antibacterial properties but, also, the ability to enhance the activity of an antibiotic in combination with it. That ability of plant active substances reflects in modification or blocking of resistance mechanism so that bacterium becomes sensitive to antibiotic or the antibiotic acts when in lower concentrations. Such an approach, besides reducing the effective dose of antibiotics on one side, also reduces the side effects of antibiotics as medicine on the other.

Numerous *in vitro* researches have confirmed synergistic effects of plant extracts and antibiotics with a significant reduction of minimum inhibitory concentration (MIC) in antibiotics. Scientists have tested various types of extracts of numerous plants in combination with different antibiotics. These were primarily antibiotics from the group of inhibitors of cell wall synthesis and protein synthesis. The tests included both Gram-positive and Gram-negative bacteria.

The ethanol extract of *Punica granatum* rind showed very good synergistic activity with ciprofloxacin resulting in upto 34-fold reduction of MIC and re-sensitization of *Klebsiella pneumoniae* resistant strain [27]. The antibacterial and modulatory potential of the ethanol extracts obtained from leaves and bark of *Azadirachta indica* in combination with aminoglycosides and carbapenems against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* was tested. The association between the ethanol bark extract and amikacin against *P. aeruginosa* PA 24 strain showed synergistic effect. Synergistic effect was also combined with ethanol bark extract and imipenem, amikacin or gentamicin against *E. coli* strains and with imipenem against *S. aureus* strains [28]. Results of combination assay between grape pomace extract and antibiotics showed that the extract combined with representatives of different classes of antibiotics as β-lactam, quinolone, fluoroquinolone, tetracycline and chloramphenicol acted in synergy in all *S. aureus* and *E. coli* strains tested with fractional inhibitory concentration index (FICI) values varying from 0.031 to 0.155. The MIC of antibiotics was reduced 4- to 75-fold.

The most abundant phenolic compounds identified in the extract were quercetin, gallic acid, protocatechuic acid and luteolin. It was also shown that combinations of grape pomace extract with antibiotics are not toxic for the HeLa cell line at concentrations in which the synergistic effect was observed [29]. Different interactions (synergistic, additive and indifference) were observed between *Thymbra spicata* L. extracts and certain antibiotics, including ampicillin, cefotaxime, amikacin and ciprofloxacin. The FICI ranged from 0.02 to 1.5 for *S. aureus* and 0.25 to 2 for *K. pneumoniae* strains. The best synergistic capacity appeared with cefotaxime against *S. aureus* strains, and the activity of cefotaxime was increased from 8- to 128-fold [30]. The hydroalcoholic extracts obtained from the leaves of *Psidium guajava* L. and *Psidium brownianum* Mart ex DC synergistically acted with gentamicin, amikacin and ciprofloxacin against *E. coli*, *P. aeruginosa* and *S. aureus* [31]. The antibiotic potentiating property of *Vangueria madagascariensis* (fruit and leaf extracts) against clinical isolates of *Acinetobacter* spp. was observed. The extracts were found to potentiate the activity of chloramphenicol and ciprofloxacin in a ratio of 50% extract: 30% antibiotic [32]. Thakur et al. [33, 34] analyzed the synergistic antibacterial potential of hydroethanolic extracts of the stem bark of *Berberis aristata* and *Camellia sinensis* with third-line antibiotics against carbapenem-resistant *E. coli*. The analysis of *Berberis aristata*/antibiotics combinations revealed synergistic behavior (FICI < 1) with colistin, tigecycline and amoxicillin/clavulanate potassium, whereas antagonism (FICI > 1) was seen with ertapenem and meropenem [33]. *Camellia sinensis*/antibiotics combinations showed synergism with tigecycline, ertapenem, meropenem, colistin and amoxicillin/clavulanate potassium [34]. Active substances of water extract of tea (*Camellia sinensis*) modified the resistance of methicillin-resistant *S. aureus* (MRSA) as well as the resistance of β-lactamases-producing *S. aureus*. The extract diluted 40- to 100-fold reduced MIC of methicillin from ≥256 to ≤0.12 μg/ml, whereas the extract diluted 40-fold reduced MIC of penicillin up to ≤0.12 μg/ml [35].

Different scientific papers reported results of synergistic antibacterial activity of various plant extracts in the presence of different antibiotics, such as oxacillin, tetracycline, nalidixic acid, ofloxacin, chloramphenicol, gentamicin, erythromycin, penicillin, ampicillin, kanamycin and ciprofloxacin. *Piper betle* L. extracts/antibiotics combination indicated additive and synergistic effects. The greatest synergy was observed against *P. aeruginosa* (FICI 0.09) in the 70% acetone extract—30% chloramphenicol combination. Synergy was also observed against *S. aureus*, *Propionibacterium acnes*, *S. epidermidis* and *Streptococcus pyogenes* [36]. The extracts from *Beilschmedia acuta* leaves and bark and those from the leaves of *Newbouldia laevis* and *Polyscias fulva*, at their concentration of 1/2 MIC and 1/5 MIC, were enhanced activity of tetracycline, chloramphenicol, ampicillin, kanamycin and ciprofloxacin against multi-drug resistant bacteria [37]. In the case of *Juglans regia* extract, 10-fold reduction in MICs was observed against *S. aureus* when it used in combination with oxacillin. In this combination, oxacillin was able to inhibit MRSA strains at concentration of 0.312 μg/ml, MIC in combination was 64-fold lower than the MIC of oxacillin alone and indicated a reversion of methicillin resistance [38]. *Indigofera suffruticosa*, a popular plant used to treat infections, was investigated as modulator of antibiotic effectiveness against *S. aureus*. Acetone extract and erythromycin showed synergistic effects (55.56%; FICI values ranged from 0.3 to 0.5), additive effects (0.6 ≤ FICI ≤0.8) in three and an indifferent effect in only one (ratio of 1:9, drug: extract; FICI = 1.7). For the chloroform extract and erythromycin combinations, both synergistic (0.2 ≤ FICI ≤ 0.4) and additive (0.7 ≤ FICI ≤ 0.9) effects were equally found in four ratios and only one ratio gave a non-interaction (1:9, drug:

extract; FICI = 1.7). No synergistic effect was seen with ether extracts, but eight ratios resulted in additive effects (0.6 ≤ FICI ≤0.9) and one ratio an indifferent (3:7, drug: extract; FICI = 1.2) [39]. Ethanol extract of *Hyptis martiusii*, with concentration of 32 μg/ml, reduced the effective concentrations of antibiotics (amikacin, gentamicin, kanamycin, neomycin and tobramycin) over 100-fold, where the tested concentrations of antibiotics ranged from 8 to 256 μg/ml, and they were reduced upto ≤1 μg/ml [40]. Sub-inhibitory concentrations of water extract of *Catha edulis* (5 mg/ml) enhanced the activity of tetracycline two- to fourfold, against resistant strains of periodontal bacteria (*Streptococcus sanguis* TH-13, *S. oralis* SH-2 and *Fusobacterium nucleatum*) [41]. Nostro et al. [42] showed that combinations of propolis and *Zingiber officinale* with clarithromycin intensified controlling of *Helicobacter pylori*. Sibanda and Okoh [43] detected the synergism of acetone extract of *Garcinia kola* nuts with amoxicillin, ciprofloxacin, tetracycline and chloramphenicol, whereas the ethanol extracts from *Aegopodium podagraria* L. and *Torilis anthriscus* in combination with streptomycin and chloramphenicol exhibit synergistic and additive effects [44, 45]. Synergistic effect was also established between ciprofloxacin and chloroform extract of *Jatropha elliptica* at the level of concentration of 1/8 MIC of antibiotics. It was found that the extract contained active substances inhibiting NorA efflux mechanism [46].

In addition to the synergistic effects observed for the plant extracts, *in vitro* studies reported the capacity of pure compounds to potentiate the activity of antibiotics. The carnosic acid, the main bioactive compound of *Rosmarinus officinalis* extracts, was capable of acting synergistically with gentamicin against *S. aureus* clinical isolates. In addition, the carnosol, γ-lactone derivative of carnosic acid, isolated from a crude extract from *Salvia officinalis* L. reduced the MICs of aminoglycosides in vancomycin-resistant enterococci. Carnosic acid (8 μg/ml) or carnosol (16 μg/ml) reduced the MICs of several aminoglycosides in vancomycin-resistant *Enterococcus faecium* and *E. faecalis* round 8- to 128-fold [47]. Combinations of tetracycline or β-lactam antibiotics with baicalein (5,6,7-trihydroxyflavone) exhibit synergistic effects against MRSA [48]. Moreover, it has been reported that epigallocatechin gallate is synergistically active in combination with β-lactams, tetracycline, oxytetracycline [49, 50]. The geranylated flavanones from *Paulownia tomentosa* fruits showed a promising synergistic potential with antibiotics [51]. Curcumin, a flavonoid isolated from the rhizome of a plant, *Curcuma longa* L., markedly reduced the MICs of the antibiotics oxacillin, ampicillin, ciprofloxacin and norfloxacin used against MRSA. The combined activity of curcumin and antibiotics resulted in a 2- to 128-fold reduction in MIC values [52]. Allicin, antibacterial compound from garlic (*Allium sativum*), potentiated the action of cefazolin (4- to 128-fold) and oxacillin (32- to 64-fold), against *Staphylococcus* sp. and cefoperazone (8- to 16-fold) against *P. aeruginosa* [53].

2.3. Mechanisms of synergistic antibacterial activity of plant extracts and antibiotics

When a number of scientific researches have confirmed the synergistic activity of plant extracts and antibiotics certainly, the next step was to investigate the mechanisms of the synergistic action. It is believed that active compounds from plants modify and inhibit the mechanisms of acquired resistance in bacterial cell and thus exhibit a synergistic effect with antibiotics [54, 55]. The mechanism of synergistic action is explained by: (i) modification of active sites on bacterial cell, (ii) inhibition of enzymes, which catalyze degradation or modification of antibiotics, (iii) increase of membrane permeability and (iv) inhibition of efflux pumps (**Figure 1**).

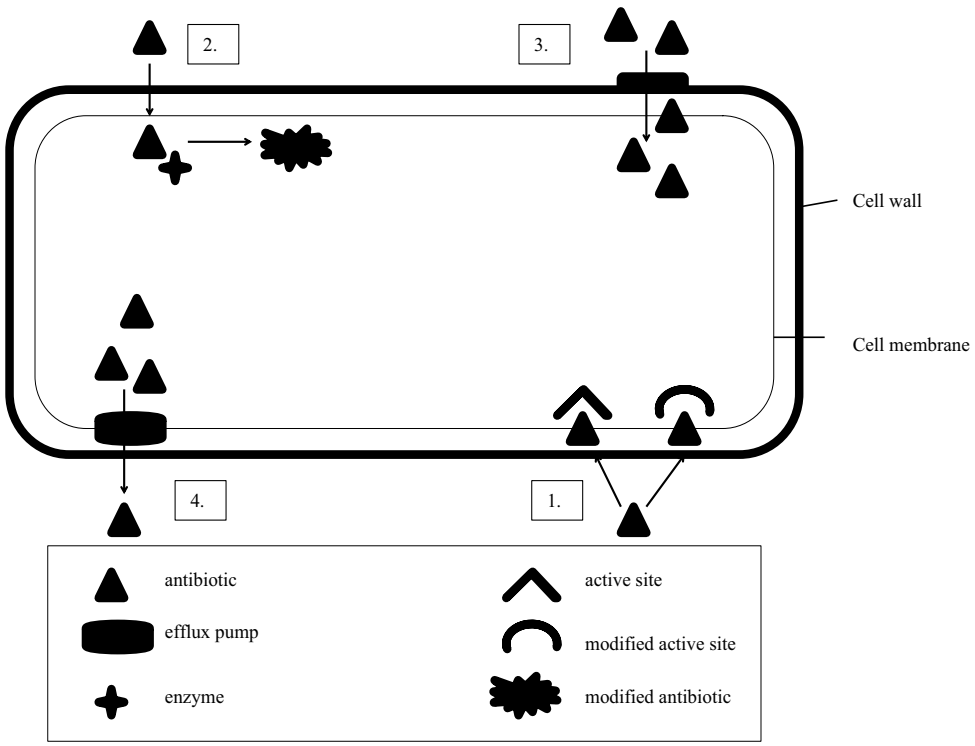

Figure 1. Mechanisms of synergistic antibacterial activity: (1) modification of the active site, (2) enzymatic degradation of antibiotic, (3) increase of membrane permeability and (4) inhibition of efflux pump.

2.3.1. Modification of active sites on bacterial cell

Modification of active sites is a common mechanism of resistance and may occur for diverse classes of antibiotics. Resistance to β-lactam antibiotics is especially present in Gram-positive bacteria. β-lactam antibiotics inhibit metabolism of peptidoglycan binding themselves to penicillin-binding proteins (PBPs), which catalyze cross linking of peptidoglycan in mesh structure. Without the mesh structure, cell wall becomes mechanically weak, which undermines integrity of the bacteria cell. Resistance occurs due to reduced affinity of PBPs for antibiotics or due to reduced production of these proteins. All the factors modifying structure, activity or synthesis of PBPs influence the reduction of resistance. Numerous scientific papers confirmed the change in resistance to β-lactam antibiotics through synergistic acting of antibiotics and plant secondary metabolites. Flavonoid baicalin isolated from *Scutellaria amoena* [56], polyphenol corilagin from *Arctostaphylos uva-ursi* [57], tellimagrandin and rugosin B from wild rose (*Rosa canina*) [58] and epigallocatechin gallate from green tea (*Camellia sinensis*) [59] significantly reduce MIC of β-lactam antibiotics, especially in case of MRSA. Methicillin resistance is due to the expression of an additional penicillin-binding protein (PBP2a), regulated by *mecA* gene, which has a low β-lactams-binding affinity, and it enables cell wall synthesis. Methicillin inhibits the transpeptidational activities of other PBPs, but PBP2a remains active ensuring the cross-linking of the glycan chains in peptidoglycan. PBP2a is not able to

completely compensate for the other PBPs because cells grown in the presence of methicillin exhibit a marked reduction in the degree of cross-linking. However, the limited degree of cross-linking is enough to ensure survival of the cell [60].

2.3.2. Enzymatic degradation or modification of antibiotics

Bacterial cell consists of various enzymatic systems, which inactivate antibiotics. It occurs through the processes of hydrolysis, replacement of active groups (acetylation, phosphorylation glycosylation and adenylation) and processes of oxidation-reduction [61]. One of the well-examined groups of enzymes is the group of β-lactamases. β-lactamases (penicillins and cephalosporins) are the enzymes from the group of lyases, which destroy the amino link of β-lactam ring turning it into inactive form. On grounds of scientific studies, it has been showed that active plant compounds may inhibit these enzymes preventing degradation of antibiotics. For example, it is well known that epigallocatechin gallate from green tea in synergistic interactions with antibiotics increases the effect of antibiotics by inhibiting β-lactamases [62, 63]. *Stephania suberosa* extracts possess multiple mode of action, inhibit β-lactamases activity and increase cell membrane permeability against ampicillin-resistant *S. aureus* [64].

2.3.3. Increase of membrane permeability

Cell wall is a first barrier, which antibiotics and other compounds must overcome to achieve their targets and demonstrate their inhibitory activity. In Gram-positive bacteria, the cell wall is composed of several layers of peptidoglycan, which are mostly permeable to different compounds, while in Gram-negative bacteria, the cell wall is complex. It is constructed of a single layer of peptidoglycan and a layer of lipoproteins and lipopolysaccharides known as outer membrane. The outer membrane is significant barrier for many compounds including antibiotics because of several reasons: (i) polysaccharides restrict or completely prevent penetration of antibiotics with high molecular weight, (ii) a lipid layer limits penetration of hydrophilic molecules and (iii) porins enable transport of hydrophilic molecules. Entrance to the periplasmic space might occur via diffusion through porins or through the lipid bilayer by solubilization. After crossing the outer membrane, compounds can be taken out from the periplasmic space by the efflux pumps or inactivated by enzymes, before effectively reaching cell membrane. The cell membrane is another barrier, which restricts influx of compounds in cytoplasm. Phenolic compounds and terpenes change function and structure of membrane. They affect membrane permeability mainly due to perturbation of the lipid bilayer causing decrease in lipid density in the bilayer. Reduced density of the lipids results in a permeable membrane [65]. Probably, the increased membrane permeability resulted in increased level of the antibiotics inside bacterial cells and their better interaction with intracellular targets. Hemaiswarya et al. [66] noticed synergistic interactions of eugenol from plant *Eugenia aromatic* with 10 different hydrophobic and hydrophilic antibiotics in case of five Gram-negative bacteria. Synergism occurred due to ability of eugenol to increase the permeability of cell membrane, and the concentration of 1 mM caused up to 50% of damage of cell membrane.

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2.3.4. Inhibition of efflux pumps

One of the mechanisms of resistance is also utilized for developing efflux pumps by bacteria to expel antibiotics from cells. Efflux pumps work through ATP hydrolysis or on grounds of difference in concentration of ions. Numerous plant-derived compounds with significant activity as inhibitors of efflux pumps were discovered. Primarily, these compounds are active against Gram-positive bacteria [67]. For examples, carnosic acid and carnosol, isolated from chloroform extract of *Rosmarinus officinale*, acted as inhibitors of efflux pumps. A 10 μg/ml of carnosic acid and carnosol increased by the activity of tetracycline two- to fourfold in case of a *S. aureus* strain that had Tet (K) pump. Active compounds inactivated efflux pump and prevented expelling of tetracycline from the cell. Carnosic acid also increased the activity of erythromycin eightfold in case of a *S. aureus* strain that had Msr (A) pump by inhibiting its activity [68]. The same authors tested the active substance from herb *Lycopus europaeus* in combination with tetracycline and erythromycin and detected doubled intensity of antibiotics activity in case of *S. aureus* strains that had Tet (K) and Msr (A) pumps [69]. Baicalein isolated from the leaves of *Thymus vulgaris* demonstrated synergy with ciprofloxacin against MRSA strains and with gentamicin against vancomycin-resistant enterococci, apparently by the inhibition of the NorA efflux pump [70]. Shahverdi et al. [71] discovered that cinnamaldehyde, from *Cinnamomum zeylanicum* bark essential oil, reduced clindamycin resistance in *Clostridium difficile* inhibiting CdeA efflux pump system, the first multidrug efflux transporter, which is identified in *C. difficile*. Recently, *Punica granatum* extract inhibits efflux pump of multidrug resistant *K. pneumoniae* [27].

3. *In vitro* testing of antibacterial synergistic activity of selected plant extracts and antibiotics

In this study, different combinations of selected plant extracts and commonly used antibiotics were tested, emphasizing the potential role of phytocompounds in increasing the effectiveness of antibiotics. The experiment involved ethanol, ethyl acetate and acetone extracts from five plant species: *Cychorium intybus* L. (Asteraceae), *Salvia officinalis* L., *Clinopodium vulgare* L. (Lamiaceae), *Cytisus nigricans* L. and *Dorycnium pentaphyllum* Vill. (Fabaceae). The plant species were selected on the basis of several factors: (i) use in traditional medicine, (ii) phytochemical composition, (iii) known *in vitro* antibacterial activity and (iv) insufficient data on synergistic activity.

3.1. Materials and methods

3.1.1. Plant material

The aerial parts of *C. vulgare, D. pentaphyllum* and *C. nigricans* were collected from the different regions of Serbia, while *S. officinalis* (leaves) and *M. officinalis* (leaves) were supplied from the commercial source. Identification and classification of the plant material were performed at the Faculty of Science, University of Kragujevac. The voucher specimens are deposited at

the Herbarium of the Faculty of Science, University of Kragujevac. The collected plant materials were air-dried under shade at room temperature and then ground into small pieces, which were stored into paper bags at room temperature.

3.1.2. Preparation of samples for testing

Dried, ground plant material was extracted by static maceration with ethanol, ethyl acetate and acetone for 3 days at room temperature. Every 24 h, 30 g of plant material was soaked with 150 ml of solvent (3 × 150 ml). After filtration, the extracts were concentrated using a rotary evaporator at 40°C to obtained dry extracts without trace of solvent (duration of solvent evaporation was 20 min for acetone extract, 30 min for ethyl acetate extract and 45 min for ethanol extract). The crude plant extracts were stored at −20°C. Before the testing, the crude extracts were dissolved in dimethyl sulfoxide (DMSO) and then diluted into nutrient liquid medium to achieve a concentration of 10% DMSO. The concentrations used in the experiments were based on the dry weight of the extracts.

Four antibiotics, amoxicillin, cephalexin, gentamicin and chloramphenicol, were used. Stock solutions of antibiotics were prepared in Mueller-Hinton broth. Each extract was combined with two antibiotics of different modes of action (cephalexin/gentamicin, amoxicillin/chloramphenicol).

3.1.3. Microorganisms

The following bacteria were used: *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and clinical isolate of *S. aureus* (PMFKg-B30), *Bacillus subtilis* (PMFKg-B2), *K. pneumoniae* (PMFKg-B26), *E. coli* (PMFKg-B32), *P. aeruginosa* (PMFKg-B28) and *P. mirabilis* (PMFKg-B29). All clinical isolates were a generous gift from the Institute of Public Health, Kragujevac. Bacteria were stored in microbiological collection at −70°C (Laboratory of Microbiology, Faculty of Science, University of Kragujevac).

Bacterial suspension was prepared from overnight cultures by the direct colony method. Colonies were taken directly from the plate and suspended into 5 ml of sterile 0.85% saline. The turbidity of initial suspension was adjusted comparing with 0.5 Mc Farland standard. When adjusted to the turbidity of a 0.5 Mc Farland standard, a suspension of bacteria contains about 10^8 colony forming units (CFUs)/ml. Ten-fold dilutions of initial suspension were additionally prepared into sterile 0.85% saline to achieve 10^6 CFU/ml.

3.1.4. Combination assay

Prior to performing the synergy test, the minimum inhibitory concentrations (MICs) of plant extracts and antibiotics were determined using microdilution plate method with resazurin in Mueller-Hinton broth [72]. Briefly, 96-well microtiter plates were prepared by dispensing 100 μl of Mueller-Hinton broth into each well. A 100 μl from the stock solution of tested compound was added into the first row of the plates. Then, twofold serial dilutions were performed by transferring 100 μl of solution from one row to another, using a multichannel pipette. The obtained concentration range was from 0.156 to 20 mg/ml for plant extracts and from 0.12 to 1000 μg/ml for antibiotics. Ten microlitres of each 10^6 CFU/ml bacterial

suspension was added to appropriate wells. Finally, 10 μl of resazurin solution was added. Resazurin is an oxidation-reduction indicator used for the evaluation of microbial growth. It is a blue non-fluorescent dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. The inoculated microtiter plates were incubated at 37°C for 24 h. MIC was defined as the lowest concentration of the tested compounds that prevented resazurin color change from blue to pink.

The combined activity of plant extracts and antibiotics was evaluated by checkerboard method [73]. The testing was performed in 96-well microtiter plates using a 6-by-6 well configuration. Twofold dilutions of each antibacterial compounds were prepared. First, 100 μl of Mueller-Hinton broth was added into 36 wells of a 96-well microtiter plate. Then, 50 μl of each dilutions of extract was added horizontally into six rows, and 50 μl of each dilutions of antibiotic was added vertically into six columns. The final volume was 200 μl. The final concentration range corresponded to 1/32 MIC – MIC. Each well contained unique combination of plant extract and antibiotic concentration. Ten microlitres of each 10^6 CFU/ml bacterial suspension and 10 μl of resazurin solution were added. The microtiter plates were incubated for 24 h at 37°C. The combination of the compounds in which resazurin color change did not appear (growth inhibition) is taken as effective MIC for the combination. Each test included growth control and sterility control.

In vitro interactions between antimicrobial agents were determined and quantified by calculating the fractional inhibitory concentration index (FICI) using the following formula:

$$\text{FICI} = \frac{\text{MICa in combination}}{\text{MICa}} + \frac{\text{MICb in combination}}{\text{MICb}} \quad (1)$$

where MICa is MIC of plant extract and MICb is MIC of antibiotics.

Interpretation of the FICI was as follows: FICI ≤ 0.5 synergy; FICI > 0.5–1 additivity; FICI > 1–4 indifference and FICI > 4 antagonism. The action of antibacterial agents was considered to be:

- Synergistic, if their joint effect was stronger than the sum of effects of the individual agents
- Additive, if their joint effect was equal to the sum of effects of the individual agents
- Indifferent, if their joint effect was equal to the effect of either individual agent
- Antagonistic, if their joint effect was weaker than the sum of effects of the individual agents or weaker than the effect of either individual agent [73].

The mean FICI of all combination was used to categorize results as synergy, additivity, indifference and antagonism.

3.1.5. Statistical analysis

Statistical evaluation of the data was performed by Student's *t*-test using the SPSS statistical software package, version 20 for Windows. The results were considered to be statistically significant at $p < 0.05$. The results of antibacterial activity of plant extracts were statistically analyzed.

3.2. Results and discussion

3.2.1. Antibacterial activity

The antibacterial activity of tested plant extracts was previously evaluated, and results were reported in [68]. Intensity of antibacterial activity depended on the species of bacteria, plant species and the type of extract. The MIC values were in range from 0.019 to >20 mg/ml (**Table 1**). In general, the significant antibacterial activity was obtained with acetone extract from *S. officinalis* and ethyl acetate and acetone extract from *C. intybus.* Other tested extracts exhibited moderate activity. Statistically significant difference in activity between extracts of *C. intybus*, *S. officinalis* and *C. vulgare* was noticed (**Table 1**). Antibacterial activity of ethyl acetate (p = 0.001) and acetone extract (p = 0.002) of *C. intybus* was statistically higher than the activity of ethanol extract. Acetone extract was the most active (p = 0.018). Moreover, the activity of acetone extract of *S. officinalis* was higher than activity of ethanol (p = 0.004) and ethyl acetate extract (p = 0.001). Ethyl acetate (p = 0.015) and acetone extract (p = 0.018) of *C. vulgare* acted better than ethanol extract. Between ethyl acetate and acetone extract, no statistically significant difference in activity was noted (p = 0.756). There is no statistically significant difference in action between extracts of *C. nigicans* and *D. pentaphyllum* ($p < 0.05$) (**Table 1**).

The tested bacterial strains showed different level of sensitivity to the antibiotics (**Table 2**). The resistance profile of bacteria was determined according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints [74]. In relation to chloramphenicol and amoxicillin, all clinical isolates were resistant. Gentamicin and cephalexin were active against clinical isolates of *B. subtilis, S. aureus* and *E. coli.* Isolates of *K. pneumoniae, P. aeruginosa* and *P. mirabilis* were resistant to all tested antibiotics.

3.2.2. Synergistic activity of plant extracts and antibiotics

In vitro testing of combined activity of ethanol, ethyl acetate and acetone extracts of five plant species (*C. intybus*, *S. officinalis*, *C. vulgare, C. nigricans* and *D. pentaphyllum*) and four antibiotics (cephalexin, amoxicillin, gentamicin and chloramphenicol) showed three types of interaction: synergism, additivity and indifference in relation to tested strains of bacteria. In general, according to obtained results, the following remarks could be made:

- *S. officinalis*, *C. vulgare* and *C. nigricans* acted synergistically with tested antibiotics, while *C. intybus* and *D. pentaphyllum* showed additive effect.
- The activity of tested antibiotics was increased up to 32-fold depending on the kind of extract and type of bacteria.
- The concentrations of tested extracts, corresponding to values ranging from 1/4 MIC to 1/32 MIC, increased the activity of antibiotics.
- The synergism was observed almost in case of all tested bacteria, with exception of *E. coli* for which there was no synergism observed in any of the combinations considered. For other bacteria, this ratio was shown in the following decreasing order: *P. mirabilis* > *K. pneumoniae* > *B. subtilis* > *P. aeruginosa* ATCC 2785 > *S. aureus* > *S. aureus* ATCC 25923 >

Plant species		1	2	3	4	5	6	7	8	9
		MIC (mg/ml)								
Cichorium intybus	E[a]	5	10	10	10	20	20	20	5	2.5
	Et[b]	2.18	2.18	2.18	8.75	8.75	2.18	2.18	2.18	1.09
	Ac[c]	2.5	2.5	2.5	5	5	2.5	2.5	2.5	2.5
Salvia officinalis	E[a]	5	5	5	>20	10	>20	>20	>20	2.5
	Et[a]	10	20	20	>20	>20	>20	>20	>20	2.5
	Ac[b]	0.03	0.15	0.31	20	1.25	20	0.31	0.156	0.019
Clinopodium vulgare	E[a]	1.25	>20	>20	>20	20	>20	20	2.5	20
	Et[b]	0.625	20	10	10	10	10	10	2.5	10
	Ac[b]	1.25	20	10	10	10	20	10	0.625	10
Cytisus nigricans	E[a]	2.5	20	20	10	10	20	20	5	1.25
	Et[a]	5	20	20	20	20	20	20	5	5
	Ac[a]	2.5	20	20	10	20	>20	20	2.5	10
Dorycnium pentaphyllum	E[a]	5	10	20	10	5	20	20	2.5	10
	Et[a]	1.25	20	20	10	10	>20	20	1.25	20
	Ac[a]	1.25	20	20	5	5	20	10	1.25	10

1. *B. subtilis*; 2. *K. pneumoniae*; 3. *S. aureus*; 4. *P. aeruginosa*; 5. *P. mirabilis*; 6. *E. coli*; 7. *E. coli* ATCC 25922; 8. *S. aureus* ATCC 25923; 9. *P. aeruginosa* ATCC 27853; E: ethanol extract; Et: ethyl acetate extract; Ac: acetone extract; Superscript with different letters are significantly different at $p < 0.05$, for every plant species separately.

Table 1. Antibacterial activity of tested plant extracts.

P. aeruginosa > *E. coli* ATCC 25922. Extracts of *Salvia officinalis*, together with amoxicillin and chloramphenicol, have synergistically acted to most bacteria.

- The ethanol and ethyl acetate extracts of *S. officinalis* and *C. nigricans* intensified the activity of amoxicillin, gentamicin, cephalexin and chloramphenicol reducing the effective concentration by 32-fold against Gram-positive bacteria *B. subtilis* and *S. aureus* and Gram-negative bacteria *K. pneumoniae* and *P. mirabilis.*
- The extracts of *C. vulgare* in combination with cephalexin and gentamicin showed synergistic effect of less intensity. The most active was the combination of acetone extract and gentamicin against *B. subtilis* in which case the MIC of antibiotics was decreased by 16-fold.
- This was the first observation of synergistic effect of *C. vulgare* and *C. nigricans* and additive effect of *C. intybus* and *D. pentaphyllum* with tested antibiotics.

3.2.2.1. Interaction between S. officinalis extracts and antibiotics

The results of combined acting of ethanol, ethyl acetate and acetone extract of *S. officinalis* and antibiotics (chloramphenicol and amoxicillin) expressed in FICI are indicated in **Table 3**. Synergistic, additive and indifferent effects were observed. FICI was ranged in intervals from 0.30 to 1.37.

The extracts showed a better synergistic capacity with amoxicillin than with chloramphenicol (**Table 4**). In reference with initial MIC values, activity of amoxicillin was increased by 4- to 32-fold depending on the species of bacteria. Of nine tested bacteria, amoxicillin acting with ethanol and acetone extract showed synergism against eight bacteria and in case of ethyl acetate extract against two bacteria. Only in case of *E. coli*, there was no synergistic, but only indifferent effect observed. On grounds of FICI values, it may be noticed that intensity of synergistic effect was different and that extracts of *S. officinalis* significantly increased the activity of amoxicillin (FICI 0.31–0.35) (**Table 4**).

Bacteria	CEF	AMO	GEN	CHL
	MIC (μg/ml)			
B. subtilis	12.5	31	3.125	250
K. pneumoniae	500	250	6.25	250
S. aureus	1.56	500	0.39	500
E. coli	1.56	>1000	1.56	>1000
P. aeruginosa	>1000	>1000	>1000	>1000
P. mirabilis	>1000	>1000	>1000	>1000
E. coli ATCC 25922	6.25	6.25	0.39	250
S. aureus ATCC 25923	6.25	1.9	0.19	125
P. aeruginosa ATCC 27853	>1000	0.12	0.098	250

CEF: cephalexin; AMO: amoxicillin; GEN: gentamicin; CHL: chloramphenicol.

Table 2. Antibacterial activity of tested antibiotics.

Bacteria	Ethanol extract		Ethyl acetate extract		Acetone extract	
	CHL	AMO	CHL	AMO	CHL	AMO
B. subtilis	0.44 (S)	0.32 (S)	0.40 (S)	0.35 (S)	1.37 (I)	0.50 (S)
K. pneumoniae	0.44 (S)	0.32 (S)	0.35 (S)	1.37 (I)	1.37 (I)	0.35 (S)
S. aureus	0.44 (S)	0.39 (S)	0.49 (S)	1.37 (I)	1.37 (I)	0.35 (S)
E. coli	1.37 (I)	1.25 (I)	1.37 (I)	1.37 (I)	1.37 (I)	1.37 (I)
P. aeruginosa	1.37 (I)	0.5 (S)	1.37 (I)	1.37 (I)	1.37 (I)	1.37 (I)
P. mirabilis	1.37 (I)	0.37 (S)	1.37 (I)	0.53 (A)	1.37 (I)	0.49 (S)
E. coli ATCC 25922	0.61 (A)	1.18 (I)	0.61 (A)	0.42 (S)	1.37 (I)	0.38 (S)
S. aureus ATCC 25923	0.61 (A)	0.37 (S)	1.37 (I)	1.37 (I)	1.37 (I)	0.35 (S)
P. aeruginosa ATCC 27853	0.30 (S)	0.31 (S)	0.56 (A)	1.37 (I)	0.61 (A)	0.35 (S)

CHL: chloramphenicol; AMO: amoxicillin.

Table 3. Interaction between *S. officinalis* extract and antibiotics expressed as FICI.

Chloramphenicol showed synergism with ethanol and ethyl acetate extract (**Table 4**). In case of combination of chloramphenicol/ethanol extract, synergism was observed against four bacteria, while in case of combination of chloramphenicol/ethyl acetate extract, synergism was observed against three bacteria. These combinations reduced the MICs of chloramphenicol even 32-fold in case of mention strains of bacteria. In case of other bacteria, as

Bacteria	Amoxicillin			Chloramphenicol	
	Ethanol extract	Ethyl acetate extract	Acetone extract	Ethanol extract	Ethyl acetate extract
	MIC*				
B. subtilis	$1/16_E + 1/8_A$	$1/16_E + 1/32_A$	$1/4_E + 1/4_A$	$1/4_E + 1/8_A$	$1/8_E + 1/32_A$
K. pneumoniae	$1/8_E + 1/16_A$	/	$1/32_E + 1/16_A$	$1/4_E + 1/8_A$	$1/16_E + 1/32_A$
S. aureus	$1/4_E + 1/16_A$	/	$1/32_E + 1/16_A$	$1/4_E + 1/8_A$	$1/4_E + 1/32_A$
P. aeruginosa	$1/4_E + 1/4_A$	/	/	$1/32_E + 1/8_A$	/
P. mirabilis	$1/32_E + 1/4_A$	/	$1/32_E + 1/4_A$	/	/
E. coli ATCC 25922	/	$1/8_E + 1/16_A$	$1/8_E + 1/16_A$	/	/
S. aureus ATCC 25923	$1/8_E + 1/8_A$	/	$1/32_E + 1/16_A$	/	/
P. aeruginosa ATCC 27853	$1/8_E + 1/16_A$	/	$1/32_E + 1/16_A$	/	/

"/" no synergism; E – extract; A – antibiotic.
*The most active combination.

Table 4. Synergism between *S. officinalis* extracts and antibiotics.

well as in combination of chloramphenicol/acetone extract, additive and indifferent effects were observed. Horiuchi et al. [47] observed that acetone extract of *S. officinalis* and isolated components, carnosol and carnosic acid, increased the activity of aminoglycosides against vancomycin-resistant enterococci. These herbal components reduced the MIC of antibiotics by 8- to 128-fold depending on the type of bacteria.

3.2.2.2. Interaction between C. vulgare extracts and antibiotics

The results of combined acting of ethanol, ethyl acetate and acetone extract of *C. vulgare* and antibiotics (cephalexin and gentamicin) expressed in FICI are presented in **Table 5**. Synergistic and indifferent effects were observed. FICI was ranged in intervals from 0.39 to 1.67. The ethanol extract exhibited the best synergistic capacity with antibiotics.

For most tested bacteria, interactions of extracts and antibiotics were indifferent. Synergism was observed against four bacteria: *B. subtilis*, *K. pneumoniae*, *P. aeruginosa* and *P. mirabilis*. There was a synergistic acting of gentamicin with all three extracts in relation to *B. subtilis,* while cephalexin acted synergistically with ethanol and ethyl acetate extract (**Table 6**). In these combinations, FICI was 0.39 and 0.44. As for *K. pneumoniae,* the strain that was resistant to cephalexin, the combination of cephalexin with ethanol and acetone extracts showed a synergism. The concentrations of extracts of 1/4 MIC intensified the activity of cephalexin, and MIC was decreased fourfold.

The ethanol extract showed with both tested antibiotics a synergistic activity against *P. aeruginosa* and *P. mirabilis.* The test results were interesting because the strains showed resistance to cephalexin and gentamicin. In synergistic combinations, it was observed that sub-inhibitory concentrations of extracts (1/4 MIC and 1/8 MIC) modified activity of antibiotics by reducing effective concentrations of antibiotics up to 16-fold.

Bacteria	Ethanol extract		Ethyl acetate extract		Acetone extract	
	GEN	CEF	GEN	CEF	GEN	CEF
B. subtilis	0.44 (S)	0.44 (S)	0.44 (S)	0.44 (S)	0.39 (S)	1.29 (I)
K. pneumoniae	1.33 (I)	0.50 (S)	1.33 (I)	1.67 (I)	1.33 (I)	0.50 (S)
S. aureus	1.04 (I)	1.23 (I)	1.24 (I)	1.23 (I)	1.19 (I)	1.07 (I)
E. coli	1.56 (I)	1.67 (I)	1.67 (I)	1.67 (I)	1.33 (I)	1.67 (I)
P. aeruginosa	0.50 (S)	0.50 (S)	1.33 (I)	1.33 (I)	1.33 (I)	1.33 (I)
P. mirabilis	0.44 (S)	0.50 (S)	1.34 (I)	1.39 (I)	1.34 (I)	1.39 (I)
E. coli ATCC 25922	1.56 (I)	1.45 (I)	1.56 (I)	1.33 (I)	1.35 (I)	1.33 (I)
S. aureus ATCC 25923	1.24 (I)	1.34 (I)	1.12 (I)	1.19 (I)	1.24 (I)	1.19 (I)
P. aeruginosa ATCC 27853	1.19 (I)	1.24 (I)	1.14 (I)	1.24 (I)	1.19 (I)	1.24 (I)

GEN: gentamicin; CEF: cephalexin.

Table 5. Interaction between *C. vulgare* extracts and antibiotics expressed as FICI.

Bacteria	Gentamicin			Cephalexin		
	Ethanol extract	Ethyl acetate extract	Acetone extract	Ethanol extract	Ethyl acetate extract	Acetone extract
	MIC*					
B. subtilis	$1/8_E + 1/4_A$	$1/4_E + 1/8_A$	$1/4_E + 1/16_A$	$1/8_E + 1/4_A$	$1/4_E + 1/8_A$	/
K. pneumoniae	/	/	/	$1/4_E + 1/4_A$	/	$1/4_E + 1/4_A$
P. aeruginosa	$1/4_E + 1/4_A$	/	/	$1/4_E + 1/4_A$	/	/
P. mirabilis	$1/4_E + 1/8_A$	/	/	$1/4_E + 1/4_A$	/	/

"/" no synergism; E – extract; A – antibiotic.
*The most active combination.

Table 6. Synergism between *C. vulgare* extracts and antibiotics.

3.2.2.3. Interaction between C. nigricans extracts and antibiotics

The results of combined acting of ethanol, ethyl acetate and acetone extract of *C. nigricans* and antibiotics (cephalexin and gentamicin) expressed in FICI are presented in **Table 7**. Synergistic and indifferent effects were observed. FICI was ranged in intervals from 0.30 to 1.56. The ethanol extract exhibited the best synergistic capacity with antibiotics.

The ethanol extract synergistically acted with both antibiotics against *B. subtilis*, *K. pneumoniae* and *P. mirabilis* (**Table 8**). The strain of *Proteus mirabilis* was resistant to gentamicin and cephalexin, but in combination with ethanol and ethyl acetate extract, the MIC of antibiotics was reduced by 32-fold.

Bacteria	Ethanol extract		Ethyl acetate extract		Acetone extract	
	GEN	CEF	GEN	GEN	CEF	GEN
B. subtilis	0.38 (S)	0.38 (S)	1.29 (I)	1.29 (I)	1.29 (I)	1.29 (I)
K. pneumoniae	0.40 (S)	0.37 (S)	1.47 (I)	1.33 (I)	1.56 (I)	1.33 (I)
S. aureus	1.2 (I)	1.07 (I)	1.27 (I)	1.23 (I)	1.29 (I)	1.23 (I)
E. coli	1.35 (I)	1.44 (I)	1.47 (I)	1.33 (I)	1.56 (I)	1.33 (I)
P. aeruginosa	1.33 (I)	1.33 (I)	1.33 (I)	1.33 (I)	1.33 (I)	1.33 (I)
P. mirabilis	0.30 (S)	0.37 (S)	0.30 (S)	1.07 (I)	0.40 (S)	0.40 (S)
E. coli ATCC 25922	1.41 (I)	1.56 (I)	1.41 (I)	1.56 (I)	1.41 (I)	1.41 (I)
S. aureus ATCC 25923	1.12 (I)	1.24 (I)	1.32 (I)	1.12 (I)	1.42 (I)	1.24 (I)
P. aeruginosa ATCC 27853	0.44 (S)	1.24 (I)	1.12 (I)	1.20 (I)	1.12 (I)	1.24 (I)

GEN: gentamicin; CEF: cephalexin.

Table 7. Interaction between *C. nigricans* extracts and antibiotics expressed as FICI.

Bacteria	Gentamicin			Cephalexin	
	Ethanol extract	Ethyl acetate extract	Acetone extract	Ethanol extract	Acetone extract
	MIC*				
B. subtilis	$1/8_E + 1/8_A$	/	/	$1/8_E + 1/8_A$	/
K. pneumoniae	$1/4_E + 1/16_A$	/	/	$1/32_E + 1/4_A$	/
P. mirabilis	$1/8_E + 1/32_A$	$1/8_E + 1/32_A$	$1/16_E + 1/4_A$	$1/4_E + 1/32_A$	$1/4_E + 1/16_A$
P. aeruginosa ATCC 27853	$1/4_E + 1/8_A$	/	/	/	/

"/" no synergism; E – extract; A – antibiotic.
*The most active combination.

Table 8. Synergism between *C. nigricans* extracts and antibiotics.

3.2.2.4. Interaction between C. intybus extracts and antibiotics

The results of combined acting of ethanol, ethyl acetate and acetone extract of *C. intybus* and antibiotics (amoxicillin and chloramphenicol) expressed in FICI are presented in **Table 9**. Additive and indifferent effects were observed. FICI was ranged in intervals from 0.56 to 1.37. The results demonstrated that extracts increased activity of amoxicillin better than activity of chloramphenicol. The most active combinations were with ethanol extract in relation to *B. subtilis* and *P. mirabilis*. For these combinations, additive effects were observed, and MICs of antibiotics decreased twofold. On the other side, Ahmad and Aquil [75] noticed synergism between ethanol extract and tetracycline, chloramphenicol and ciprofloxacin.

Bacteria	Ethanol extract		Ethyl acetate extract		Acetone extract	
	CHL	AMO	CHL	AMO	CHL	AMO
B. subtilis	0.56 (A)	0.61 (A)	1.37 (I)	1.37 (I)	0.91 (A)	0.86 (A)
K. pneumoniae	0.68 (A)	0.68 (A)	1.37 (I)	0.84 (A)	0.91 (A)	0.68 (A)
S. aureus	1.29 (I)	0.7 (A)	1.37 (I)	0.62 (A)	0.74 (A)	0.59 (A)
E. coli	1.37 (I)	1.37 (I)	1.37 (I)	0.7 (A)	1.37 (I)	0.7 (A)
P. aeruginosa	1.37 (I)	1.37 (I)	1.37 (I)	1.37 (I)	1.37 (I)	1.37 (I)
P. mirabilis	1.37 (I)	0.56 (A)	1.37 (I)	0.7 (A)	1.37 (I)	0.67 (A)
E. coli ATCC 25922	1.37 (I)	1.37 (I)	1.37 (I)	1.37 (I)	1.37 (I)	0.87 (A)
S. aureus ATCC 25923	1.37 (I)	0.7 (A)	1.37 (I)	1.37 (I)	1.37 (I)	1.37 (I)
P. aeruginosa ATCC 27853	1.37 (I)	1.37 (I)	1.37 (I)	1.37 (I)	1.37 (I)	1.37 (I)

CHL: chloramphenicol; AMO: amoxicillin.

Table 9. Interaction between ***C. intybus*** extracts and antibiotics expressed as FICI.

Bacteria	Ethanol extract		Ethyl acetate extract		Acetone extract	
	GEN	CEF	GEN	CEF	GEN	CEF
B. subtilis	1.13 (I)	1.29 (I)	1.031 (I)	1.24 (I)	1.29 (I)	0.56 (A)
K. pneumoniae	1.30 (I)	1.33 (I)	0.75 (A)	1.37 (I)	1.24 (I)	1.30 (I)
S. aureus	1.29 (I)	1.23 (I)	0.56 (A)	1.12 (I)	1.07 (I)	1.18 (I)
E. coli	1.56 (I)	1.75 (I)	2.00 (I)	1.67 (I)	1.67 (I)	1.33 (I)
P. aeruginosa	1.33 (I)	1.33 (I)	1.031 (I)	1.34 (I)	1.37 (I)	1.37 (I)
P. mirabilis	1.17 (I)	1.07 (I)	0.84 (A)	1.37 (I)	0.68 (A)	0.91 (A)
E. coli ATCC 25922	1.18 (I)	1.30 (I)	0.63 (A)	1.33 (I)	1.33 (I)	1.35 (I)
S. aureus ATCC 25923	1.07 (I)	1.12 (I)	0.75 (A)	1.33 (I)	1.19 (I)	1.24 (I)
P. aeruginosa ATCC 27853	1.12 (I)	1.20 (I)	1.25 (I)	1.33 (I)	1.37 (I)	1.19 (I)

GEN: gentamicin; CEF: cephalexin.

Table 10. Interaction between *D. pentaphyllum* extracts and antibiotics expressed as FICI.

3.2.2.5. Interaction between D. pentaphyllum extracts and antibiotics

The results of combined acting of ethanol, ethyl acetate and acetone extract of *D. pentaphyllum* and antibiotics (cephalexin and gentamicin) expressed in FICI are presented in **Table 10**. Additive and indifferent effects were observed. FICI was ranged from 0.56 to 2.0. The ethanol extract indifferently acted with antibiotics. Additive effect was noticed in combination with ethyl acetate and acetone extract. In these combinations, MIC values of antibiotics were decreased two times in presence of 1/4 MIC of extracts.

4. Concluding remarks

The problem of bacterial resistance is growing, and the outlook for the use of antibacterial drugs in the future is still uncertain. Even though pharmacological industries have produced a number of new antibiotics in the last few decades, resistance to these drugs by bacteria has increased. Plants are valuable sources of new and biologically active molecules possessing antibacterial properties. This activity can be attributable both to direct action against bacteria or as synergistic activity with antibiotics. The *in vitro* synergistic activity of plant active compounds against multidrug-resistant bacteria has been widely shown by the numerous scientific studies. This progress in synergy research enhances the possibility of designing new antibacterial agents of plant origin for the treatment of infections. However, the mechanisms underlying these synergy effects are still poorly explored. Only with exact knowledge of these mechanisms, it will be possible to develop a new generation of standardized, effective preparations. Furthermore, *in vivo* testing of activity, toxicity and bioavailability will determine

their actual relevance for treatment of human infection diseases. Finally, an excellent database of active compounds is formed, and future studies on bioavailability, pharmacodynamics and mechanism of action will contribute in the development of new antibacterial agents.

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Section 2

Control by Novel Technologies

Chapter 3

Antibacterial Activity of Metallic Nanoparticles

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Additional information is available at the end of the chapter

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Abstract

Metallic nanoparticles (NPs) for medical applications have been documented since earlier times. Advancement in modern medicines results in an increase in utilization of NPs for medical purposes due to their antibacterial, antiviral, antifungal, anti-inflammatory, and antiangiogenic properties. In this chapter, three metallic NPs are studied extensively as powerful nanoweapons for the destruction of bacteria. Recent research gives evidence that metallic NPs are very effective in supporting antimicrobial activities. The chemically and laser-ablated silver, gold, and copper NPs exhibited enhanced antibacterial activity than previously reported. The antibacterial mechanism was found dose- and size-dependent and was more profound for Gram-negative bacterium as compared to Gram-positive ones. The dose calculations of minimum inhibitory concentration (MIC) with NPs have been calculated for both Gram-positive and Gram-negative bacteria. The maximum zone of inhibition by disk diffusion was also experimented against various bacteria. These NPs exhibit excellent performance physically, catalytically, and chemically. Present study will be beneficial in areas of environment, information technology, health, cosmetics, and food department. This chapter will cover the details of fabrication and antibacterial activity results of silver, gold, and copper NPs. This chapter endeavors to demonstrate the use of metallic NPs as an alternative antibacterial nanobiotics.

Keywords: laser ablation synthesis, wet chemical technique, antibacterial mechanism, Gram-positive bacteria, Gram-negative bacteria

1. Introduction

Recently, metallic nanomaterials have become the most enormous and rapid emerging materials of science areas. The increased attention of nanomaterial fabrication, especially nanoparticles (NPs), is due to their fascinating properties revealed by their size, high surface area, and extraordinary surface activity exhibiting outstanding catalytic, electrical, and optical properties. Thus, metallic NPs have participated in extensive applications of research methodology

and in advance micro- and nanotechnologies. They are proved as excellent heterogeneous catalysis, used in thin-film fabrication technology, in electronics, and in the manufacturing of microelectronic devices [1]. For controlled synthesis of NPs, the traditional colloid techniques are shared with modern techniques for [1]. Metallic NPs have powerful absorption spectrum in visible range, which is due to electron coherent oscillations on the surface of particles, called surface plasmon resonance (SPR). The SPR spectrum of metallic NPs has multiple applications in the field of biotechnology and has drawn enormous attraction recently [2, 3].

Silver is one of the widely studied metals during the last decades in nanotechnology research. Its distribution of size, stability, morphology, and surface charge/modification each plays a vital role in many scientific fields [1]. Its main features provide improvement in photography, microelectronics, photonics, photocatalysis, lithography, and antimicrobial and antifungal activity [4–6].

Gold (Au) NPs are also famous candidates for medical therapy, cancer treatment, gene therapy, diagnostics, drug delivery, and biological purposes [7–9]. Its main advantage is its simple formation by chemical reduction and exhibiting low toxicity. To improve the capability of gold NPs, different techniques have been employed that provide various dimensions of NPs and its functionalization [10–12]. Due to inert characteristics of gold NPs, it is well known for biocompatibility, but its cytotoxicity mostly depends on nanoparticle's size having precise concentration [13].

The next prominent metal is copper (Cu) that has similar optical, electrical, and thermal characteristics like silver and gold. The only disadvantage is its oxidation state during the synthesis process; thus, it is the major challenge for any scientist. Although gold is costly as compared to silver and copper, consequently the fabrication of the latter NPs develops into more complimentary in recent research [14]. Copper NPs provide an ideal compromise between its novel properties and cost, thus proving an important material for industry. In fact, its vast field of applications is magnetic media for storage devices, solar energy transformations, electronics, and catalysis, and in addition, it has revealed a promising antimicrobial action [15].

1.1. Synthesis methods for metallic NPs

Fabrication of metallic NPs can be achieved by three ways: (i) physical methods in which metallic aggregates subdivided mechanically such as by laser ablation, vapor deposition, wire discharge, and mechanical milling; (ii) chemical methods in which metallic atoms produce nucleation and then growth occurs; and (iii) biological methods in which metallic NPs are formed by the reduction of metallic salts with any plant serum. The physical techniques yield dispersions with broad distribution of particle size. Chemical methods such as salt reduction are convenient method for the controlled sized particles [16].

1.1.1. Laser ablation in liquid (LAL)

One of the advance and toxic-free techniques for nanoparticle fabrication is laser-ablated solid metal targets. In this technique, bulk target is positioned in any liquid environment and ablated by a pulsed laser. From ablated portion of the target, a dense plume of vapor and atomic clusters is ejected into the liquid environment and, thus, has rapid formation of NPs. This technology generates small, surfactant-free, and monodisperse NPs, having many advantages on chemical reduction technique [17–19] due to the use of toxic reducing agents. The size, dispersion, and composition of NPs generated by LAL method dispersed in liquid

medium can be well controlled [20–22] by adjusting the target, liquid type, and laser parameters, like wavelength, fluence, and pulse duration [23].

1.1.2. Wet chemical method for nanoparticle production

The second traditional method is wet chemistry mostly utilized to fabricate metallic NPs. This technique also has the ability to produce NPs of controlled morphology, composition, and crystallinity. Here, chemical reduction of salts generates comparatively larger NPs as compared to that of LAL technique [24, 25]. In wet chemical method, the formation of NPs has undergone by the following processes:

(A) Nucleation: The main process of synthesis initiates with the process of nucleation, in which a new phase particle called "seed" has been formed in a previous system of single phase (a homogenous solution of salt) [26]. With the additional attachment of metal atoms to this seed from solution, further growth of NPs is done. The shape of the NPs and its growth depends on the varying conditions of reaction [27–31].

(B) Seeding process: During the reduction process of salt, the metallic atom concentration enhances with the decomposition of precursor till a supersaturated state has been reached [32]. At this stage, the atoms begin to agglomerate and form nuclei of precise dimension called seed which further grow into crystallite [32]. Typically, precursors are metallic salts, mostly used as reducing agents [25, 32]. Capping agents are used to stabilize the metallic seed by preventing agglomeration and maintaining the nuclei size [33]. Seed-mediated nuclei growth process is one of the most efficient and effective processes because of low activation energy requirement for metal reduction process on to the preformed seeds to get NPs of controlled size and shape [34, 35].

(C) Nanoparticle formation: The nanoparticles are formed by the controlled competition of surface energy enhancement and a bulk energy diminution. An enhancement in the surface energy helps in dissolution, while decrease in bulk energy favors the process of growth [32]. Continued addition of atoms allows the seed to increase in size in a uniform manner. Thus, solutions of suitable metallic precursor, reducing agent and capping agent, are the basic necessity for the formation of metallic nanoparticles via chemical reduction method.

1.2. Antimicrobial mechanism of metallic NPs

Metal and metallic salts are well known for antibacterial mechanism for centuries as silver pots were used for drinking water from 4000 BCE [36, 37]. Recent research in nanophysics capable the scientist to study the antibacterial properties on various metallic NPs [38, 39].

Basic toxic antibacterial mechanism of metallic NPs is still under debate, but three main mechanisms are supposed which include, firstly, the formation of reactive oxidative species (ROS); secondly, releasing process of ions; and, finally, interaction of NPs with the cell membrane (**Figure 1**). Metallic NPs as compared to their salts have enhanced potential to combat bacterial infections [40–43]. Mostly, the size of NPs influences the antibacterial mechanism [44–49].

1.2.1. Entering the cell

The first step of antibacterial mechanism is the metallic ions of nanometer range attached to the cell via transmembrane protein. After attaching to bacterial cells, producing structural

changes in the cell membrane and blocking the transport channels [6, 50], the whole process is size dependent. Small NPs are more efficient, while larger NPs have a higher absolute surface area permitting for better adhesion property of van der Waals forces. Then, NPs may be internalized, produce ionization within the cell, and damage intracellular structures resulting in cell death (**Figure 1**) [40].

1.2.2. Reactive oxidative species (ROS) generation

The production of reactive oxidative species (ROS) by metal NPs plays a large role in their antibacterial effectiveness (**Figure 1**). ROS consist of short-lived oxidants, such as superoxide radicals (O^{-2}), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^{-1}), and singlet oxygen (O^{-2}) [51, 52]. Due to the high reactivity of these species, ROS can cause damage to peptidoglycan and cell membranes, DNA, mRNA, ribosomes, and proteins [42]. ROS can also inhibit transcription, translation, enzymatic activity, and the electron transport chain [42, 51]. Some metal oxide NPs rely on the generation of ROS as a main mechanism of toxicity [6, 41, 50].

1.2.3. Protein inactivation and DNA destruction

Metal atoms have the tendency to attach with thiol group of enzymes and finally deactivate the function of enzymes. It is also suggested that metal ions attach themselves between the pyrimidine and purine base pairs disturbing the bonding of hydrogen between two strands of antiparallel and destruct the molecule of DNA (**Figure 1**). Although this has to be further investigated, but this is true that metal ions have tendency to attach with DNA, once they go into the cell [53].

1.3. Detection methods

There are various direct and indirect methods for the microbial growth measurement. In direct method, mostly microbial effects are evaluated by viable technique of plate count, serial dilution,

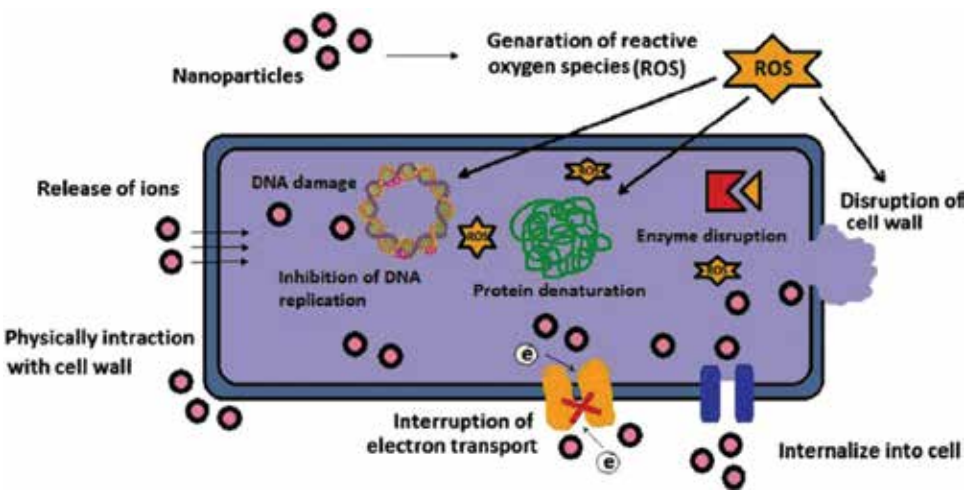

Figure 1. Various mechanisms of antimicrobial activity of the metal NPs [40].

and disk diffusion method, while indirect methods are analyzed by turbidity, dry weight, and by metabolic activity. In brief, short description of direct and indirect methods is as follows.

1.3.1. Viable plate count method

In the plate count techniques, Petri dishes are used for each reading. Agar plate is prepared, and one plate of inoculums and other plates with inoculum and nanoparticle solutions are spread on it with the help of sterile spreader; this plate is incubated for 24 hours at 37°C and then counted the colonies of each plate. Then, the inhibition percentage growth with each reading is calculated [54, 55].

1.3.2. Disk diffusion method

The prepared culture of bacteria is mixed in nutrient broth to form liquid culture. Then, sterile nutrient agar solution is prepared and put into dishes and waited to be solidified. After that, holes are generated with the help of cork borer. Bacterial culture is spread on the agar with sterile cotton bud. Holes are filled with solution of metallic NPs and stabilized; after that, the plates are incubated for 24 hours at 37°C. The zones of inhibition are observed the next day and calculated with standard error. For accurate analysis, proper repetition of the experiments is done for microbial study [16].

1.3.3. Estimating bacterial numbers by indirect methods

Indirect methods are basically referred as time-consuming methods, and the large number of samples is prepared at a time. A spectrophotometer is utilized to analyze the turbidity process by measuring the quantity of light that transmits through a bacterial cell suspension. As more transmission occurs through suspension, it means that turbidity decreases, indicating the reduction in bacterial cells and vice versa [56, 57].

2. Antibacterial mechanism of metallic nanoparticles

2.1. Antibacterial mechanism of silver nanoparticles (SNPs)

In recent research, Zafar et.al. [57] have worked on the antibacterial mechanism of silver NPs fabricated by laser ablation and by chemical method having different sizes and examined these NPs against Gram-positive (+) bacteria (*Bacillus subtilis* and *Staphylococcus aureus)* and Gram-negative (−) bacteria *(Salmonella*, *Escherichia coli*, and *Klebsiella pneumonia)* by turbidity method and by well diffusion method.

2.1.1. Turbidity method

A medium (nutrient broth) (5.00 ml) was arranged in the labeled tubes and then sterilized, thus forming broth media in transparent form. The bacterium culture was then ready by shifting an identified Gram-positive [*S. aureus* and *B. subtilis*] and Gram-negative bacteria's culture

[*Salmonella* and *E. coli*] into four (4) tubes of liquid nutrient broth with platinum wire. After the process of inoculation, tubes were incubated at 37°C for 1 day. The next day, a visible growth was observed in the tubes of culture. Then, these tubes of bacterium culture were strictly checked by various identification tests. Already measured doses (low, medium, and high) of sterilized SNPs were tested against this bacterium growth. Each tube had facilitated with medium (5 ml), inoculums (200 μl), plus the measured dose of SNP stock solution. Three tubes were arranged for each reading. Negative control tube had broth and NPs only, and positive control tube had broth and inoculums only. All tubes were incubated at 37°C for 24 hours.

Turbidity of tubes was confirmed by Elisa reader, a spectrophotometer having a filter of 600 nm wavelength. The values of optical density (OD) from Elisa reader in absorption mode represented the bacterial growth of labeled samples. Three OD values of each dose were taken, and their mean was calculated with standard deviation (St. Dev.). Three designed doses of SNPs were taken. Bacterial growth in nutrient broth revealed the total percentage (%) of bacterium growth. The percentage (%) change in the growth of bacterium was measured against OD value of pure nutrient media of broth (reference OD value). Similarly, the maximum calculated growth of *S. aureus*, *B. Subtilis*, *Salmonella*, and *E. coli* was 75.19, 68.5, 74.3, and 71.9%, respectively [57]. Contaminant in microorganism's growth was only 1.5% which is ignorable against growth of bacterium (**Table 1**).

Same process had been applied for the calculation of all doses. The growth of bacteria decreases with increasing dose of NPs [57]. Chemically synthesized SNPs inhibited 67.02% *S. aureus*, whereas *E. coli* was inhibited up to 87.9%. It indicated that more doses should design for full inhibition of *S. aureus*. *B. subtilis* was inhibited 39.9%, and *Salmonella* was inhibited only 80.2%. Laser-ablated SNPs inhibited the bacterium with higher efficiency having low dose (2.10 μg/ml). For example, 67.02% growth calculated with dose (2.35 μg/ml) for *S. aureus* diminished up to 92.0% with dose (2.10 μg/ml), whereas no growth appeared for *E. coli* with designed high doses. Similarly, the calculated growth of *Salmonella* is 18.0% that shows that the growth is reduced up to 82% [57].

Two sized SNPs were considered for antibacterial evaluation. The chemically synthesized SNPs have size range ~30–40 nm (Sample S1), while the laser-ablated SNPs had size range ~20–30 nm (Sample S2). Equal doses of laser-ablated NPs provided enhanced inhibition against each bacterium. Chemically synthesized SNPs exhibit less efficiency with respect to laser ablated SNPs. The first reason was slightly larger-sized nanoparticles, and, secondly, it might be possible that some chemical species may be adsorbed on the surface of NPs which declined the antibacterial

Inoculum	**OD value of culture (S)**	**OD value for culture** **R=S – 0.065±0.001**	**Percentage growth** $=\frac{R}{S} \times 100$ **(%St. Dev)**
S. aureus	0.262 ± 0.004	0.197 ± 0.005	75.19 ± 0.9
E. coli	0.253 ± 0.003	0.188 ± 0.004	74.3 ± 0.7
B. subtilis	0.207 ± 0.001	0.142 ± 0.002	68.5 ± 0.3
Salmonella	0.232 ± 0.001	0.167 ± 0.002	72 ± 0.3

Table 1. Percentage growth of *S. aureus*, *E. coli, B. subtilis*, and *Salmonella* observed in broth medium [57].

performance. Hence, for the pureness of sample, chemical fabrication has many restrictions to employ these NPs for the biological, catalytic, and sensing applications.

For statistical analysis and regression line, along X-axis, dose of SNPs was taken, whereas along Y-axis, percentage growth was plotted. Regression line has been plotted by using the values from **Table 2** in given equations:

$$b = \frac{\sum XY - \frac{\sum X \sum Y}{N}}{\sum X^2 - \frac{(\sum X)^2}{N}} \quad (1)$$

$$a = \frac{\sum Y}{N} - b\frac{\sum X}{N} \quad (2)$$

$$Y = a + bX \quad (3)$$

From regression lines (**Figure 2**), the calculated minimum inhibitory concentration (MIC) of chemically synthesized NPs (30–40 nm) was estimated as ~2.8, 13.5, 4.37, and 2.81 μg/ml for *E. coli*, *B. subtilis*, *S. aureus*, and *Salmonella*, respectively, whereas the calculated MIC for laser-ablated NPs (20–30 nm) were ~2.10, 2.68, and 2.36 μg/ml for *E. coli*, *Salmonella*, and *S. aureus*, respectively. Student's t-test with $p < 0.05$ was applied to all experimental data.

2.1.2. Well diffusion method for silver nanoparticles

Antibacterial mechanism was further evaluated against *S. aureus* and *E. coli* with same doses of SNPs by well diffusion method [57]. The results [57] obviously determined the zone of inhibition for laser-ablated and chemically synthesized SNPs, and no zone appeared in the control. Further, the inhibition zones due to laser-ablated NPs are considerably enhanced as compared to chemically synthesized SNPs as shown in **Figure 3** and **Table 3**.

In previous studies, Laszlo Korosi fabricated SNPs by LAL method and concluded that size of SNPs strongly effected on the antibacterial mechanism. The antibacterial activity of SNPs

Strain	**Percentage growth inhibition of chemically synthesized SNPs**			**Percentage growth inhibition of laser-ablated SNPs**		
	(% + standard error)			**(% + standard error)**		
	Low dose (0.78μg/ml)	**Medium dose (1.57 μg/ml)**	**High dose (2.35μg/ml)**	**Low dose (0.78μg/ml_**	**Medium dose (1.57 μg/ml_**	**High dose (2.10μg/ml)**
S. aureus	41.5 ± 0.5	53.8 ± 1.7	67.02 ± 0.9	52.3 ± 0.5	76.3 ± 1.7	92.0 ± 0.9
E. coli	46.81 ± 1.7	63.2 ± 0.9	87.9 ± 0.9	57.1 ± 1.7	84.5 ± 0.9	99.81 ± 0.9
Salmonella	38.1 ± 0.3	58.9 ± 0.3	80.2 ± 0.3	40.5 ± 0.3	68 ± 0.3	82 ± 0.3
B. subtilis	31.5 ± 0.3	33.7 ± 0.3	39.9 ± 0.3	-	-	-

Table 2. Low-, medium-, and high-dose effects of laser-ablated SNPs and chemically synthesized SNPs on *S. aureus, E. coli, Salmonella,* and *B. subtilis* [57].

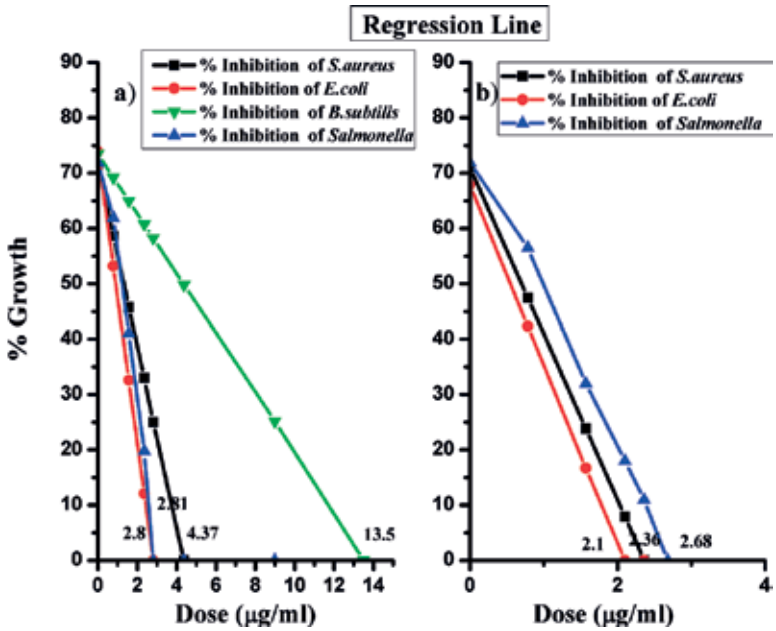

Figure 2. Regression line and percentage inhibition of *S. aureus*, *E. coli*, *B. subtilis*, and *Salmonella* by silver nanoparticles synthesized by (a) chemical and (b) laser-ablated method [57].

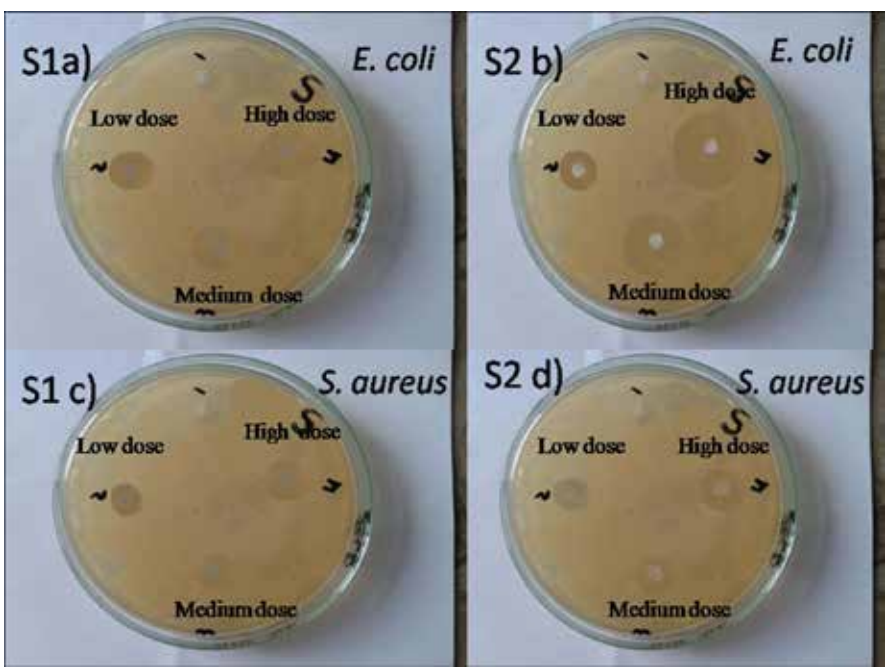

Figure 3. Well diffusion method: chemically synthesized (S1) SNPs against (a) *E. coli* and (b) *S. aureus* and laser-ablated (S2) SNPs against (c) *E. coli* and (d) *S. aureus* [57].

Strain	Zone of inhibition (mm) of chemically synthesized Ag nanoparticles (% + standard error)			Zone of inhibition (mm) of laser-ablated Ag nanoparticles (% + standard error)		
	Low dose (0.78 μg/ml)	Medium dose (1.57 μg/ml)	High dose (2.35 μg/ml)	Low dose (0.78 μg/ml)	Medium dose (1.57 μg/ml)	High dose (2.10 μg/ml)
S. aureus	11 ± 0.2	12 ± 0.3	13 ± 0.2	11 ± 0.5	13 ± 0.2	15 ± 0.3
E. coli	16 ± 1.7	19 ± 0.9	21 ± 0.9	18 ± 1.7	21 ± 0.3	23 ± 0.2

Table 3. Zone of inhibition for low, medium, and high doses of chemically synthesized and laser-ablated SNPs on *S. aureus* and *E. coli* [57].

(3 nm) was very much high against *E. coli* and SNPs (20 nm) revealed low antibacterial performance even though with concentration of 5 mg/L. However, *S. aureus* was partially with stand SNPs (3 nm) up to 5 mg/L [58]. In present study, SNPs give the best maximum zone (~ 15 and 23 mm for *S. aureus* and *E. coli*).

2.2. Antibacterial mechanism of gold nanoparticles

The antibacterial efficiency of two samples of chemically synthesized gold nanoparticles (GNPs) was evaluated by Shamaila et.al. [59]. The first sample of GNPs, G1, has a size range of ~ 6–34 nm, and the second sample, G2, has a size range ~ 20–40 nm. Their potential efficacy was checked against Gram-negative bacteria *(E. coli*, *K. pneumonia*) and Gram-positive bacteria (*S. aureus*, *B. subtilis*) by the method of turbidity and well diffusion technique [59].

2.2.1. Turbidity method

Same process was used for turbidity method as described in Section 2.1.1. Three doses were selected, low dose of ~ 1.35 μg, medium dose of ~ 2.03 μg, and High dose of 2.70 ~ μg GNPs (**Table 4**). The mean OD values with standard deviation of all test tubes for all doses were calculated carefully. The antimicrobial potential of GNPs against *S. aureus*, *K. pneumonia*, *B. subtilis*, and *E. coli* was statistically worked out. The percentage (%) declined, or enhancement in the bacterium growth was anticipated against OD value of pure (nutrient broth) media as a reference.

Observing the calculations of **Table 4**, it was concluded that GNPs of G1 sample (size range 6–34 nm) which is smaller than NPs of G2 sample revealed the reduction in percentage (%) growth. For *S. aureus*, maximum growth was calculated ~75.19% without dose [57] which declined up to 22.4%, whereas in the case of *E. coli*, maximum percentage (%) growth (74.3%) was diminished up to 6.2%. Thus, for complete percentage (%) growth inhibition for *S. aureus*, further dose will be required. For *B. subtilis*, reduction in percentage (%) growth was 45.2% only, and *K. pneumonia* was diminished up to 10%. The second set of sample G2 of GNPs with size ~ 20–40 nm intended the following behavior. *S. aureus* was reduced up to 23.7%, whereas *E. coli percentage (*%) growth decreased up to 6.2%. Percentage (%) growth reduction of *B. subtilis* was only 49.4% and percentage (%) inhibition of *K. pneumonia* was up to 13.8%.

Strain	Percentage reduction in growth of sample G1 of gold NPs (% + standard error)			Percentage reduction in growth of sample G2 of gold NPs (% + standard error)		
	Low dose (1.35μg/ml)	Medium dose (2.03 μg/ml)	High dose (2.7μg/ml)	Low dose (1.35μg/ml)	Medium dose (2.03 μg/ml)	High dose (2.7μg/ml)
S. aureus	46.4 ± 0.4	33.75 ± 1.2	22.4 ± 0.8	53.1± 0.6	40 ± 1.4	23.7 ± 0.8
E. coli	45.2 ± 1.4	26.6 ± 0.8	4.2 ± 0.8	47.6 ± 1.2	32.2 ± 0.8	6.2 ± 0.8
K. pneumonia	38.3 ± 0.4	22.8 ± 0.4	10.0 ± 0.6	42.7 ± 0.4	26.8 ± 0.4	13.8 ± 0.4
B. subtilis	57.8 ± 0.4	52.1 ± 0.6	45.2 ± 0.4	60.7 ± 0.4	56.3 ± 0.6	49.4 ± 0.4

Table 4. Antibacterial effects of two sized G1 and G2 GNPs against four bacteria [59].

The comparison of antibacterial results of two sets of samples of NPs having different sizes is given in **Table 4**. Equal doses of these samples were taken which give inhibition for each human pathogen. Sample G1 NPs exhibited more antibacterial mechanism with respect to sample G2 NPs due to size difference. There is difference in the antimicrobial results of GNPs for Gram-negative and Gram-positive bacterium. This is due to the difference in its membrane structure, i.e., the thickness of layer peptidoglycan and wide variation range between both types of bacteria [58]. Thus, inhibition of Gram-positive bacteria is achieved with higher dose of NPs.

Regression line has been plotted between doses and percentage (%) growth, exactly like silver NPs with given equations in Section 2.1.1. Curves of growth inhibition were plotted for G1 and G2 NPs. From **Figure 4**, the MIC values of sample G1 (size 7–34 nm) are calculated ~ 2.93, 3.92, 3.15, and 7.56 μg/ml for *E. coli*, *S. aureus*, *K. pneumonia*, and *B. subtilis*, respectively, whereas the MIC readings of sample G2 (size 20–40 nm) reveal 2.96, 3.98, 3.3, and 8.61 μg/ml for *E. coli*, *S. aureus*, *K. pneumonia*, and *B. subtilis*, *respectively*. A. Nanda et al. [60] observed the bactericidal potential of GNPs (40–80 nm) by agar method of diffusion and examined inhibition zone of *K. pneumonia* and *E. coli.* No noteworthy conclusions have been reported earlier for GNPs with respect to performance of SNPs [61, 62]. However, in the current project, GNPs reveal the best performance, and maximum zone was observed with dose measurement against human pathogens.

2.2.2. Well diffusion method of gold nanoparticles

The doses were further tested to examine the zone of inhibition of *S. aureus* and *E. coli* by well diffusion method. Agar plates were used to evaluate the antibacterial action of GNPs against human pathogen bacteria *E. coli* and *S. aureus*, with same method as described for SNPs. The suspension of NPs (5, 15, and 30 μl) was poured into wells named 2, 3, and 4. The inhibitory zones were measured after incubation at 37°C for 24 hours.

G1 NPs and G2 NP results from method of well diffusion against *E. coli* and *S. aureus* bacteria are given in **Figure 5** and in **Table 5**, where inhibition zones for GNPs are obvious. The inhibition zone of G1 NPs is larger than G2 NPs as confirmed (**Table 5**).

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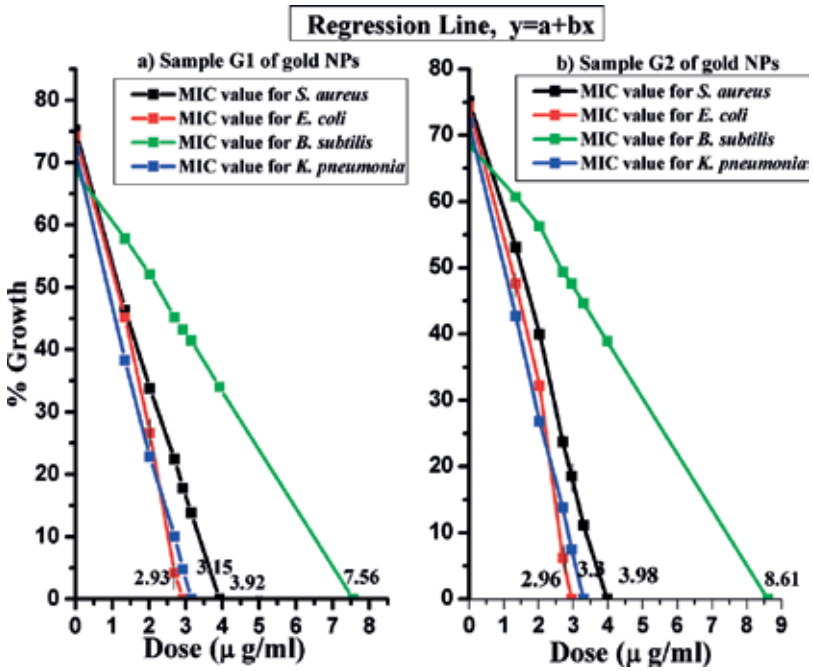

Figure 4. Calculation of doses for each bacterium: (a) sample G1 and (b) sample G2 of GNPs [59].

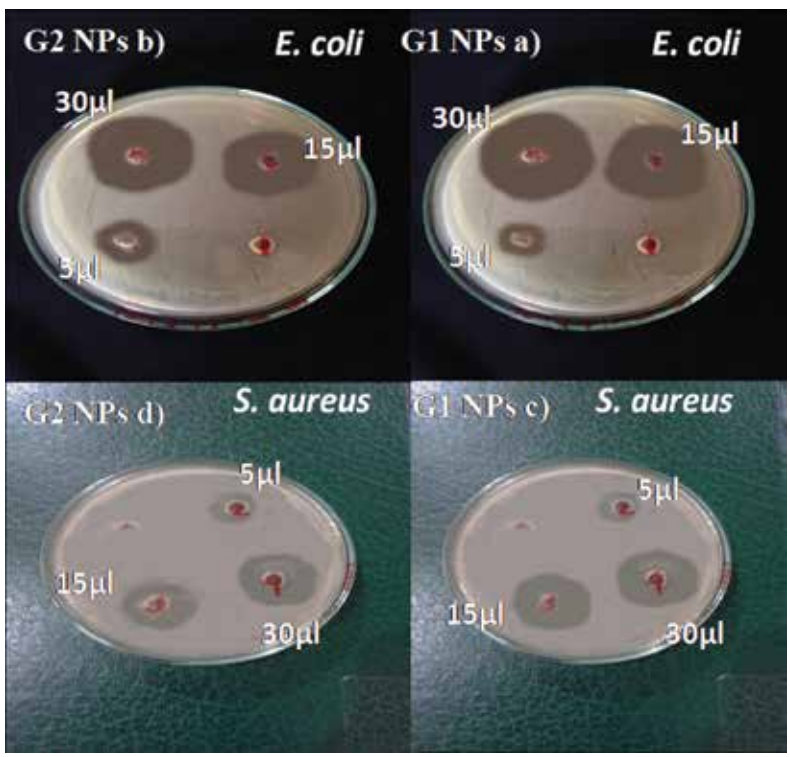

Figure 5. Well diffusion method of two sized GNPs for *E. coli* (a, b) and *S. aureus* (c, d) [59].

Micro organisms	Organism category	Dose	Zone of inhibition (mm)		
		(μL)	Control	G2 NPs	G1 NPs
S. aureus	Gram-positive	05	0	13	12
		15	0	20	23
		30	0	22	25
E. coli	Gram-negative	05	0	10	11
		15	0	28	32
		30	0	31	35

Table 5. Zone of inhibitions for samples G1 and G2 of GNPs [59].

The current research examined the calculated inhibition zone for *E. coli* which was ~ 35 mm with sample G1 and ~ 31 mm with sample G2. Similarly, the zone for *S. aureus* is ~ 25 and 22 mm with G1 and G2 samples, respectively. Previously, Nazari et al. calculated the inhibition zone ~ of 14 and 13 mm, for *E. coli* and *S. aureus* with dose of 4000 μg [63]. In present experimentation, GNPs give the best maximum zone (~ 25 and 35 mm for *S. aureus* and *E. coli*).

2.3. Antibacterial mechanism of copper nanoparticles

In the present research of Khalid et al. [64], copper nanoparticles (Cu NPs) were used to evaluate the antibacterial mechanism. The size of chemically synthesized Cu NPs without any stabilizer was rapidly increased. Antibacterial mechanism of laser-ablated Cu NPs (with filter and without filter of MCE membrane) was examined against *E. coli* and *S. aureus* by the turbidity and disk diffusion method [24].

2.3.1. Turbidity method

Three concentrations of Cu NPs were used. Cu NPs were proved to be very toxic for bacteria *E. coli* and *S. aureus*. Three factors are important in the toxicity of Cu NPs such as concentration of NPs, particle size, and bacterial growth [64, 65]. Thus, those experimental conditions were selected so that they exhibited the best curve of dose-response. Three concentrations low (1.96 μM), medium (3.92 μM), and high (5.88 μM) were prepared for Cu NPs. With these doses, the antimicrobial action of Cu NPs was observed with and without filtration of MCE by same turbidity method as described in Section 2.1.1. The MCE membrane allows the NPs to pass up to ~ 0.22 μM size. Four test tubes of each dose of Cu NPs inoculated aseptically in which one contains only control. These all test tubes were incubated for 24 h at 37°C. The percentage growth inhibitions of *S. aureus* and *E. coli* are shown in **Table 6**.

To find out how much maximum dose was required for complete reduction in the bacterial growth, statistical analysis was calculated by the equations given in Section 2.1.1. **Figure 6** represents regression line.

By chemical reduction, Cu NP doses required for maximum growth inhibition of *E. coli* calculated by regression line were 6.07 μM for with membrane and 8.33 μM for without membrane. Similarly, maximum growth inhibition for *S. aureus* was 8.32 μM with membrane and

Dose (μM)	Copper nanoparticles by chemical reduction method					
	With MCE membrane growth inhibition (%)			Without MCE membrane growth inhibition (%)		
	Low dose	Medium dose	High dose	Low dose	Medium dose	High dose
S. aureus	65.1± 0.6	48.06± 1.0	31.8± 0.8	69.6± 0.8	57.1± 1.2	42.1± 0.6
E. coli	58.3± 1.4	34.6± 0.8	1.9± 0.8	68.1± 0.6	49.8± 0.8	27.4± 1.0

Table 6. Antibacterial effects of doses of copper NPs on *E. coli* and *S. aureus*.

11.76 μM without membrane. The significant enhancement in concentration of NPs attributes higher toxicity and reduction in percentage growth of bacteria to minimum values.

2.3.2. Well diffusion method of copper nanoparticles

The zone of inhibition of laser-ablated Cu NPs was further observed [24] by agar method of well diffusion (**Table 7**). The calculations of zone of inhibition for *E. coli* and *S. aureus* are for high doses 28 ± 0.01 mm and 21 ± 0.02 mm, respectively [24]. No zone of inhibition is observed for positive control (**Figure 7**).

2.4. Antibacterial mechanism of metallic nanoparticles

Antimicrobial performance of metallic NPs depends on the features of bacterial species. The main dissimilarity between Gram-negative and Gram-positive bacteria is its membrane structure such

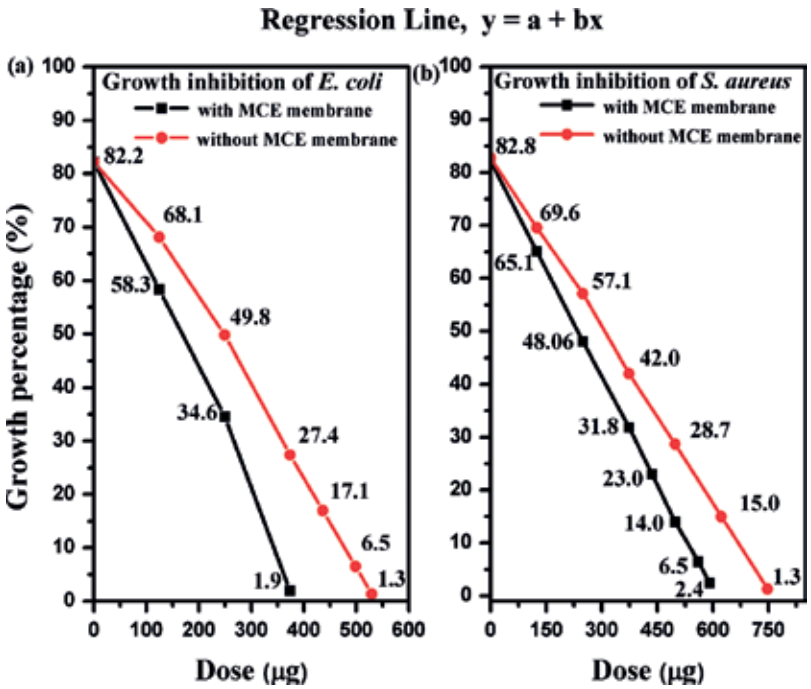

Figure 6. Graphically representation of growth inhibition by Cu NPs for (a) *E. coli* and (b) *S. aureus* estimated by regression line with and without MCE membrane.

Sr. no.	Concentration of nanoparticles	Growth of *E. coli* (%)	Growth of *S. aureus* (%)	Inhibition zone of *E. coli* (in mm)	Inhibition zone of *S. aureus* (in mm)
1	1.9 μM	52.6	60.8	16	14
2	3.9 μM	23.7	42.5	19	16
3	5.8 μM	0.9	19.3	32	-
4	6.9 μM	-	1.3	-	22

Table 7. Effects of copper NPs having low, medium, and high doses on *E. coli* and *S. aureus* [24].

that peptidoglycan layer thickness has the main features of polymer along with amino acid and sugar, thus forming an outside layer of plasma membrane (cell wall) which offers the stability to structure which is responding to the osmotic pressure of cytoplasm. Its range of thickness is higher (50%) in Gram-positive (+) and lower (8%) in Gram-negative (−) bacterium. Gram-positive bacterium comprises peptidoglycan layer in multiple steps having long teichoic acid chain. Conversely, Gram-negative bacteria contain a single and thin peptidoglycan layer with the absence of teichoic acid. Outer membrane has periplasmic and lipopolysaccharide layer [58].

Thus, the main process is first the attachment of metal ions or NPs to the bacterium outer cell wall and then accumulation of protein precursor's layer, which ultimately disables the proton motive force. Metallic NP destabilizes the outer membrane, produces crack in the membrane of plasma, and reduces the synthase activities of the depletion layer of intracellular adenosine triphosphate (ATP) [31], thus reducing the process of metabolism. It destructs the ribosome subunit by t RNA binding and thus finally the total collapse of biological mechanism.

It has been further concluded that the small-sized metallic NPs with larger surface area exhibited some electronic effects which were beneficial to improve the surface attraction of NPs. In addition, the percentage of enhanced surface area straightly interacted with the membrane of microorganism with vast extent and hence bridged an improved relation with bacteria. These two vital factors powerfully enhanced the antimicrobial action of the NPs with high surface

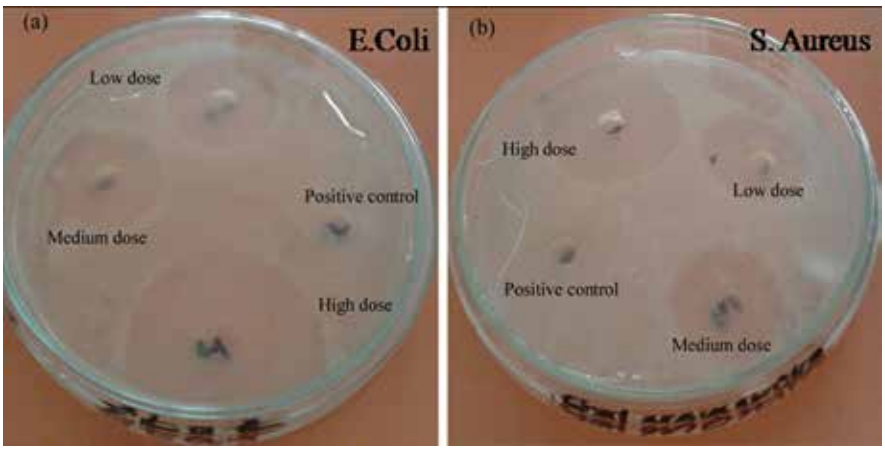

Figure 7. Zone of inhibition by Cu NPs in mm for (a) *E. coli* and (b) *S. aureus* [24].

area. Bacterial proteins (cell wall and cytoplasm) were accountable for the cell performance. These NPs disrupted the normal performance of protein function, which causes the cell's death. NPs basically interacted with soft bases having sulfur or phosphorus components. So, the sulfur of proteins and phosphorus belonging to DNA molecules are favorite attacking sites of NPs. Then, these NPs attached themselves to enzymes (thiol groups), i.e., nicotinamide adenine dinucleotide (NADH) dehydrogenases and destructed its respiratory chain by releasing of oxygen species, thus creating oxidative stress. Consequently, a major damage is occurred in the cell structures and lastly leads to cell loss [21, 59].

Shrivastava et al. [66] have reported that the SNPs affected *S. aureus* in lesser amount. The bacterium *B. subtilis* has been considered a most powerful bacterium (Gram-positive). The reason is that it can stay alive easily in any rigid condition due to endospore formation (stress-resistant) and its formation of DNA externally which can be produced by recombination with original DNA at its last stage and can tolerate under adverse situations. *Salmonella* also exhibits more resistance with respect to *E. coli*.

In the present research work, SNPs exhibited effective antibacterial behavior against *E. coli* bacterium with respect to other bacterium. *S. aureus* exhibited more resistance than *E. coli*; so, selected dose (high ~2.10 μg/ml) is quite enough for maximum inhibition of *E. coli*. Due to variation in peptidoglycan layer in Gram-positive bacteria, more doses of SNPs would be recommended for full inhibition of *Salmonella* and *S. aureus*. The laser-ablated SNPs have demonstrated and elevated antibacterial behavior due to small size and purity of NPs which may be beneficial in wood flooring, coatings, and cotton textile industries.

It is demonstrated that the small-sized GNP sample ~ 6–34 nm displays good bacterial performance than the second GNP sample ~ 20–40 nm. This advantage is by reason of its minute size, enhanced surface-area-to-volume ratio, and fine penetration power. Finally, the small-sized NPs effortlessly bind to the prominent parts of the outer membrane, so causing damages in structure, deprivation, and ultimately cell death. Various GNP doses displayed the best antibacterial action against *E. coli* with respect to other tested bacteria. Similarly, dose of 2.70 μg/ml was quite sufficient for *K. pneumonia* and for *E. coli's* complete inhibition. More NP doses were recommended for total inhibition of *S. aureus* and *B. subtilis* because of their difference of peptidoglycan layer. Here, *B. subtilis* had been considered the most resistive Gram-positive bacteria in the performed experimentation.

It is evident that for both Gram-positive and Gram-negative bacteria, the increase in the concentration of Cu NPs revealed the decrease in percentage growth. With high dose of Cu NPs, a noticeable antibacterial action (growth inhibition ~ 1.9%) was recorded for *E. coli* with filtered NPs (smaller in size); however, at the same time for nonfiltered dose (larger in size), the percentage growth decreases up to only 27%. Similarly, the percentage growth of *S. aureus* for filtered NPs decreased up to 31.8% and for non-filtered up to ~ 42.1% only. The clear difference in percentage (%) growth inhibition for both strains of bacteria was deeply attributed to the concentration of NPs and size of NPs. These outcomes concluded that the further doses of Cu NPs are required to show full antibacterial action against these bacterial strains. The cell wall of Gram-positive bacteria is composed of heavy layer of peptidoglycan (about ~ 20–80 nm), forming a three-dimensional structure of rigid tissues. This rigidity protects the cell wall, provides only fewer sites for Cu NPs, and does not allow to penetrate the NPs [24].

In current research, the observed antibacterial mechanism of all metallic NPs is tested on behalf of concentration and size of NPs. The basic mechanism is that how many metal ions are released because they attributed to their large surface-area-to-volume ratio. These ions directly interact with each bacterium's outer membrane. One possible justification is nanometer size pores of bacteria cell membranes that can give a pathway for the NPs [59]; secondly, the ions released by NPs may fasten to the cell wall of bacteria and crack it. Ions of NPs which are released produce radicals of hydroxyl that may deform the basic function of essential proteins and finally its DNA for the cell death [57–59].

3. Conclusion

The two techniques for fabricating metallic nanoparticles were compared here to specify a broad range of achievable characteristics. This aptitude to give variation in properties of controllable metallic nanoparticles would be eventually profitable for future research relaying on the antibacterial properties of nanoparticles. A direct assessment between metallic NPs generated either by wet chemical technique or LAL method will be supportive in finding the exact trends and mechanisms of antimicrobial, which are currently hot issues of recent advance research. The antibacterial activity was size and dose dependent and was more explicit against Gram-negative bacterium than Gram-positive ones. The fabricated nanoparticles are spherical in shape with polycrystalline nature having various size ranges in nm. Antibacterial behavior of metallic nanoparticles against pathogenic bacteria demonstrates that they can act as an efficient tool for antibacterial. Silver, gold, and copper nanoparticles exhibit their excellent performance physically, catalytically, and chemically because of its larger surface-area-to-volume ratio. Turbidity provides an efficient and fast technique for the estimation of the bacterial development in a liquid. The calculations of MIC are further supportive in calculation of inhibition zone by agar method of diffusion. The present study will be beneficial in environment, information technology, health, cosmetics, and food department fields. Additionally, silver nanoparticles have reduced the life-threatening facts because of their flexible nature with respect to conventional antibiotics. Gold nanoparticles provide benefit in the field of biomedicine, chemistry, and genetics due to their characteristics of functionally active power. In biomedicine, gold NPs can become a vital revolution in the research of drug delivery and cancer therapy. They also proved to be nontoxic and a safe antimicrobial representative due to their powerful functional nature instead of antibiotics. Antimicrobial nanomaterials are potentially eye-catching for a variety of medical applications, i.e., dental procedures. Copper nanoparticles perform best as an antibiotic, antifungal, and antimicrobial agent when utilized for plastics, textiles, and coatings. Copper and its oxide are used for the coating of cellulose of nanofibrillar, a promising nanobiomaterial. The metallic nanoparticles showed potential performance as an antimicrobial against *E. coli*, *S. aureus*, *B. subtilis*, and *Salmonella* and are suggested as an alternative antibacterial nanobiotics.

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Chapter 4

Green-Synthesized Silver Nanoparticles and Their Potential for Antibacterial Applications

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Additional information is available at the end of the chapter

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Abstract

The prevalence of infectious diseases is becoming a worldwide problem, and antimicrobial drugs have long been used for prophylactic and therapeutic purposes, but bacterial resistance has creating serious treatment problems. The development of antibiotic resistance makes scientists around the world to seek new drugs that would be more effective. The use and search for drugs obtained from plants and other natural products have increased in recent years. It is well known that silver and its compounds have strong antibacterial activity. Silver, compared to the other metals, shows higher toxicity to microorganisms, while it exhibits lower toxicity to mammalian cells. The progress in the field of nanotechnology has helped scientists to look for new ways in the development of antibacterial drugs. Silver nanoparticles (AgNPs) are interesting for their wide range of applications, e.g. in pharmaceutical sciences, which include treatment of skin diseases (e.g. acne and dermatitis) and other infectious diseases (e.g. post-surgical infections). Various antibacterial aids, such as antiseptic sprays, have also been developed from AgNPs. In this chapter, we have focused on various synthesis methodologies of AgNPs, antibacterial properties, and the mechanism of action.

Keywords: silver nanoparticles, plant extracts, antibacterial activity, green synthesis, biosynthesis

1. Introduction

Frequent use of antibiotics results in resistance of pathogens against them. This is the health and the life-threatening reality. It is therefore necessary to look for new sources of effective potent drugs. Nature is an inexhaustible source of health-promoting substances. Combination of knowledge in natural medicine with modern technology leads to the discovery of new drugs. One of the most promising sources in recent years has been shown to be plant extracts,

which are rich in antioxidant and antimicrobial compounds that have been used as a nanoparticle synthesis agent [1–3]. Nanotechnology and nanoscience have been established recently as an interdisciplinary subject dealing with biology, chemistry, physics, and engineering. The term "*nano*" is derived from the Greek word dwarf in the meaning of extremely small. The size of nanoparticle is between 1 and 100 nm [4, 5]. Unique biological, chemical, and physical properties of silver nanoparticles (AgNPs) lead to the wide range of applications in spectroscopy, sensors, electronics, catalysis, and pharmaceutical purposes [6]. It is well known that silver has an inhibitory effect toward many bacterial strains and microorganisms commonly presented in medical and industrial properties [7], e.g. in the medical industry including creams containing silver to prevent local infections of burns or open wound, dental work, catheters, plastics, soaps, pastes, and textiles [8–11]. Many authors confirmed that AgNPs show efficient antimicrobial properties and kill bacteria at low concentrations (mg L^{-1}) [12] without toxic effect on human cells [13, 14].

The presented review deals with the possibilities of the synthesis of AgNPs with respect to green synthesis as well as their antimicrobial activity and its determination. Finally, we focused on the mechanism of action theories of silver nanoparticles.

2. Synthesis of silver nanoparticles

Production of nanoparticles can be achieved through different methods. Generally, we can divide these methods into physical, chemical, and biological. Some of these methods are simple and make good control of nanoparticle size by affecting the reaction process. On the other side, there are still problems with stabilization of the product and in obtaining monodisperse nanosize using achieved method [15]. In **Table 1**, we show list of some synthesis techniques of AgNPs and some of them are further briefly described.

2.1. Physical and chemical methods

For a physical approach, the nanoparticles are prepared using evaporation-condensation, which could be carried out in a tube furnace at atmospheric pressure [16]. This method has

Silver nanoparticles synthesis			
Physical methods	**Chemical methods**	**Green synthesis methods**	
		***In vitro* methods**	***In vivo* methods**
Pulsed laser ablation	Reduction	Using microorganisms	Using plant
Evaporation-condensation	Sonochemical	Using plant extracts	Using microorganisms
Spray pyrolysis	Photochemical	Using biomolecules	Using yeast
Ball milling	Electrochemical	Using algae	Using algae
Vapor and gas phase	Microwave	Using mushroom extracts	
Arc discharge		Using essential oils	

Table 1. Selected techniques for the preparation of AgNPs.

some disadvantages due to large space of tube furnace, high energy consumption, and long time for achieving thermal stability. For these reasons, various methods of synthesis of AgNPs by physical methods were developed, for example, a thermal decomposition for the preparation of nanoparticles in powder form [17]. Conventional physical methods, such as pyrolysis, were used by Pluym et al. [18]. The advantages of physical methods are speed and no use of toxic chemicals, but there are also some disadvantages, such as low yields and high energy consumption [19].

Chemical methods provide an easy way to prepare AgNPs in solution (water or organic solvent could be used). The most common method of synthesis of silver nanoparticles is reduced by organic and inorganic reducing agents. Generally, various reducing compounds, such as sodium ascorbate, sodium borohydride, hydrogen, Tollens reagent, and *N,N*-dimethylformamide, could be used for Ag^+ ion reduction. The reduction leads to the formation of metallic silver Ag^0, which is followed by agglomeration into oligomeric clusters. These clusters eventually lead to the formation of metallic colloidal silver particles [20]. It is essential to use stabilizing agents during the preparation of AgNPs to avoid their agglomeration [21]. It is necessary to note that polymeric compounds such as poly (vinyl alcohol), poly (vinyl pyrrolidone), and polyethylene glycol are effective protecting agents for the stabilization of nanoparticles [22, 23].

2.2. Green synthesis

As we described, there are various chemical and physical methods of synthesis of silver nanoparticles. These methods are cost and toxic for the environment [24]. These facts lead to the look for new, simple, and eco-friendly alternatives that would not harm human and animal health. The revolution in the world of synthesis of silver nanoparticles has brought the development of the green synthesis techniques. The biologically provided synthesis of nanoparticles has been shown to be simple, low cost, and environmentally friendly. In the green synthesis, the reduction procedure is performed by a natural-based material including bacteria, fungi, yeast, plants and plant extracts, or small biomolecules (e.g. vitamins, amino acids, or polysaccharides) [25–27]. With the development of reliable methodology to produce nanoparticles, several attempts of *in vivo* and *in vitro* synthesis of AgNPs have been realized. In this chapter, we divided the green synthesizing methods into *in vivo* synthesis, where for the biogenic production of silver nanoparticles, whole cells were used. In this approach, silver reduction can happen intracellularly or extracellularly with the formation of silver nanoparticles on the cell walls. Another case of synthesis of AgNPs is *in vitro* method. These procedures perform outside of a living organism with a cell-free extract, since the nanoparticles do not need whole cells for their synthesis. Generally, the process of biosynthesis involves three steps. The first step is, the phase of reduction of Ag^+ ions; the second is growth step, where larger aggregates are observed; and finally, the third, in which the stabilization of nanoparticles with capping agents is proceeded [28]. In **Table 2**, we reported the most recently published green synthesis of silver nanoparticles.

2.2.1. In vitro synthesis of AgNPs

In the so-called green approach, the reduction procedure is performed by a natural-based material, most commonly a plant extract containing substances with the antioxidant and

Species	Reducing/ capping agent	Precursor	Charac. techn.	Tested bacteria	Method	Ref.
Plants						
Pelargonium endlicherianum	Root extract	5 mM $AgNO_3$	SEM UV/Vis DLS Zeta pot.	**G-:** *E. coli*, *Pseudomonas aeruginosa* **G+:** *Staphylococcus epidermidis*	Microdilution method, MIC	[66]
Lantana camara	Terpenes-rich extract of leaf	1 mM $AgNO_3$	XRD UV/Vis FTIR SEM Zeta pot.	**G-:** *E. coli*, *Pseudomonas aeruginosa* **G+:** *Staphylococcus aureus*	Agar well-diffusion method	[63]
Diospyros sylvatica	Root extract	10 mM silver acetate	UV/Vis SEM TEM XRD	**G-:** *E. coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus vulgaris* **G+:** *Bacillus subtilis, Bacillus pumilis, Streptococcus pyogenes, Staphylococcus aureus*	Macrodilution broth method, MIC	[67]
Adhathoda vasica	Leaf extract	50 mM $AgNO_3$	UV/Vis XRD SEM TEM	**G-:** *Salmonella typhimurium* **G+:** *Bacillus subtilis*	Agar diffusion method	[68]
Carica papaya	Peel extract	0.25–1.25 mM $AgNO_3$	UV/Vis FTIR XRD DLS AFM Zeta pot.	**G-:** *E. coli, Klebsiella pneumoniae* **G+:** *Staphylococcus aureus, Bacillus subtilis*	Disc-diffusion method	[61]
Alstonia scholari	Leaf extract	1 mM $AgNO_3$	UV/Vis XRD EDS SEM AFM FTIR	**G-:** *E. coli*, *Pseudomonas* sp., *Klebsiella* sp. **G+:***Bacillus* sp., *Staphylococcus* sp.	Agar well-diffusion method	[69]
Ocimum tenuiflorum, Solanum tricobatum, Syzygium cumini, Centella asiatica, Citrus sinensis	Leaves/peel extract	1 mM $AgNO_3$	UV/Vis XRD AFM SEM	**G-:** *E. coli*, *Pseudomonas aeruginosa, Klebsiella pneumoniae* **G+:** *Staphylococcus aureus*	Agar well-diffusion method	[70]

Species	Reducing/ capping agent	Precursor	Charac. techn.	Tested bacteria	Method	Ref.
Ficus religiosa	Leaf extract	1 mM $AgNO_3$	TEM UV/Vis EDX FTIR DLS DSC Zeta pot.	**G-:** *E. coli, Pseudomonas fluorescens, Salmonella typhi* **G+:** *Bacillus subtilis*	Disc-diffusion method	[71]
Tamarindus indica	Fruit extract	5 mM $AgNO_3$	UV/Vis XRD EDX SEM TEM FTIR	**G-:** *Pseudomonas aeruginosa, Salmonella typhi, E. coli, Klebsiella pneumonia* **G+:** *Bacillus cereus, Staphylococcus aureus, Micrococcus luteus, Bacillus subtilis*	Agar diffusion method	[72]
Alfalfa	*In vivo* seeds	$AgNO_3$	XRD TEM	—	—	[52]
Mushrooms						
Pleurotus ostreatus	Water extract	1 mM $AgNO_3$	UV/Vis SEM TEM EDX FTIR	**G-:** *E. coli, Pseudomonas aeruginosa* **G+:** *Bacillus subtilis, Bacillus cereus, S. aureus*	Disc-diffusion method, MIC	[34]
Ganoderma neo-japonicum (*Imazeki*)	Water extract	1 mM $AgNO_3$	UV/Vis XRD TEM	—	—	[35]
Pleurotus florida	Water extract	1 mM $AgNO_3$	UV/Vis XRD FTIR TEM AFM	**G-:** *Salmonella typhi, Proteus mirabilis, Providencia alcalifaciens* **G+:** *S. aureus*	MIC	[36]
Larvae						
Oecophylla smaragdina	Tissue extract	1 M $AgNO_3$	UV/Vis TEM EDX	**G-:** *E. coli* **G+:** *S. aureus*	Disc-diffusion method, MIC MBC	[73]
Algae						
Gracilaria corticata	Water extract	1 M $AgNO_3$	UV/Vis XRD FTIR TEM EDS	—	—	[74]

Species	Reducing/ capping agent	Precursor	Charac. techn.	Tested bacteria	Method	Ref.
Pterocladiella capillacea	Water extract	1 mM $AgNO_3$	UV/Vis FTIR TEM EDX	**G-:** *E. coli, Pseudomonas aeruginosa* **G+:** *S. aureus, B. subtilis*	Agar well-diffusion technique	[64]
Sargassum longifolium	Water extract	1 mM $AgNO_3$	UV/Vis XRD FTIR SEM TEM	—	—	[51]
Bacteria						
Acinetobacter spp.	Cell-free extract	0.5–5 mM $AgNO_3$	UV/Vis XRD SEM TEM EDS DLS	**G-:** *E. coli, Pseudomonas aeruginosa, Enterobacter aerogenes, Shigella sonnie, Salmonella typhi* **G+:** *S. aureus, Streptococcus mutans*	Disc-diffusion method, MIC	[62]
Bacillus subtilis	Culture supernat	1 mM $AgNO_3$	UV/Vis TEM	—	—	[40]
Klebsiella pneumonia, Escherichia coli, Enterobacter cloacae	Culture supernat	1 mM $AgNO_3$	UV/Vis TEM EDS	—	—	[41]
Escherichia coli	Culture supernat	1 mM $AgNO_3$	UV/Vis TEM FTIR DLS	—	—	[25]
Pseudomonas stutzeri	*In vivo*	50 mM $AgNO_3$	TEM EDX	—	—	[53]
Bacillus cereus	Biomass	1 mM $AgNO_3$	UV/Vis SEM TEM EDX	**G-:** *E. coli, Pseudomonas aeruginosa, Klebsiella pneumoniae Salmonella typhi* **G+:** *S. aureus*	Agar well-diffusion technique	[55]
Fungi						
Verticillium	Fungal biomass	0.2 mM $AgNO_3$	UV/Vis TEM XRD SEM EDX	—	—	[57]

Species	Reducing/ capping agent	Precursor	Charac. techn.	Tested bacteria	Method	Ref.
Penicllium brevicompactum	Water extract	1 mM $AgNO_3$	UV/Vis SEM TEM FTIR XRD	—	—	[39]
Biomolecules						
β-Glucan	Solution	0.5–1 mM $AgNO_3$	UV/Vis EDX TEM DLS	**G-:** *E. coli, Methylobacterium* spp., *Sphingomonas* spp.	Disc-diffusion method	[75]
Pectin	Water solution	1–10 mM $AgNO_3$	TEM XRD DSC TGA DLS FTIR Zeta pot.	**G-:** *E. coli* **G+:** *Staphylococcus epidermidis*	MIC	[26]
Glutathione	1 mM water solution	1 mM $AgNO_3$	FTIR AFM SEM	**G-:** *E. coli*	Optical density	[46]

DSC: differential scanning calorimetry; EDX: energy dispersive X-ray spectroscopy; TEM: transmission electron microscopy; XRD: X-ray diffraction; TGA: thermogravimetric analysis; DLS: dynamic light scattering; Zeta pot.: Zeta potential; SEM: scanning electron microscopy; EDS: energy dispersive spectroscopy; AFM: atomic force microscopy; FTIR: Fourier-transform infrared spectroscopy; MIC: minimum inhibitory concentration; UV/Vis: ultraviolet and visible spectroscopy; G−: Gram-negative bacteria; G+: Gram-positive bacteria.

Table 2. Green synthesized AgNPs and their antibacterial activity, if determined.

reducing properties, e.g. aldehydes, ketones, terpenoids, flavones, or carboxylic acids [29]. In the field of plant-mediated nanotechnology, various plant extracts of specific parts such as root, bark, stem, leaves, seed, fruit, peel, and flower have been used for the synthesis of silver nanoparticles [30–32]. In general, silver nitrate aqueous solution is used for the reaction with the plant extract leading to rapid formation of stable nanoparticles. The plant extract is usually prepared by suspending dried powdered parts of plant in distilled or deionized water or organic solvent, most often ethanol and methanol. Extraction is carried out in various ways (different temperature, time, extract concentration, and pH). After extraction, the solid residues are removed, and the filtrate is used for the synthesis of silver nanoparticles. It is investigated that green synthesis using plant extracts is faster than microorganisms, such as bacteria or fungi [30]. The biosynthesis of silver nanoparticles from different parts of plants is schematically described in **Figure 1**.

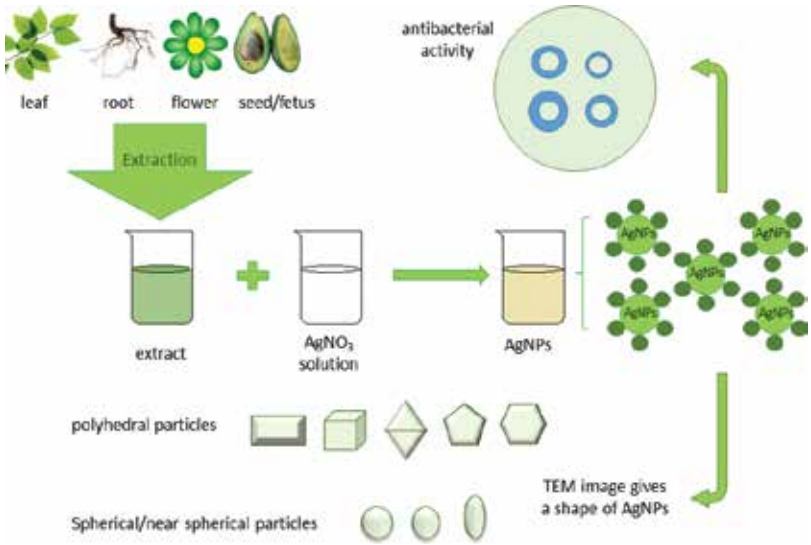

Figure 1. Schematic representation of synthesis of silver nanoparticles.

It is well known that edible mushrooms are rich in proteins, saccharides, vitamins, amino acids and other compounds, which can be used as reducing agents in biosynthesis of silver nanoparticles. The aqueous extract of edible mushroom *Volvariella volvacea* was used for the synthesis of AgNPs. Author thought that as mushrooms are rich in proteins, there is increased productivity of nanoparticles compared to other biosynthesis routes already reported [33]. In recent time, there has been published another papers that described green synthesis from mushroom extracts, for example, *Pleurotus ostreatus* [34], *Ganoderma neo-japonicum* [35], and *Pleurotus florida* [36].

Interestingly, the use of microorganism extract has resulted in an easy method to synthesize nanoparticles with characteristic shapes, size, and morphology. Extracts from microorganisms (fungi, bacteria, yeasts, actinomycetes) may act as reducing and capping agents for the synthesis of AgNPs. The reduction of Ag^+ ions is proceeded by biomolecules found in extract (enzymes, proteins, amino acids, polysaccharides or vitamins). In case of microorganisms, the extracts can be made by two methods. The first is by washing the biomass and dissolving the cells in water or a buffer [37], and the second is by used medium in which biomass has grown [38]. The aqueous extract from a fungus *Penicillium brevicompactum* was attempted [39]. Of course, specific bacteria can be used for the synthesis of nanoparticles. An interesting approach of green biosynthesis of AgNPs using supernatant of *Bacillus subtilis* and microwave irradiation in water solution has been studied [40]. Authors reported extracellular biosynthesis of silver nanoparticles and avoid the aggregation of microwave radiation they used. The rapid biosynthesis of AgNPs using the bioreduction of aqueous Ag^+ ion by the culture supernatants of *Klebsiella pneumonia*, *Escherichia coli*, and *Enterobacter cloacae* was reported [41]. An extensive volume of literature reported successful biosynthesis of AgNPs using microorganisms including bacteria, fungi, yeasts, and actinomycetes.

Reduction of Ag^+ ions is achieved using biomolecules that also serve as a capping agent. Reaction is mostly performed in water solution, which is considered as environmentally friendly solvent system. The extraction and purification of biomolecules require one or more steps in production process, and for this reason, it could be needed more time and would be not economic. The solution of this problem is using simple molecules, such as saccharides and polysaccharides as reducing agents. A simple method for silver nanoparticles was described by Raveendran et al., who used α-D-glucose as reducing agent in gently heated system [42]. In another work, the AgNPs were synthesized using pectin from citrus [26]. Authors found optimal conditions and some advantages, such as short reaction time, almost 100% conversion of Ag^+ ion to Ag^0 and very good reproducibility and stability of the product. There were used many approaches using polysaccharides for synthesis of AgNPs, e.g. dextrin [43], cellulose [44], or polysaccharides isolated from marine macro algae [45]. Preparation of silver nanoparticles using other isolated or purified biomolecules has also been studied. For example, as reducing and capping agents were used glutathione [46], tryptophan residues of oligopeptides [47], natural biosurfactants [48], oleic acid [49], etc.

Essential oils could be one of the alternative methods for biosynthesis of AgNPs. With respect to chemical composition of essential oils (phenols, flavonoids, terpenes), essential oils have been successfully used for the preparation of silver nanoparticles. Usually, reduction is used for aqueous solution of silver nitrate, and essential oils serve as reducing and capping agents [50].

Algae extracts have great efficiency in green synthesis of nanoparticles. There have been a few articles published reporting this method. Rajeshkumar and his co-workers published an algae-mediated preparation of AgNPs using purified brown algae *Sargassum longifolium* water extract. The extract was mixed with silver nitrate water solution and kept at the room temperature [51]. Bioreduction of Ag^+ ions by algae extracts is similarly proceeded due to content of phytochemicals (carbohydrates, alkaloids, polyphenols, etc.).

2.2.2. In vivo synthesis of AgNPs

Under the term *in vivo*, we understand the biosynthesis of nanoparticles in living organisms, either extracts or isolated biomolecules. Gardea-Torresdey published the very first article discussing about synthesis of silver nanoparticles using living plant Alfalfa (*Medicago salvia*). They found that Alfalfa root is able to absorb silver in neutral form (Ag^0) from agar medium and transport it into the shoots of plant where Ag^0 atoms arrange themselves to produce AgNPs [52]. Marchiol et al. [28] reported the *in vivo* formation of silver nanoparticles in plants *Brassica juncea*, *Festuca rubra,* and *Medicago sativa.* The rapid bioreduction was performed within 24 h of exposure to $AgNO_3$ solution. TEM analyses indicated the *in vivo* formation of AgNPs in the roots, stems, and leaves of the plants, which had a similar distribution but different sizes and shapes. The contents of reducing sugars and antioxidant compounds were proposed to be involved in the biosynthesis of AgNPs.

Some microorganisms resistant to metal can survive and grow in the presence of metal ions. The first evidence of bacteria synthesizing silver nanoparticles was observed using

Pseudomonas stutzeri AG259 strain [53]. Since the first evidence of bacteria producing AgNPs in 1999, different bacteria were used, e.g. *Lactobacillus* strains [54], *E. coli* [25], or *Bacillus cereus* [55]. Fungi have been observed as good producers of silver nanoparticles due to their tolerance and capability of bioaccumulation of metals. When fungus is exposed to the Ag^+ ions, it produces enzymes and metabolites, which protect it from undesirable foreign matters resulting in production of AgNPs [56]. Many reports dealing with biosynthesis of silver nanoparticles using fungi or yeasts have been published. For example, fungus-mediated synthesis of silver nanoparticles was described by Mukherjee [57]. They isolated the fungus *Verticillium* from the *Taxus* plant, and after mycelia growth and separation, they suspended dry mycelia in distilled water and added the Ag^+ ions to prepare silver nanoparticles. They found that the AgNPs were formed below the cell wall surface due to reduction of silver ions (Ag^+) by enzymes presented in the cell wall membrane. Extracellular production of silver nanoparticles was described, for example, by Sadowski, who prepared nanoparticles from *Penicillium* fungi isolated from the soil [58].

3. Antibacterial activity

The discovery of the first antibiotics has dramatically changed the quality of human life, but the development of the natural mechanism of bacterial resistance has been forced scientists to develop more effective antimicrobial drugs. The interest about the use of nanoparticles as antibacterial agents has seen a dramatic increase in the last few decades. The unique properties of silver nanoparticles have allowed exploiting in medicinal field. The most studies have been attended to their antimicrobial nature. Since silver nanoparticles show promising antimicrobial activity, researchers use several techniques to determine and quantify their activity on various Gram-positive and Gram-negative bacteria.

3.1. Methods of evaluation of antibacterial activity

To evaluate the antimicrobial activity different methods are currently used, the results of which are given in different ways. Commonly used techniques to determine the antimicrobial activity of biogenic silver are the minimal inhibitory concentration (MIC), the minimal bactericidal concentration (MBC), time-kill, the half effective concentration (EC_{50}), well-diffusion method, and disc-diffusion method. The most commonly used is disc-diffusion method developed in 1940. These well-known procedures are comprised of preparation of agar plates incubated with a standardized inoculum of test microorganism. Then, the sterile discs (about 6 mm in diameter) impregnated with AgNPs at a desired concentration are placed on the agar surface. According to agar well-diffusion method, the tested concentration of AgNPs is introduced into the well with a diameter of 6–8 mm punched into agar. Cultured agar plates are incubated under conditions suitable for tested bacteria, and the sensitivity of the tested organisms to the AgNPs is determined by measuring the diameter of the zone of inhibition around the disc or well. This method is contributed and beneficial for its simplicity and low cost and is commonly used in antibacterial activity of Ag nanoparticles evaluation [59].

The antibacterial properties of silver nanoparticles are often studied by employing dilution methods, quantitative assays, the most appropriate ones for the determination of MIC values. Minimal inhibitory concentration (MIC) is usually expressed in mg mL^{-1} or mg L^{-1} and represents the lowest concentration of the AgNPs, which inhibits the visible growth of the tested microorganism. Either broth or agar dilution method may be used for quantitative measurement, the *in vitro* antimicrobial activity against bacteria. The minimum bactericidal concentration (MBC) is less common compared to MIC determination and is defined as the lowest concentration of antimicrobial agent killing 99.9% of the final inoculum after incubation for 24 h. The most appropriate method for determining the bactericidal effect is the time-kill test and can be also used to determine synergism for combination of two or more antimicrobial agents. These tests provide information about the dynamic interaction between different strains of microorganism and antimicrobial agents. The time-kill test reveals a time-dependent or a concentration-dependent antimicrobial effect. The varied time intervals of incubation are used (usually 0, 4, 6, 8, 10, 12, and 24 h), and the resulting data for the test are typically presented graphically [59].

3.2. Antibacterial activity of AgNPs

Antimicrobial activity of silver is well known. Silver has been used for treatment of several diseases since from ancient time [60]. The AgNPs synthesized by different methods were widely tested against number of pathogenic bacteria with evidence of strong antimicrobial activity against a broad-spectrum bacteria including both Gram-negative and Gram-positive. Some researchers have been reported that the AgNPs are more effective against Gram-negative bacteria [61–63], while opposite results have also been found [64]. The difference in sensitivity of Gram-positive and Gram-negative bacteria against AgNPs may result from the variation in the thickness and molecular composition of the membranes. Gram-positive bacteria cell wall composed of peptidoglycan is comparatively much thicker than that of Gram-negative bacteria [2, 65].

The importance of antibacterial activity study on different bacterial strains becomes from the importance of understanding the mechanism, resistance and future application. The latest studies on antimicrobial properties are summarized in **Table 2**.

Although the antibacterial effect of silver nanoparticles has been widely studied, there are some factors affecting the antimicrobial properties of AgNPs, such as shape, size, and concentration of nanoparticles and capping agents [30]. Nakkala et al. [71] analyzed AgNPs with the average size of 21 nm, and the size distribution was found to be 1–69 nm prepared by medicinal plant *Ficus religiosa*. These nanoparticles showed excellent antibacterial activity in *P. fluorescens*, *S. typhi*, *B. subtilis,* and *E. coli*. Bacterial cells exposed to lower concentration of AgNPs exhibited delays of growth which may be due to the bacteriostatic effect, while at the higher concentration (of 60 and 100 μg), the AgNPs were found to exhibit bactericidal effect as no growth was observed.

The smaller particles with a larger surface-to-volume ratio were able to reach bacterial proximity most easily and display the highest microbicidal effects than larger particles [19, 69, 76]. Normally, a high concentration leads to more effective antimicrobial activity, but particles of small sizes can kill bacteria at a lower concentration. Furthermore, apart from size and concentration, shape

also influences the interaction with the Gram-negative organism *E. coli* [19, 77]. Pal et al. [78] discussed about depending of nanoparticles' shape and size on antibacterial activity against Gram-negative bacteria *E. coli*. They found that observed interaction between nanoparticles of silver with various shapes and *E. coli* was similar, and the inhibition results were variable. They speculated about the fact that AgNPs with the same surface areas, but different shapes, may have unequal effective surface areas in terms of active facets [78]. Sadeghi et al. [79] found different antimicrobial effects of nanosilver shapes (nanoparticles, nanorods, and nanoplates) for *S. aureus* and *E. coli*. SEM analysis indicated that both strains were damaged and extensively inhibited by Ag-nanoplates due to the increasing surface area in AgNPs.

3.3. Mechanism of action

In the past decade, silver nanoparticles as antimicrobial agents have attracted much attention in the scientific field. Although several reviews have described the AgNPs' mechanism in detail, the exact mechanism of the antibacterial effect of silver and AgNPs remains to be not fully elucidated. Most studies considered multiple mechanisms of action but simplified the main tree of different mechanisms determine the antimicrobial activity of silver nanoparticles: (1) irreversible damage of bacterial cell membrane through direct contact; (2) generation of reactive oxygen species (ROS); and (3) interaction with DNA and proteins [80–83]. The damage of cell membranes by AgNPs causing structural changes renders bacteria more permeable and disturbs respiration function [84]. Interestingly, Morones et al. [84] demonstrated the existence of silver in the membranes of treated bacteria as well as in the interior of it by transmission electron microscopy (TEM) analysis. Another aspect of mechanism is the role of Ag^+ ions release. Research has shown that the Ag^+ ions at a lower concentration than that of AgNPs can exert the same level of toxicity [60]. Several evidences suggest that the silver ions are important in the antimicrobial activity of silver nanoparticles [81, 85]. Durán et al. [81] discussed that silver ions react with the thiol groups of proteins, producing bacterial inactivation, and inhibit the multiplication of microorganisms. Ag^+ in μmol L^{-1} levels had weakened DNA replication due to uncoupling of respiratory electron transport from oxidative phosphorylation, which inhibits respiratory chain enzymes and/or interferes with membrane permeability. On the other side, silver ion can interact with the thiol groups of many vital enzymes and inactivate them and generate reactive oxygen species (ROS) [29]. The AgNPs can act as a reservoir for the monovalent silver species released in the presence of an oxidizer. [85] Ag^+ release was found to correlate with AgNP size, the silver nanoparticles antibacterial activity below 10 nm is mainly caused by the nanoparticle itself, while at larger sizes, the predominant mechanism occurs through the silver ions [81]. Lee et al. [86] studied the mechanism of antibacterial action on *Escherichia coli*. A novel mechanism for the antibacterial effect of silver nanoparticles, namely the induction of a bacterial apoptosis-like response, was described. They observed accumulation of reactive oxygen species (ROS), increased intracellular calcium levels, phosphatidylserine exposure in the outer membrane which indicate early apoptosis, disruption of the membrane potential, activation of a bacterial caspase-like protein and DNA degradation which is the sign of late apoptosis in bacterial cells treated with silver nanoparticles (**Figure 2**).

Antimicrobial activity of silver nanoparticles combined with various antibiotics is currently being studied, and the synergistic antibacterial effect has been found. Singh et al. [62] studied

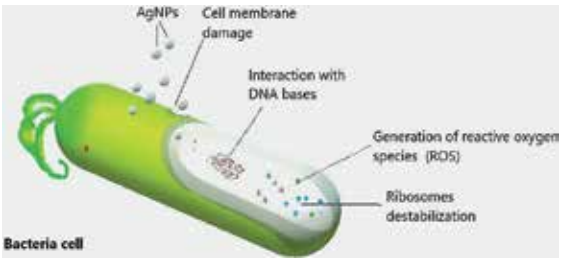

Figure 2. Schematic visualization of AgNPs' mechanism of action.

individual and combined effects of AgNPs with 14 antibiotics. They found that synergistic action of AgNPs and antibiotics resulted in enhanced antibacterial effect. Exposure of bacteria in combination of AgNPs and antibiotics reduced the MICs significantly, and the bacteria were found to be susceptible to all of the tested antibiotics, except cephalosporins, where no change was observed. The significant reduction of required antibiotic dose up to 1000-fold in combination with small amount of AgNPs could achieve the same effect. The study on bacterial strains resistant to one or more antibiotics belonging to the β-lactam class indicated that the addition of AgNPs decreased MIC on the susceptibility range, therefore, addition of AgNPs not only reduced MIC, but also caused bacteria sensitivity to antibiotic. Briefly, simultaneous action of AgNPs with antibiotics could prevent the development of bacterial resistance. These results are in accordance with findings reported by Gurunathan [76], who observed synergistic effects of silver nanoparticles in the presence of conventional antibiotics on Gram-negative bacteria *E. coli* and *K. pneumoniae*. The viability of bacteria was significantly reduced by more than 75% in combination of sublethal dose of meropenem and AgNPs. Evidence of a synergistic effect resulting from the combination of silver nanoparticles with five different antibiotics was declared by reducing MIC against multiresistant, β-lactamase, and carbapenemase producing Enterobacteriaceae [87]. The resistance on antibiotic treatment of *S. aureus* is fast growing global problem due to slow development of new effective antimicrobial agents. Akram et al. [88] investigated synergic effect of five various antibiotics and AgNPs applied in combination with blue light against methicillin-resistant *S. aureus* (MRSA). These triple combinations of blue light, AgNPs, and the antibiotic considerably enhanced the antimicrobial activity against MRSA, in comparison with treatments involving one or two agents.

The biofilm formation is adjunctive problem of resistance on antimicrobial agents and chronic bacterial infections. It was proposed that Ag-NPs can impede biofilm formation [89]. Hwang et al. [90] found that combination of AgNPs with ampicillin, chloramphenicol, and kanamycin against various pathogenic bacteria inhibits the formation of biofilm. Deng et al. [91] examined the synergistic antibacterial mechanism of four different classes of conventional antibiotics in combination with AgNPs against the multidrug-resistant bacterium *Salmonella typhimurium*. The antibiotics enoxacin, kanamycin, neomycin, and tetracycline interact with AgNPs strongly and forming antibiotic-AgNPs complex, while no such effects were observed for ampicillin and penicillin. This complex with AgNPs interacts more strongly with the *Salmonella* cells and causes more Ag^+ release, thus creating a temporal high concentration of Ag^+ near the bacterial cell wall that ultimately causes cell death.

4. Conclusion

The use of silver nanoparticles provides an opportunity to solve a global problem of bacterial resistance toward antibiotics. The possibilities of silver nanoparticles synthesis are very broad. In the last decade, there has been dramatically grown scientific interest in nanoparticles biosynthesis by various reducing and capping agents presented in biological sources including plants, plant extracts, microorganism, or larvae. The natural green synthesis approach is an eco-friendly and cost-effective due to the fact that no toxic and dangerous chemicals are used.

One of the key aspect in the design of more potent antibacterial system is the understanding its mode of action. Generally, nanoparticles are well established as promising alternative to antibiotic therapy or combinational therapy because they possess unbelievable potential for solving the problem with the development of pathogens resistance. Finally, from this point of view, silver nanoparticles represent product with potential application in medicine and hygiene, and the green synthesis of AgNPs can pave a way for the same.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 5

Superhydrophobic Surfaces Toward Prevention of Biofilm-Associated Infections

Gabriela Morán and Rachel Méallet-Renault

Additional information is available at the end of the chapter

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Abstract

In this chapter, we briefly review the different strategies for surface modification as a method to fight against bacteria adhesion and biofilm formation. We focus on superhydrophobic materials and biofilm medical infections. We give some insights into common materials and preparation techniques for superhydrophobic surfaces before discussing recent bacteria interacting with superhydrophobic surfaces. These surfaces have indeed demonstrated great potential in preventing bacterial adhesion and biofilm formation due to the presence of micro- and nanostructures. Although much work has been done, further investigations are still required to improve the surface mechanical properties over time and to understand the underlying mechanism behind their antimicrobial and antifouling capability. Moreover, there is a lack of standard methodology for evaluating antibacterial properties, and biofilm prevention should be studied with longer incubation times. We strongly believe in the potential of superhydrophobic surfaces, and we encourage more research on its magnificent properties, especially for their advantages over other antimicrobial surfaces.

Keywords: superhydrophobic surfaces, biofilm-associated infections, antibacterial surface, bacterial adhesion, biofilm formation, anti-biofilm surface

1. Strategies on surface modification for antibacterial properties

The rapid proliferation of pathogenic bacteria, which is responsible for nosocomial infections, is becoming a major public health problem because of bacterial resistance to antimicrobial treatments (antibiotics and biocides) [1]. It is now well established that bacterial populations attach to solid substrates for survival, forming biofilms. Biofilms are dense microbial communities, adhering to surfaces, which secrete an extracellular matrix mainly composed of water, polysaccharides, DNA and proteins [2]. Many different strategies on surface modification have been studied over the last few years to reduce bacterial adhesion and to avoid biofilm formation.

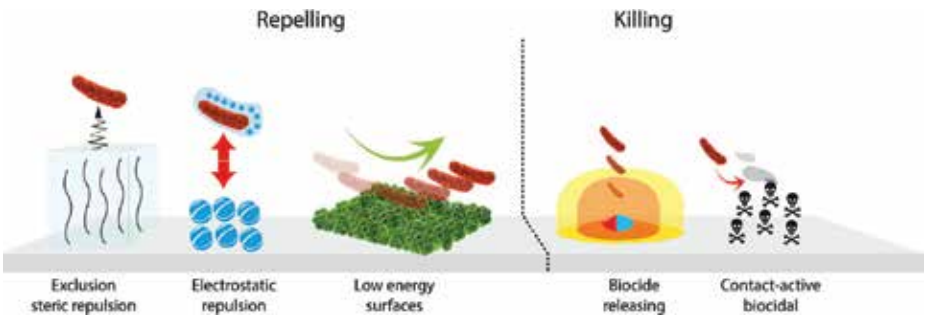

Figure 1. Antibacterial surfaces.

In general, two different global strategies can be distinguished (**Figure 1**). The first strategy relies on killing bacteria through antimicrobial compounds (either release or direct contact), such as silver ions [3] and antibiotics [4]. However, this strategy can involve tedious preparations and might represent a threat to the environment and biological systems. The second strategy relies on repelling bacterial attachment through morphological or physical-chemical interactions such as steric impediment, electrostatic interactions and low surface energy [5]. Many of the steric and electrostatic repulsion techniques proposed until now show no persistence, and surface hydrolysis may occur. Wet surfaces can provide the ideal conditions for biofilm formation; therefore, wettability properties of surfaces play a crucial role on biofilm formation. Low-surface-energy surfaces have a great potential since their antibacterial properties depend mostly on surface roughness [6]. In the present chapter, our discussion focuses on superhydrophobic surfaces. For further information regarding other techniques, useful information can be found in the review by Griesser et al. [7] and in the work by Siedenbediel and Tiller [8].

2. Superhydrophobicity

Surface wettability is an interface phenomenon reflecting the behavior of a liquid in contact with a solid surface. The control of surface wettability is present in nature and in our daily life in many applications such as waterproof coatings, cooking utensils and bathroom accessories. A material's property can be evaluated by the measurement of the contact angle (CA) between a droplet of liquid and the material surface [9].

It is well known that the interface liquid-gas (LG) area tends to be minimized due to the surface tension. When a drop is in contact with a solid surface, a balance between three-phase surface tension occurs (**Figure 2**), and it can be described by the Young's equation as follows:

$$\gamma_{SG} = \gamma_{SL} + \gamma_{LG} cos\theta \tag{1}$$

where γ_{SG}, γ_{SL} and γ_{LG} are the solid – gas (SG), solid – liquid (SL) and liquid – gas (LG) surface tensions and θ the contact angle, also referred to as the intrinsic contact angle or smooth surface contact angle [10].

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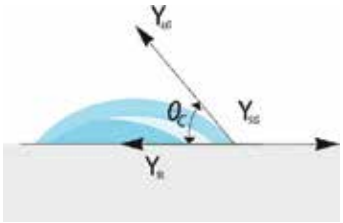

Figure 2. Liquid droplet on solid surface.

Depending on the CA value, different materials are said to be either hydrophilic or hydrophobic. For instance, if the liquid is water (or oleophilic/oleophobic when the liquid is an oil), a surface in contact with water is said to be hydrophilic if the CA is below 90°. For a CA >90°, it is hydrophobic, and for a CA >150°, it is referred to as a superhydrophobic surface. This last term refers to the repellent capability of such surfaces toward water [10].

Lotus leaves are an excellent example of superhydrophobic surfaces, as they are known to be self-cleaning. On a self-cleaning surface, particles can 'roll-off' it by their adhesion to water droplets [11]. With the technological development over the last decade, lotus leaves have been the subject of many studies. It has been found that its repellent properties are due to a hierarchical micro- and nanostructured topography. As nature demonstrates, the design of nonwettable surfaces must take parameters such as the roughness and the chemical nature of the material into consideration. The aim of this chapter is to highlight the use of superhydrophobic surfaces to prevent bacterial adhesion and biofilm formation. The main models to determine CA measurement for non-flat surfaces are described in the following section.

2.1. Wenzel and Cassie-Baxter model

As for the lotus leaf, the presence of micro- and nanostructures will contribute to the wettability of the surface, playing a crucial role on superhydrophobicity. Higher apparent CA values cannot be achieved by chemical modification, but by changing the roughness. According to Wenzel [12], the real contact area of the solid–liquid interface could be increased by changing the roughness more than changing the microscale apparent area. Wenzel's model considers that there is no gas layer between the solid–liquid interface, so the liquid fills the grooves on the surface (**Figure 3**). This angle can be referred to as the wetted contact with the surface.

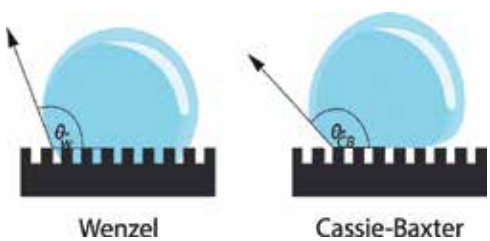

Figure 3. Wenzel and Cassie-Baxter models.

The relationship between the apparent CA θ_W and the intrinsic CA at equilibrium is:

$$\cos\theta_W = r\cos\theta \tag{2}$$

where r is the ratio of the surface area over the apparent area, defined as the Roughness r of the material. By increasing the roughness (a bigger height difference between the posts and grooves, and the density of posts), superhydrophobicity can be achieved.

Cassie and Baxter [13] studied composite materials present in nature, finding that if the surface is hydrophobic enough, the gas phase between the solid-liquid interface will remain and the droplet will not fill the grooves on the rough surface (**Figure 3**, right). For this case, the relationship is as follows:

$$\cos\theta_{CB} = f\left(1+\cos\theta_W\right) - 1 \tag{3}$$

The Cassie-Baxter equation adapts to any composite surface contact, which is represented as follows:

$$\cos\theta_{CB} = f_1\cos\theta_1 + f_2\cos\theta_2 \tag{4}$$

where θ_{CB} is the apparent CA of the composite surfaces, θ_1 and θ_2 are the intrinsic CA of two materials, and f_1 and f_2 are the fractions of two materials of the composite surfaces. f is the solid fraction of the substrate. For very rough surfaces, f will tend to zero, while θ_{CB} will tend to 180°.

Bittoun et al. [14] theoretically studied different types of surface topographies by changing roughness scales, on drop 2D systems. They studied sinusoidal, flat-top pillars and triadic Koch curves finding that Cassie-Baxter state is thermodynamically more stable, in comparison with the Wenzel state. In addition, they concluded that multi-scale roughness increases the mechanical stability of the surface and is beneficial for superhydrophobicity. Among the three topographies, round-top protrusions (sinusoidal) were shown to be the best for non-wettability, as nature has already proved.

It has been suggested that by changing the surface topography from flat to structured and by including a hierarchical organization, the water CA will be modified and superhydrophobicity may be achieved [9]. Lotus leaves have proved this theory, regardless of hydrophilic coating on their surface, the presence of hierarchical micro- and nanoscale features are responsible for their superhydrophobicity [15]. **Figure 4** shows different structures varying from flat to hierarchical.

2.2. Hysteresis

For many medical and industrial applications, the interest of a self-cleaning surface is very attractive; nevertheless, on many occasions, the CA hysteresis has been ignored in investigations. For real-life applications of repellent surfaces, the dynamic movement of the liquid drops has to be taken into consideration because it depends on the CA hysteresis. A high hydrophobicity in static CA does not imply a high hydrophobicity in dynamic CA, as proved by Oner et al. A low hysteresis CA is ideal [16].

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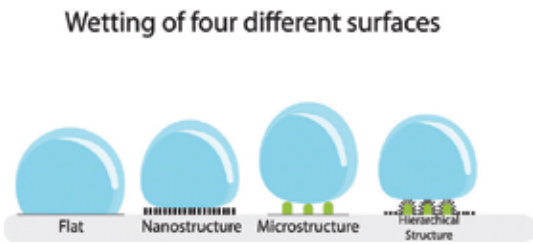

Figure 4. Different types of surface roughness and wettability.

Furthermore, CA hysteresis is important for the removal of surface contaminants when water droplets are moved along a tilted surface [9]. As we showed previously, the uniform flow of the droplet will allow or not allow the transport of the contaminant along the surface. When the flow is uniform, the material can be considered to behave as a self-cleaning surface. On the contrary, if the CA hysteresis is too high, the transport will not be very efficient and is possible that not all the contaminants will be carried by the drop (**Figure 5**) [9].

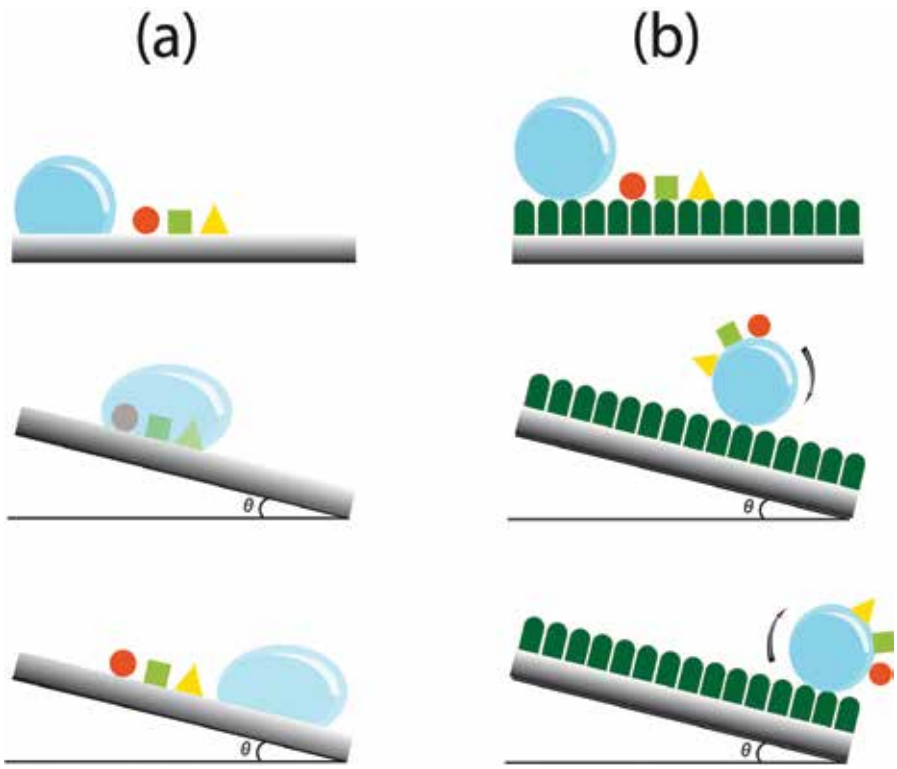

Figure 5. Dirty flow on surfaces with high (a) and low (b) CA hysteresis. While the drop is moving on a high CA hysteresis surface, it will not be efficient enough to transport the contaminants on the surface.

2.3. Superhydrophobic surfaces toward biofilm prevention

Biofilms represent a high risk for nosocomial infections in which different pathogenic bacteria are involved in biofilm-associated infections (BAI) [1]. Remarkable efforts have been made toward superhydrophobic surfaces, as they are considered self-cleaning. As we have previously mentioned, superhydrophobicity is usually achieved by a combination of surface structure, often at a micro/nanoscale, with low-surface-energy compounds. Numerous studies have been published over the last decade, regarding the use of superhydrophobic surfaces or coatings to reduce bacterial adhesion and thus to prevent biofilm formation.

Figure 6 shows how the interest in the development and study of antibacterial superhydrophobic surfaces has increased dramatically over the last decade. Despite all the efforts made until now, there is still a lack of standard methodology to assess the effectiveness of materials against biofilm formation. Incubation times and bacterial strains differ dramatically from publication to publication. In the following sections, we try to review the most relevant publications, first discussing the main preparation techniques and then describing some of the results obtained according to the bacterial strains studied and the incubation times. The aim of this review is to give the reader general tools to be able to compare and understand the key conclusions from different studies, since the conditions change from one to another.

2.4. Common materials and preparation techniques for superhydrophobic surfaces

As previously mentioned, superhydrophobic surfaces can be obtained by a wise combination of factors, among which, surface roughness and low surface energy are of particular importance. These features can be achieved using a wide variety of materials, involving organic and inorganic substrates. Among the most common techniques to modify the surface of the substrate, we find laser ablation, vapor deposition, electrochemical polymerization, lithography, sol-gel processing and layer-by-layer deposition (**Figure 7**). As a result, a vast range of surfaces are available. Unfortunately, only a few of these surfaces could be used for practical applications due to the high cost of production and feasibility.

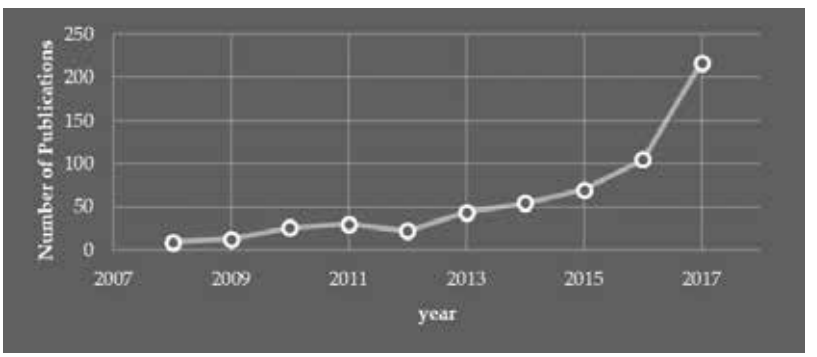

Figure 6. Number of publications per year based on the keywords: Superhydrophobic and antibacterial, from science direct database.

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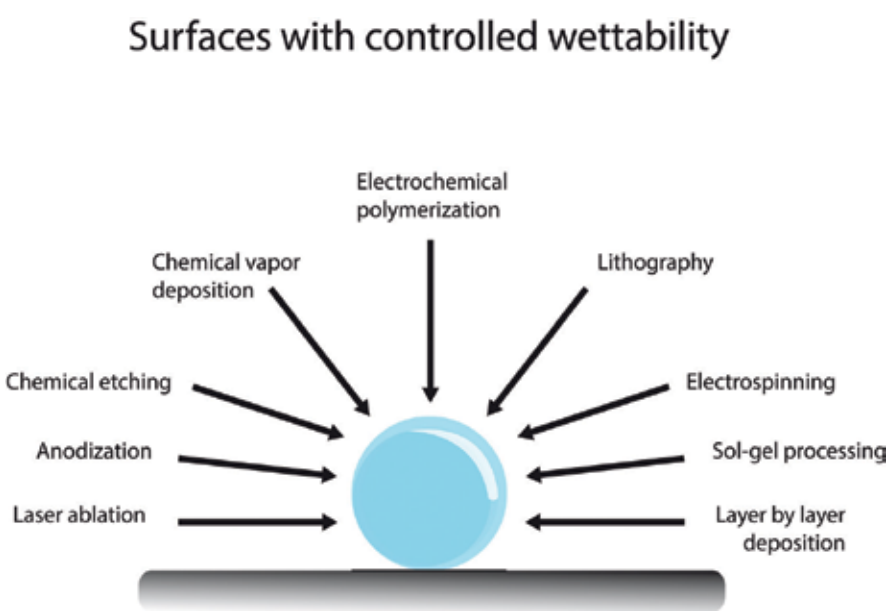

Figure 7. Most used techniques to obtain superhydrophobic surfaces and coatings.

A brief summary of different substrate materials and processing techniques is given below (**Table 1**) on superhydrophobic surfaces where bacterial interaction was tested (nonexhaustive list).

Among these techniques, we can distinguish two categories: the first category concerns the intrinsic superhydrophobic surfaces, in particular, where the surface structuration is applied directly on the substrate. In the second category, we can find the films and surface coatings, such as polymeric or oleic coatings deposited on a substrate. We focus here on biomedical infections and biomedical applications. Medical implants require a very homogenous and stable surface. The main materials used for medical implants are titanium, stainless steel, cobalt-chromium and some polymers. For this reason, many of the superhydrophobic studies made until today concern titanium surfaces [28]. In most of the cases, titanium surfaces belong to the first category, since their main application is in medical implants. Infections due to medical implants represent about 60–70% of nosocomial infections. BAI have been found in almost 100% of medical implants, such as prosthesis (heart valves, orthopedic, vascular, ocular), urinary catheters, contact lenses and intrauterine contraceptive devices [29]. Biofilms are found to be 100–1000 times more resistant to antibiotics [30]. BAI can cause a number of health complications such as chronic inflammation, antibiotic resistance, chronic and recurrent infections and, in the worst cases, sepsis [29]. Nowadays, BAI are related not only to medical implants and intensive care units but also to non-intensive care hospital areas and many other healthcare settings [29]. Thus, the implementation of self-cleaning surfaces, which facilitate the removal of microorganism on any common surfaces such as desks, tables, walls and clothes, has become a key step in preventing BAI in nosocomial settings.

Transfer from the laboratory to real-life applications, from an applied material point of view, requires testing the mechanical stability over time, in both dry and wet conditions. Even if a

Substrate material	Material processing and coating	Ref.
Titanium	Electrochemical anodization. TiO_2 nanoscale tubes and after silanization deposition.	Tang et al. [17]
	Laser ablation self-organized micro- and nanostructures.	Fadeeva et al. [18]
	Laser ablation self-organized micro- and nanostructures.	Truong et al. [19]
Polyurethane	Soft lithography micro and nano pillar-structured surface.	Xu and Siedlecki [20]
Silicon	Plasma etching and Teflon/oil coating micro- and nanostructured porous surface.	Epstein et al. [21]
Silica	Based-catalyzed hydrolysis and condensation. Nanostructured silica fluorinated colloids with xerogel coating.	Privett et al. [22]
Aluminum	Anodization, post-etching process and Teflon coating. Al_2O_3 nanoporous and nanopillared surface obtained.	Hizal et al. [23]
Steel, glass, polystyrene	Thermal deposition of n-paraffin and fluorinated waxes.	Pechook et al. [24]
Stainless steel	Multilayer depositions of polydopamine (PDA) and silver (Ag) nanoparticles followed by post-modification with 1H, 1H, 2H, 2H–perfluorodecanethiol.	Qian et al. [25]
Aluminum wafer	Electrochemical deposition of silver coating.	Che et al. [26]
Poly-dimethylsiloxane (PDMS)	Aerosol assisted chemical vapor deposition of copper nanoparticles.	Oskan et al. [27]

Table 1. Example of most common substrates and techniques used to obtain superhydrophobic surfaces.

surface is considered self-cleaning, mechanical stress will be applied under normal conditions through disinfection and wear. Besides mechanical stability, thermal and chemical resistance would be required due to the oxidation in the environmental conditions of almost any application. There are many other fields that would benefit from self-cleaning surfaces, such as naval, the food industry, the energy industry and also for fuel storage. **Figure 8** shows some examples of medical devices that could implement superhydrophobic surfaces.

Figure 8. Example of medical devices which could implement superhydrophobic surfaces.

2.5. Bacterial adhesion and biofilm formation

We can find in the literature tests on several bacterial strains, among which we can highlight *Staphylococcus aureus* and *Staphylococcus epidermidis* (Gram positive, coccus), and *Pseudomonas aeruginosa* and *Escherichia coli* (Gram negative, rod shape), as well as all facultative anaerobic bacteria. *S. aureus* and *P. aeruginosa* recently have been cataloged by the World Health Organization (WHO) as priority pathogens to be considered as threat because of their resistance to antibiotics [31]. *S. epidermidis* is among the leading causes of nosocomial sepsis [29], and *E. coli* can be found on almost any surface, besides being the most prevalent microbe identified from positive blood cultures [32].

As it is known, biofilm formation can be described in five different stages as follows (**Figure 9**) [33]:

Stage I: Reversible planktonic cell landing on a surface and initial attachment.

Stages II and III: Bacterial growth and microcolony formation, irreversible attachment.

Stage IV: biofilm maturation.

Stage V: dispersion of planktonic cells capable of forming new colonies.

It is important to highlight the fact that the formation of a mature biofilm will be dependent on the bacterial strain and incubation conditions. For discussion, as an example, we can consider *P. aeruginosa* biofilm as a representative. Rasamiravaka et al. [33] studied the development of *P. aeruginosa* biofilm over time by using fluorescence microscopy. After 2 h of incubation, the bacteria culture could be cataloged at Stage I; after 8 h, bacteria attachment was considered irreversible. Microcolony formation was observed after 14 h and biofilm formation and maturation from 1 to 4 days. Stage V was observed after 5 days. Although much research has been made so far, many studies ignored the biofilm formation, focusing only on a few

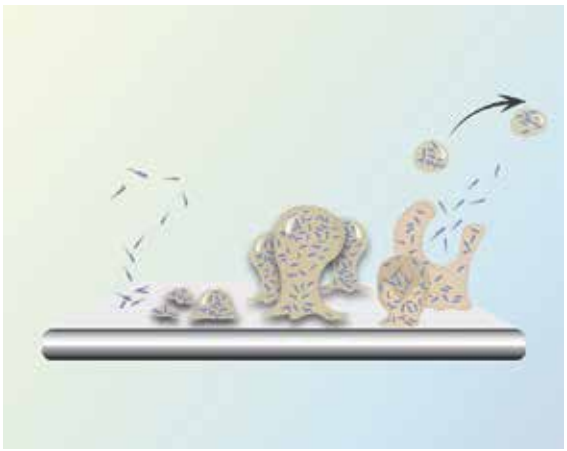

Figure 9. Dirty flow on surfaces with high (left) and low (right) CA hysteresis. While the drop is moving on a high CA hysteresis surface, it will not be efficient enough to transport the contaminants on the surface.

hours of incubation (<24 h). In such cases, we must say that only bacterial adhesion has been studied, without any knowledge as to whether the attachment is either reversible or irreversible. Nevertheless, to study biofilm prevention properties of surfaces, longer incubation times should be considered and for real-life application even longer times where all the phases should be observed. In **Table 2**, we present recent examples of relevant studies of bacterial interactions on superhydrophobic surfaces.

Although **Table 2** does not summarize all the studies to date on antibacterial superhydrophobic surfaces, we can observe that most of the studies have focused only on bacterial adhesion rather than the prevention of biofilm formation. Qian et al. [25] recently published a remarkable work on superhydrophobic multilayer film deposition on stainless steel with antibacterial properties,

Ref.	Bacterial strain	Incubation time	Mode	Biofilm prevention
Tang et al. [17]	*S. aureus*	2, 4 h	Stationary	Not tested
Fadeeva et al. [18]	*S. aureus*	18 h	Stationary	No for *S. aureus*
	P. aeruginosa			Yes for *P. aeruginosa*
Truong et al. [19]	*S. aureus*	1 h	Stationary	Not tested
	S. epidermidis			
	P. maritimus			
Xu and Siedlecki [20]	*S. epidermidis*	1, 2 h	Flow	Not tested
Epstein et al. [21]	*P. aeruginosa*	24, 48 h and 7 days	Flow	Yes
	S. aureus			
	E. coli			
Privett et al. [22]	*S. aureus*	1.5 h	Flow	Not tested
	P. aeruginosa			
Hizal et al. [23]	*S. aureus*	1 h	Both	Not tested
	E. coli			
Pechook et al. [24]	*B. cereus*	24 h and 7 days	Stationary	Yes
	P. aeruginosa			
Qian et al. [25]	*S. aureus*	1, 3 days	Stationary	Yes
	E. coli			
Che et al. [26]	*E. coli*	12 h	Stationary	Reduced
Oskan et al. [27]	*S. aureus*	1 h	Stationary	Not tested
	E. coli			

Table 2. Recently published studies on antibacterial properties of superhydrophobic surfaces, summarizing the bacterial strains, incubation mode and times and more importantly, if biofilm formation prevention was evaluated or not (nonexhaustive list).

evaluating the incubation with *E. coli* and *S. aureus* at 1 or 3 days. The surfaces were prepared with hierarchical micro/nanostructures using polydopamine (PDA) and silver (Ag) nanoparticles. These were compared against non-structured surfaces. For both strains, no cells were observed after day 1, and until day 3, cell quantity was by far less than the other surfaces studied. They proved that the biofilm formation could be prevented using these hierarchical micro/nanostructured superhydrophobic surfaces. They studied two different bacterial strains, gram positive and negative, and also long incubation times to thoroughly assess if biofilm formation is prevented or not.

Fadeeva et al. [18] prepared microstructured superhydrophobic titanium surfaces by femtosecond laser ablation. They incubated samples with *S. aureus* or *P. aeruginosa* for 18 h, finding significant reduction for *P. aeruginosa*, but not for *S. aureus*. The aim of the study was to investigate further the behavior of two different shaped pathogens on their surfaces in order to evaluate antifouling properties; nevertheless, they were not able to determine the mechanism by which this was achieved. They suggested that the ability of *S. aureus* to colonize the superhydrophobic surfaces could be dependent on their shape. However, this assumption cannot be generalized as other studies [21, 22] have shown that some surfaces are effective independent of the shape of bacteria. It is important to insist on the need of testing superhydrophobic surfaces with different bacteria (shape, gram positive and negative). Moreover, it would be even more interesting to test a mixture of bacteria to see if the antibacterial capability of the surface is independent of these multiple factors.

2.6. Insights into the mechanisms below prevention of biofilm formation by superhydrophobic surfaces

It has been proved that the Lotus leaf superhydrophobicity originates from the hierarchical micro/nanostructures on their surface even though a hydrophilic layer covers these structures [15]. Micro-/nanostructuration allows the surface to encapsulate air between, creating an air cushion which reduces the water adhesion onto the surface, thus preventing the adhesion of microorganisms and other fouling molecules.

Studying bacterial distribution on the surfaces by imaging techniques has provided important insights on the mechanisms used by the bacteria to attach to the surfaces. With SEM images, Truong et al. [19] observed the distribution of different bacterial strains on superhydrophobic titanium surfaces, finding that the upper regions of the surfaces were not covered by bacteria as much as the crevices between the upper regions. This suggested that the capability to reduce bacterial adhesion of superhydrophobic surfaces can be reduced over time after the trapped air is completely excluded under complete submersion conditions. However, Ma et al. [34] compared the adhesion of bacteria (*P. aeruginosa*) and non-biological adhesion under partially and completely submersion conditions on superhydrophobic *Colocasia esculenta* leaves. They found that the adhesion was dependent on the nanostructure density on the surface rather than the air-cushions trapped on micro/nanostructures. These results demonstrate the efficacy of superhydrophobic surfaces in reducing bacterial adhesion and preventing biofilm formation even under submerged conditions, when they are properly designed at the nanoscale.

3. Perspectives

Much work has been carried out to date on superhydrophobic surfaces, allowing us to understand a little more clearly about the mechanism behind their antimicrobial properties. These surfaces have demonstrated great potential in preventing bacterial adhesion and biofilm formation. Many techniques have been proposed for their preparation and application in different fields. Even so, there is still much work to be done, such as improving the surface mechanical properties over time, and mainly toward our understanding of the underlying mechanism behind their antimicrobial and antifouling capability. With the work so far, we can conclude that micro- and nanostructures have great influence on their antibacterial properties, so this requires further in-depth studies. As discussed in this chapter, there is a lack of standard methodology for evaluating antibacterial properties; however, we can highlight the need to evaluate not only bacterial adhesion but also the prevention of biofilm formation with longer incubation times. Much work has been done so far, but there is still a long way to go from the laboratory to reach real-life applications. We strongly believe in the potential of superhydrophobic surfaces and we encourage continued research on its magnificent properties, especially for their advantages over other antimicrobial surfaces.

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Section 3

Pathogenesis and Clinical Cases

Chapter 6

Chronic *Pseudomonas aeruginosa* Infection as the Pathogenesis of Chronic Obstructive Pulmonary Disease

Takemasa Matsumoto and Masaki Fujita

Additional information is available at the end of the chapter

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Abstract

Chronic obstructive pulmonary disease (COPD), resulted from tobacco smoking, has an extremely poor prognosis and is a major cause of chronic morbidity and mortality worldwide. In this chapter, we review the role of bacterial infection on the pathogenesis of COPD, with a particular focus on *Pseudomonas aeruginosa*. Chronic infection with *P. aeruginosa* has been shown to contribute to COPD pathogenesis under certain conditions. In addition, *P. aeruginosa* is a major factor influencing severe symptoms, acute exacerbation, and the progression of COPD. Treatment for chronic *P. aeruginosa* infection may become a new strategy for addressing COPD.

Keywords: chronic inflammation, emphysema, acute exacerbation, bronchiectasis, microorganism, pathogenesis

1. Introduction

Chronic obstructive pulmonary disease (COPD) is induced by mainly tobacco smoking. The patients of COPD complained of cough, sputa, or exertional dyspnea. Compared to the patients with bronchial asthma, COPD has an extremely poor prognosis. Approximately 3,200,000 COPD patients died worldwide in 2015 [1]. The mortality is estimated to be ≥8-fold that of bronchial asthma. In this chapter, we review the role of bacterial infection on the pathogenesis of COPD, with a particular focus on *Pseudomonas aeruginosa*.

2. Chronic obstructive lung disease (COPD)

COPD is a relatively common disease characterized by respiratory symptoms and airflow limitation, usually caused by smoking. A mixture of small airway disease and parenchymal destruction caused airflow limitation. COPD patients complained of cough, dyspnea, and sputum production. Acute exacerbation frequently occurs after an acute respiratory infection. The acute exacerbation leads to more severe airflow limitation [2]. Sometimes, COPD patients are accompanied with bronchiectasis. These patients caused more frequent acute exacerbations and severe airway obstruction. Also, these patients demonstrated pathogenic microorganisms and mortality [3]. At present, there is no universally effective treatment for COPD. Some treatments just relieve the symptoms, while others could slow the progression of the disease. The most effective treatment is stopping smoking. Bronchodilators are the mainstay of available treatment options for COPD. There are several types of bronchodilators. Long-acting beta agonist (LABA), long-acting muscarinic antagonist (LAMA), or a combination of these agents is used for COPD patients, since long-acting medication is preferred. For the patients complicated with bronchial asthma, known as asthma-COPD overlap (ACO), inhaled glucocorticoids (ICSs) can be used. Also, ICSs can be used for patients with frequent COPD exacerbation. Short-acting beta agonists (SABAs) and short-acting muscarinic anticholinergics (SAMAs) are used to relieve symptoms, such as exertional dyspnea. Since theophylline can be given orally, it can be used for patients who cannot inhale medications. Advanced COPD patients with hypoxemia are administered with long-term oxygen therapy (LTOT). Rehabilitation programs are also important. They can be effective; however, they do not improve the outcome. Lung volume reduction surgery is performed in select patients with COPD; however, the candidate patients are limited. Also, lung transplantation is rarely done because of lack of donors [4]. Antioxidants, mucolytics, antiproteases, and antifibrotics are sometimes used; however, these drugs are not a mainstay for COPD. Anti-inflammatory drugs such as phosphodiesterase 4 inhibitors are used to reduce airway inflammation. Also, kinase inhibitors, chemokine receptor antagonists, innate immune mechanism inhibitors, and statins are developing [5]. However, these new treatments are still insufficient to demonstrate the evidence for caring COPD. As such, a novel therapy for COPD is required.

3. *Pseudomonas aeruginosa*

P. aeruginosa is a glucose non-fermenting Gram-negative bacillus. The size of *P. aeruginosa* is 0.7 × 2 μm. *P. aeruginosa* is localized in the natural environment such as in soil and freshwater. *P. aeruginosa* does not show usually pathogenicity in healthy individuals but, however, causes infection mainly in immunocompromised patients [6]. *P. aeruginosa* is well known to cause chronic infection in bronchiectasis as well as COPD. Biofilm formation is important for chronic infection. A biofilm is a self-produced polymer matrix consisting of polysaccharides, protein, and DNA. Bacteria are embedded in biofilm. The polysaccharide alginate is the major components of the *P. aeruginosa* biofilm matrix. *P. aeruginosa* escapes from host immunity and antibacterial drugs using biofilms [7, 8].

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4. *P. aeruginosa* and lung diseases other than chronic obstructive pulmonary disease

P. aeruginosa is often detected in ventilator-associated pneumonia and nosocomial pneumonia. Moreover, bronchiectasis is easily colonized by *P. aeruginosa* [9]. *P. aeruginosa* infection is a risk factor for mortality and morbidity in cystic fibrosis patients [10]. *P. aeruginosa* sometimes produces mucinous materials, so-called mucoid *P. aeruginosa*. Gibson et al. reported that mucoid *P. aeruginosa* contributes to the progression of cystic fibrosis compared to non-mucoid *P. aeruginosa* [11]. *P. aeruginosa* infection in non-cystic fibrosis bronchiectasis patients induces more severe impairment of the pulmonary function, although the rate of decline in the pulmonary function is not affected [12].

5. *P. aeruginosa* and chronic obstructive pulmonary disease

5.1. Steady status of COPD and *P. aeruginosa*

Numerous studies have characterized the lung microbiome of healthy adult subjects using BAL samples. The most common phyla consistently observed have been *Bacteroides*, *Firmicutes*, and *Proteobacteria* in the phylum level. Prominent genera among healthy controls are *Prevotella*, *Veillonella*, *Streptococcus*, and *Pseudomonas*. Tobacco smoking alters the microbial constitution of the upper airways [13]. Erb-Downward et al. reported that *Pseudomonas*, *Streptococcus*, *Prevotella*, *Fusobacterium*, *Haemophilus*, *Veillonella*, and *Porphyromonas* were observed in COPD lungs [14]. *P. aeruginosa* is often observed in the sputum of patients with COPD. Rosel et al. reported that *P. aeruginosa* was colonized in one-quarter of patients with COPD during steady status [15]. *P. aeruginosa* was detected in 4–34.7% of sputum samples from COPD patients [16–20]. *P. aeruginosa* causes chronic infections in COPD [21], and especially COPD patients accompanied with bronchiectasis are easily colonized with *P. aeruginosa*. COPD patients with *P. aeruginosa* colonization have a worse disease activity than COPD patients without *P. aeruginosa* colonization. Desai et al. conducted a longitudinal prospective observational study of COPD. They found that the average of breathlessness, cough, and sputum scale (BCSS) score was higher during the periods of colonization compared to periods without colonization. Colonization was associated with a clinically significant worsening of daily symptoms, even in the absence of clinical exacerbation [22]. These findings suggest that novel therapies that decrease the bacterial colonization may be able to improve the daily symptoms and quality of life in COPD patients.

5.2. Acute exacerbation of COPD and *P. aeruginosa*

Acute exacerbation of COPD is defined as a worsening of the respiratory condition, such as coughing, sputum production, and dyspnea, beyond daily physiological fluctuations and requiring additional treatment. COPD patients with acute exacerbation document a worse quality of life as well as decrease of pulmonary function. Finally, COPD patients with acute

exacerbation result in lower mortality [9]. Acute exacerbation mainly occurs due to airway infections. The relationship between COPD and *P. aeruginosa* infection in acute exacerbation of COPD has already been reported [23–26]. However, bacteria other than *P. aeruginosa* can also cause acute exacerbation of COPD. Indeed, infection with a new strain of *Haemophilus influenzae*, *Moraxella catarrhalis*, or *Streptococcus pneumoniae* is strongly associated with the occurrence of exacerbation. *H. influenza* (20–30%), *S. pneumoniae* (10–15%), and *M. catarrhalis* (10–15%) were causative bacteria for acute exacerbation. *P. aeruginosa* accounted for only 5–10% of causative bacteria. Infection of respiratory viruses has also been shown to cause exacerbations. However, it is difficult to identify the specific respiratory viruses because of technical problems. *P. aeruginosa* is less frequently detected from the sputum in COPD patients with acute exacerbation than in those without exacerbation. However, acute exacerbation caused by *P. aeruginosa* generally produces a more severe clinical condition than that caused by other pathogens [26].

5.3. Progression of COPD and *P. aeruginosa*

The relationship between disease progression and *P. aeruginosa* infection in COPD patients is not fully understood. Bronchiectasis, a percentage of forced expiratory volume in 1 s (%FEV1) of <35%, systemic steroid use, and antibiotic therapy within the preceding 3 months increased the risk of *P. aeruginosa* colonization [13, 23, 24]. The detection rates of *H. influenza* and *P. aeruginosa* were reported not to be associated with the severity of emphysematous changes [26, 27]. However, chronic *P. aeruginosa* infection was recently reported to be associated with severe obstruction in COPD patients [28]. Hospitalized COPD patients with acute exacerbation by *P. aeruginosa* demonstrated worse lung function, greater dyspnea, and more hospitalizations over the previous year. Therefore, *P. aeruginosa* infection is commonly observed in COPD patients and has been found to cause severe symptoms of COPD, the development of severe acute exacerbation, disease progression, and a poor prognosis.

6. Pathogenesis of chronic obstructive pulmonary disease

The pathogenesis of COPD is considered to be chronic airflow limitation results from an abnormal inflammatory response to the inhaled particles and gasses in the lung in susceptible smokers. There is a famous hypothesis that a protease-antiprotease imbalance leads to the progression of the destruction of alveoli [2]. Alveolar cell loss through apoptosis might contribute to the pathogenesis [29–31]. There are many animal models for COPD, including elastase, cigarette, inhaled gasses, and gene-targeted models. As COPD models, administration of papain or porcine pancreatic elastase model is well known [32]. Neutrophil elastase and proteinase-3, but not non-elastolytic enzymes, such as bacterial collagenase, caused COPD-like changes [33–35]. Cigarette smoking is a major factor inducing the development of COPD. Long-term cigarette smoking caused macrophage-predominant inflammation and airspace enlargement in animal models similar to those found in humans [36]. Potential mechanisms include high concentrations of reactive oxygen species (ROS) [37], oxidative stress

[38], and matrix metalloproteinase (MMP)-12 [39, 40]. Inhaled stimuli, such as sulfur dioxide [41, 42], nitrogen dioxide [43], and oxidant [44], have been shown to induce COPD-like lesions in animal models. Ultrafine particles, such as silica, coal dust, diesel exhaust particles, and cadmium, induced focal emphysema [45, 46], chronic airway inflammation [47], and interstitial fibrosis with enlargement of the airspaces [48]. Alveolar wall apoptosis without the accumulation of inflammatory cells, resulting in emphysematous changes, was attained by active caspase-3 [31, 49]. Prednisolone also causes emphysematous changes through apoptosis [50]. Apoptosis in the alveoli resulted in airspace enlargement was also attained by a block of vascular endothelial cell growth factor (VEGF) receptor-2 [51]. Ceramide production, as the second messenger lipid, was induced by apoptosis. Ceramide played a role in induction of inflammation by the blockade of apoptosis by a VEGF receptor antibody. Since ceramide administration provoked the expression of MMP-12, it was considered to be a link between excessive apoptosis and inflammation [52]. Several gene-targeted models demonstrated COPD-like changed; however, the changes were developmental abnormalities rather than the destruction of mature lung tissue. Tight-skin mice [53, 54], pallid mice [55], and beige mice [56] were reported to be models. COPD mimic models by genetically altered techniques have been reported such as the overexpression of collagenase in the lung of transgenic mice [57], the deficiency of the microfibrillar component fibulin-5 and the deficiency of platelet-derived growth factor A (PDGF-A) [58, 59], epithelial restricted integrin $\alpha v\beta 6$ knockout mice [60], fibroblast growth factor (FGF) receptor (FGFR)-3, and FGFR-4-double knockout mice [61]. Mice lacking the surfactant protein D (SP-D) gene [62] and the tissue inhibitor of metalloproteinase-3 (TIMP-3) gene [63] showed COPD-like lesions. These gene-targeted mice provided useful information for understanding the pathogenesis of COPD. However, none of these mice showed the same pathologic changes as those seen with human COPD.

7. *Pseudomonas aeruginosa* as the pathogenesis for chronic obstructive pulmonary disease

Chronic inflammation also plays a pivotal role in its development. Administration of lipopolysaccharide (LPS) to the lungs induced severe inflammation and resulted in airspace enlargement [64, 65]. COPD-like changes, such as goblet cell metaplasia in the larger airways, thickening of the airway walls, and irreversible alveolar enlargement, were attained by repeated administration of LPS [66, 67]. Tumor necrosis factor (TNF)-alpha is a proinflammatory cytokine induced by stimuli such as LPS. We reported that TNF-alpha overexpression mice in the lungs demonstrated pulmonary emphysema-like changes [68, 69]. MMP activation induced alveolar enlargement [68]. Chronic inflammation by TNF-alpha overexpression is considered to play an important role in the development of COPD. Several reports have found that the overexpression of inflammatory cytokines, such as IL-13 and IFN-gamma, resulted in pathologic changes mimicking human COPD [70–72]. These reports also supported the hypothesis that chronic inflammation in the lungs leads to lung tissue destruction, a hallmark of pulmonary emphysema, and chronic bronchitis.

We therefore investigated the role of chronic inflammation in the pathogenesis of COPD. *P. aeruginosa* induced chronic inflammation as previously described. We hypothesized that chronic inflammation, specifically that induced by *P. aeruginosa*, contributed to the pathogenesis of COPD. To test this hypothesis, pathophysiological changes due to chronic *P. aeruginosa* infection in club cell secretory protein (CCSP)-deficient mice were investigated [73]. The nonciliary bronchial epithelium as well as the uterine and urethral ducts expresses CCSP proteins [74]. The anti-inflammatory response could be speculated as the role of CCSP [75]. Therefore, we consider CCSP-deficient mice to be a model susceptible to chronic infection. CCSP-deficient mice with single administration of *P. aeruginosa* demonstrated similar result to wild-type mice [76]. We used *P. aeruginosa*-colonized catheter methods [73]. This chronic status of *P. aeruginosa* infection continued for more than 5 weeks. As a result, these deficient mice showed more severe inflammation in response to chronic *P. aeruginosa* infection than wild-type (WT) mice, and their bronchi were markedly stenotic (**Figure 1**). The mean linear intercept, destruction index, and lung compliance in the CCSP-deficient mice were significantly higher than those in the wild-type mice (**Figure 2**). Severe inflammation leads to the destruction of the alveolar wall, and bronchial stenosis leads to air trapping (**Figure 3**). Chronic infection of *P. aeruginosa* in CCSP-deficient mice demonstrated COPD-like changes [73]. Recent studies attempting to characterize COPD have shown that CCSP is strongly related to COPD progression [77, 78]. CCSP has been reported to play a role in the modulation of pulmonary inflammation during the infection and recovery phases. Our study revealed the important role of CCSP in the chronic infection phase. Chronic *P. aeruginosa* infection might play a distinctive role in the pathogenesis of COPD. However, several limitations exist. Mice have less airway branching than humans and lack respiratory bronchioles. Major species differences between murine and human lung morphogenesis and discrepancies in both the innate and adaptive immunity between the human and murine immune systems exist [79, 80]. We should consider the limitations carefully.

Recently, macrolide treatment has been reported to protect against acute exacerbation. Among some subjects with COPD, taking azithromycin daily for 1 year, when added to the usual

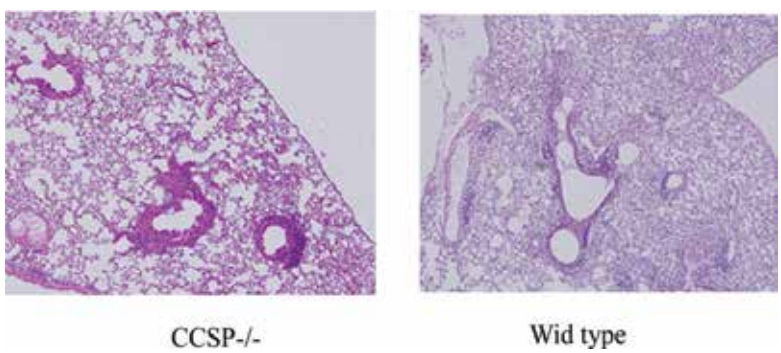

Figure 1. Histology of lungs after chronic *P. aeruginosa* infection. Inflammatory cells infiltrated around the bronchioles and the alveoli septa. The CCSP-deficient model (CCSP−/−) showed bronchial constriction and alveolar enlargement compared to the wild-type model.

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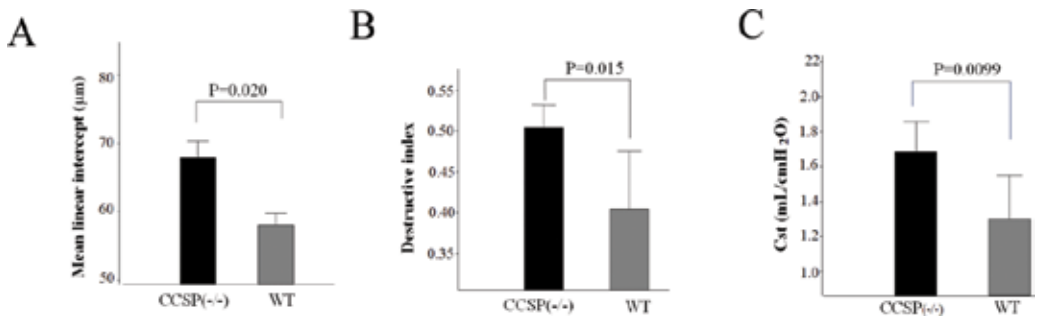

Figure 2. Pulmonary physiology after chronic *P. aeruginosa* infection. (A) Chronic *P. aeruginosa* infection in CCSP-deficient mice (CCSP−/−) resulted in an increase in the mean linear intercept compared to that in wild-type (WT) mice. Furthermore, the CCSP-deficient mice demonstrated a severe destructive index (B). (C) CCSP(−/−) induced elevated pulmonary compliance compared with WT mice. These results indicate that chronic *P. aeruginosa* infection in CCSP-deficient mice induced COPD-like changes.

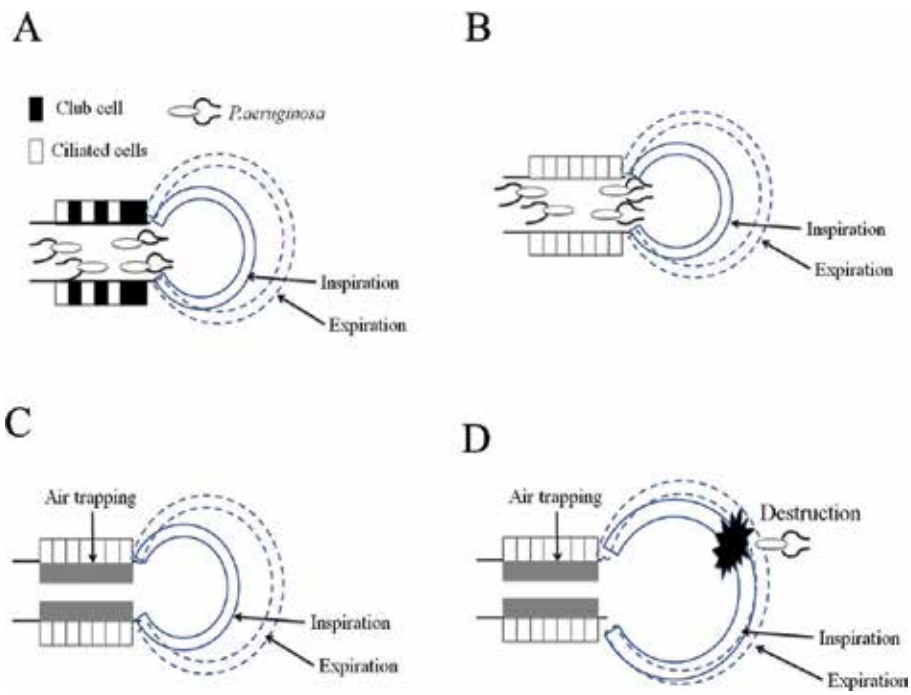

Figure 3. Schematic illustrations of COPD-like changes induced by chronic *P. aeruginosa* infection. (A) When *P. aeruginosa* infected the bronchus, CCSP was secreted from club cells to suppress the inflammation. (B) Since CCSP-deficient mice could not suppress the inflammation through CCSP secretion, serious inflammation occurred. (C) As a result, the bronchus became stenotic, and air trapping occurred. Air trapping progressed, and the alveoli were destroyed. (D) Alveolar destruction due to inflammation also occurred. Finally, the CCSP-deficient mice demonstrated COPD-like changes.

treatment regimen, reduced the frequency of exacerbations and improved the quality of life [81]. Macrolides are known to modulate the inflammation, the so-called immunomodulatory effect, besides antimicrobial effects. In Japan, macrolides are used to treat chronic infection

with *P. aeruginosa*, such as diffuse panbronchiolitis (DPB). These agents might therefore modulate the chronic inflammation induced by *P. aeruginosa*. Further studies are needed to clarify the pathogenesis of COPD. We should seek out direct evidence that chronic *P. aeruginosa* infection is related to the pathogenesis of COPD.

8. Conclusion and future directions

P. aeruginosa infection had never been reported as the pathogenesis of COPD. However, we showed that chronic infection of *P. aeruginosa* contributed to COPD pathogenesis. *P. aeruginosa* is also a major factor influencing the severity of symptoms, acute exacerbation, and progression of COPD. Treating chronic *P. aeruginosa* infection may become a new strategy for treating COPD.

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Conflict of interest

The authors declare that they have no conflicts of interest (COI).

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Chapter 7

Pathogenesis of Cholera: Recent Prospectives in Rapid Detection and Prevention of Cholera

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Additional information is available at the end of the chapter

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Abstract

Cholera, also known as "blue death" is a potentially epidemic and life-threatening secretory diarrhea characterized by numerous voluminous watery stools, often accompanied by vomiting and resulting in hypovolemic shock and acidosis. The causative agent of this water-borne disease belongs to certain members of the species *Vibrio cholerae* (*V. cholerae*) which can also cause mild or unapparent infections. *V. cholerae* is a facultative anaerobic, Gram-negative bacilli, which possess the characteristic feature of darting motility under wet-mound preparations. Other members of the species may occasionally cause isolated outbreaks of milder diarrhea, whereas others—the vast majority—are free-living and not associated with disease. The emergence of new, virulent, drug-resistant strains of *Vibrio* is the main cause of protracted outbreaks leading to high fatality rates. The subsequent loss of fluid volume causes a drop in blood pressure and circulatory shock. If the patient remains untreated, they become progressively weaker, sometimes to the point of death, within 12–24 h of the onset of symptoms. The severity and fatality of the disease depend on the strain of *Vibrio*. The cholera toxin-producing (CT-producing) *V. cholerae* manifests the most fatal disease known as cholera gravis. Throughout most of the twentieth century, cholera was caused by *V. cholerae* of the O1 serogroup, and the disease was largely confined to Asia and Africa. The emergence of a pandemic in 1992 was caused by an unknown serogroup of *V. cholerae* (O139) wherein the targets were India and Bangladesh. The pathogenesis and virulence of the bacteria are due to an enterotoxin it produces—cholera toxin (CT). The mechanism of action of CT is discussed in this chapter at a later stage. Attempts have been made to produce vaccines through a number of trial-and-error methods, and still the possibility of an effective vaccine which gives a good prophylactic measure is under consideration. The identification of the bacteria as well as toxin detection is one of the main elements in the clinical microbiology field. The identification and confirmation of this epidemic disease commend from the morphological identification of *Vibrio* and end with serotyping and biotyping in addition to toxin detection by various means of assays—both in vitro and in vivo. This chapter will cover all of the mentioned areas of clinical microbiology with respect to cholera infection in addition to the recent outbreaks and epidemics which occurred across the globe.

Keywords: *Vibrio cholerae*, enterotoxin, *Vibrio cholerae* O139, vibriocidal antibodies, secretory IgA, ELISA

1. Introduction

Cholera, a water-borne infectious disease (also known as "blue death"), is characterized by life-threatening secretory diarrhea often accompanied by numerous voluminous watery stools and vomiting, eventually leading to hypovolemic shock and acidosis. The term "blue death" is given to the disease as it changes the skin of the infected individuals to blue discoloration due to severe dehydration [1, 2]. It is caused by certain members of the species *V. cholerae* which can also cause mild or unapparent infections. These bacteria are found in water or food contaminated with feces. Countries with poor water and sewage treatment are likely to be the victims of cholera. This disease is very unlikely to be spread from person to person. The history of cholera began in the nineteenth century where millions of people across the continents were vanished due to six subsequent pandemics. South Asia in 1961 witnessed the seventh pandemic, Africa in 1971, and the Americas in 1991 [3]. The outcome of humanitarian crisis, like overcrowded camps, and lack of water and sanitation systems are some of the factors which increase the risk of cholera transmission. The presumptive confirmation of cholera is done on the primary clinical investigation of watery stools and severe dehydration. Nowadays the use of rapid identification kits has eased the detection of cholera within a few hours of receiving the sample in the laboratory [2, 3]. The failure to produce an effective oral vaccine against cholera is the area where the professionals and researchers are worried about. The different trials and attempts to produce a vaccine and the vaccines under trial are further discussed in the chapter. The clinical microbiology of cholera, its epidemiology and the laboratory investigations are discussed in detail in this chapter.

2. Clinical features

2.1. Symptoms

The symptoms of the cholera disease usually manifest 1–3 days after ingestion of contaminated food and/or water. In most cases, the symptoms range from mild to moderate, but in 20% of cases, severe life-threatening fatal conditions appear. The most severe manifestation of the disease, "cholera gravis," affects only minority of the patients infected with CT-producing *V. cholerae* [1]. It has been estimated that classical strains are more virulent than El Tor strains. The incubation period of cholera is dependent on inoculum size and ranges from several hours to 5 days [2]. The onset of the clinical manifestation may be sudden profuse watery diarrhea followed by anorexia and abdominal discomfort in some cases. Initially the stool will be dark brown with solid nature; gradually it turns to inoffensive whitish watery stool giving a rice water appearance [1]. This is the characteristic nature of cholera which helps to diagnose

from the other gastrointestinal infections. The massive fluid loss via vomiting and excessive diarrhea is due to the action of enterotoxins produced by the *V. cholerae* [3]. Enterotoxins are cytotoxic and alter the permeability of the epithelial cells of the intestinal wall by creating pores. As a result of osmosis, the water and other fluid substances flow out of the body leading to massive diarrhea and severe dehydration [4]. Vomiting is also present which develops after the onset of diarrhea. In cholera gravis the rate of diarrhea reaches a maximum to 600–1000 ml/h leading to hypotension, tachycardia and vascular collapse. Due to severe dehydration, the skin turgor will be poor, giving a dry appearance, with eyes sunken. The patient will be restless and thirsty, and the hands will show wrinkles as after a long immersion which is often referred to as "washer woman's hands." The drastic hypovolemic shock leads to hypokalemia resulting in painful muscle cramps and in severe cases hypokalemic nephropathy and focal myocardial necrosis. Body temperature will be quite normal except for 20% of the infected individuals which shows a slight temperature rise [4–6].

Lactic acidosis and stool bicarbonate losses associated with dehydration are manifested by decrease of blood pH and plasma bicarbonate and an increased serum anion gap (mean of 20.2 mmol/l in a study). Despite potassium loss, untreated acidosis usually leads to high potassium levels in serum. In children, hypoglycemia associated with coma and convulsions may occur [4, 6].

2.2. Mechanism of pathogenesis

A better understanding of the source of infection is very much necessary for the management and effective prophylaxis of any diseases. In the case of cholera, contaminated water and feces of persons infected with *V. cholerae* are the primary sources of infection. Planktons and zooplankton harboring the infectious agent and certain environmental factors such as surface change and terrestrial nutrient discharge are also among the factors that indirectly lead to the production of more hosts [7].

The mechanism of invasion begins as soon as the bacterium establishes itself in the bowel, following the colonization of the epithelial layer of the small intestine by penetrating the mucous. The penetration of the mucous is facilitated by mucolytic enzymes which help to destroy the mucous integrity, and also the long tail of the invading organism allows it to propel itself through the thick mucosal layer. The attachment *Vibrio* onto the microvilli of the small intestine is aided by the so-called pili on the bacterium which is one of the major factors which attributes to the darting motility [6, 8]. The production of an endotoxin by the bacteria, called CT, is the major key for the virulence mechanism. So it is proved that only toxigenic strains of *Vibrio*, i.e., *Vibrio* that produces CT, are capable of causing cholera. Studies suggest that this CT is encoded by a filamentous bacteriophage which is harbored by pathogenic vibrios [1].

The CT that is made is comprised of six protein subunits: one A subunit and five copies of B subunits, generally denoted as AB5. B subunit is also known as the binding factor which binds to the GM1 ganglioside receptor of the epithelial cells of the small intestine. Once bound with the target cells, it forms a toxin complex which is then endocytosed by the cell.

As soon as the process of endocytosis takes place, the enzymatic activation of A subunit occurs leading to increased adenylate cyclase activity, thereby increasing the concentration of cAMP to more than 120-folds. This in turns leads to increased permeability of the chloride channels subsequently mediating the efflux of more ATP-mediated chloride ions and secretion of mere H_2O, Na^+, K^+ and HCO_3^- into the lumen of the intestine [4, 5, 7]. The increased absorption of water as well as electrolytes is responsible for the massive dehydration leading to the clinical symptoms of cholera. The brief mechanism of pathogenesis is depicted in **Figure 1**.

2.3. Differential diagnosis

In places where cholera disease is found epidemic or endemic, treatment should never be delayed due to pending laboratory investigations; laboratory confirmation is very essential for the prophylaxis as well as for treatment in epidemic-endemic areas.

As outlined above, definitive diagnosis is required for which the most crucial step is the isolation of *V. cholerae* from the stool of the infected individuals. For an epidemic survey, a further characterization rather than the isolation of *Vibrio* may not be required. In other circumstances, isolates should be typed by specific serum agglutination for O139 and O1 serotypes [9].

2.4. Therapy

The key to therapy is adequate rehydration till the disease has come to its end course (usually 1–6 days in the absence of antibiotics). Rehydration can be accomplished by oral rehydration with oral rehydration solution (ORS) as in other cases of diarrhea and dysentery. In severe

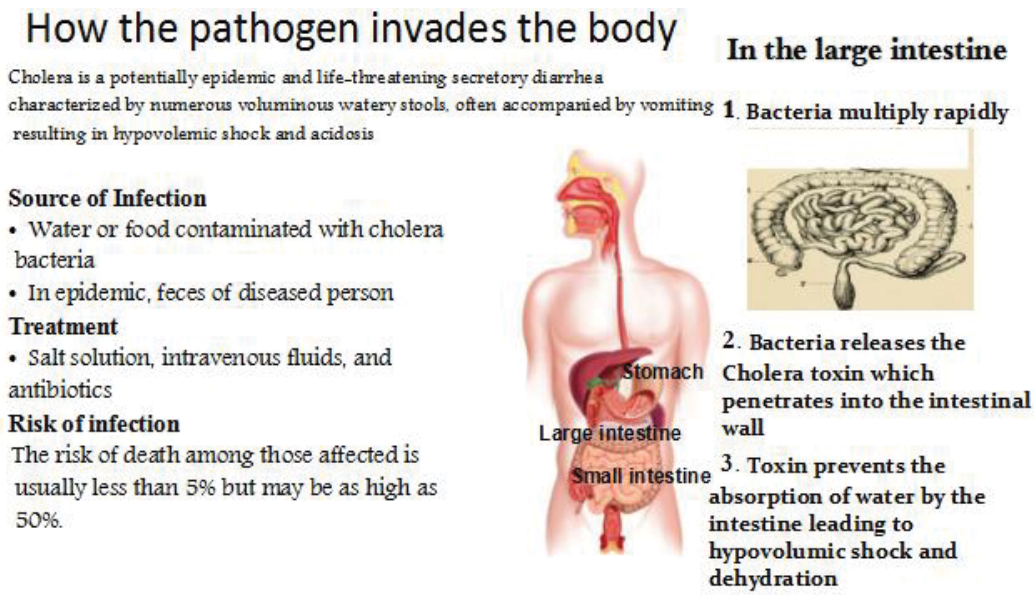

Figure 1. Mechanism of pathogenesis of *V. cholerae*.

cases, intravenous infusion of fluid will be necessary. Antibiotics help to reduce the severity of illness and the duration of excretion of the organism. In children, hypoglycemia is treated with 25 or 50% glucose administered intravenously [10].

2.5. Intravenous rehydration

For adults, intravenous rehydration is done as fast as possible; 2 liters is administered the first 30 minutes. If the condition of the patient improves at this point, the rate of infusion can be lowered to 100 ml/kg of the body weight within the first 4 hours of therapy. Children should receive 30 ml of the intravenous fluid per kg of body weight during the first hour and an additional 40 ml/kg in the following 2 hours [10, 11]. Both adults and children should receive appropriate amount of ORS during the course of illness. The rate and frequency of intravenous infusion can be varied depending on the electrolyte loss from the stool of the patient. The World Health Organization (WHO) recommends Ringer's lactate as the best rehydration solution. Isotonic saline corrects only hypovolemia, so potassium and glucose should be administered along with it for proper recovery from dehydration [12, 13].

2.6. Oral rehydration

Patients with moderate illness and dehydration can receive fluid replacement orally to compensate for the water and electrolyte depletion. In mild cases, the WHO recommends 50 ml of ORS/kg of body weight to be given in the initial 4 h. For moderate dehydration, the quantity should be increased to double, i.e., 100 ml/kg [11, 14]. Stool output should be continuously monitored depending on which the rehydration therapy is optimized. For children, who tend to have high fluid losses, it should be replaced 1:1 with ORS. The oral solution used must be the one recommended by the WHO [1]. This solution consists of appropriate amount of electrolytes to balance the fluid and electrolyte losses due to dehydration. In addition, it also contains optimum quantity of glucose to facilitate the absorption of sodium and water.

The formula for the current WHO oral rehydration solution is 2.6 grams table salt (NaCl), 2.9 grams trisodium citrate dihydrate, 1.5 grams (0.053 oz) potassium chloride (KCl) and 13.5 grams anhydrous glucose ($C_6H_{12}O_6$) per liter of fluid [1, 6, 12].

A homemade oral rehydration solution can be prepared when readymade sachet is not available. The composition of the ORS solution consists approximately of six level teaspoons of sugar and 1/2 a teaspoon of table salt in 1 liter of water. The molar ratio of sugar to salt should be 1:1, and the solution should not be hyperosmolar [9, 12].

2.7. Antimicrobial therapy

Treatment with antibiotics should start only after the initial rehydration therapy and correction of acidosis. Antibiotics play a secondary role in the treatment of cholera by shortening the length of cholera diarrhea as well as the excretion of vibrios. Though most of the *V. cholerae* strains have so far not exhibited any drug resistance, O139 strains have an intrinsic resistance to trimethoprim-sulfamethoxazole [14, 15]. Tetracycline is the usual drug of choice [7]. Rather

than the possibility of staining of teeth, tetracycline has not shown any contraindications in adult and children. Besides tetracycline, the quinolone group of antibiotics (ciprofloxacin and norfloxacin) has shown excellent in vitro activity against *Vibrio* [10, 11]. The widespread use of tetracycline as a prophylactic control is not recommended nowadays due to the emerging resistance toward it in the epidemic areas [14].

3. Epidemiology

Cholera is an indication of the prevalence of unsanitary conditions especially with the growing populations in developing countries. It is a plight that about 3–5 million cases of cholera are reported each year with the advancements of health and medical professionals across the globe [16]. When 52 countries reported 236,896 cases in 2006 with a fatality rate of 2.7%, a total of 589,854 cases were reported in 2011 from 58 countries [7]. These numbers suggest cholera being a global disease threatening worldwide.

In developing countries, where the population is high, cholera is the main cause of epidemic diarrhea. Global pandemic outbreaks are also being reported from Asia, Africa and Latin America [17]. Africa, being a country with limited medical facilities, has shown a considerable decrease in cholera outbreaks in the recent years. When 117,570 cases were reported in 2012, only 56,329 cases were reported in 2013 [16]. This shows a remarkable sanitary improvement as well as prophylactic measures adopted by the country. During 2013, Mexico and other Central African countries reported an outbreak, while 2014 witnessed a cholera outbreak in South Sudan. In an outbreak in Cuba, a total of 185 cases were reported in 2012 [18]. The WHO reports an average of 132,121 cholera cases in 2016 from 38 countries which includes 2420 fatality reports [13].

Considering the epidemiology of the different serotypes and biotypes of *V. cholerae,* serogroup O1 and O139 cause outbreaks. The biotype EL Tor and serotype Ogawa are the main causes of epidemic cholera in developing countries [18, 19]. Among *V. cholerae* serogroups, O1 is the main cause of recent outbreaks. O139 was first identified in a massive outbreak in Bangladesh in the year 1992, but as of now, it is only seen in some sporadic cases [20]. It has not been reported outside Asia. The causative strain and serotype of *Vibrio* responsible for the first four pandemic is unknown, whereas the causative agents of the fifth and the sixth pandemics were identified as classical biotypes of O1 strains and El Tor biotype, respectively [16]. In 2010, Haiti outbreak was reported which was imported by the United Nations Peacekeeping Forces from South Asia. The United Nations report says it has affected 788,000 people and claimed the lives of 9200 people [16]. These information lead us to the fact that in the progress of epidemics and pandemics, new clones of toxigenic *Vibrio* strains continue to emerge which is one of the major threat to the countries who are the victims of the life-threatening disease.

Vibrio being the native species of the aquatic environment cannot be eradicated permanently from the globe as in the case of small pox [13]. There is an increased risk of epidemic and pandemic cholera with changes in the environmental and climatic conditions. As a prophylactic measure, integrated prevention strategies and interdisciplinary actions are required.

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4. Immune response and vaccines

Over 75 years of research has been done on the development of an effective vaccine against cholera. Since *Vibrio* does not exhibit much of antigenic variation, the very realistic goal of a cholera vaccine is very much near and attainable. The advanced study of the intestinal immune system and the pathogenicity of cholera have brought the insight of vaccine very much near.

4.1. Infection-derived immunity

The immunity induced by cholera infection is strain and serotype specific. Studies show that volunteers experimentally infected with classical biotype *V. cholerae* O1 showed 100% protection against subsequent infection against classical biotype vibrios [12]. Similarly, infection with El Tor biotype exhibited 90% immunity against El Tor vibrios [2]. The immunity lasts for a minimum of 3 years for the similar biotype. Initial infection with either the Ogawa or Inaba serotype conferred protection against both the serotypes during reinfection.

4.2. Components of the immune system

Infection with *Vibrio* can range from a fatal diarrheal disease to a mild non-clinical/unapparent case where only serological diagnosis is only possible [1, 12]. The reason for this remains unclear. But there have been certain assumptions regarding the symptomatic difference in the infected patients. One of them is the availability of the intestinal receptors for the toxins and the cholera vibrios in patients. After natural infection, circulating antibodies are detected against most of the cholera antigens including flagellar (H) antigen, toxin and somatic (O) antigen [21, 22]. Antibodies raised against vibrios are referred to as "vibriocidal antibodies" since they lyse the bacteria in the presence of complement and other serum components. The parenteral administration of vaccines causes an apparent rise in the "vibriocidal antibodies" [2, 12]. The immune response consists of both antibacterial and antitoxic immunities, antibacterial immunity being the most predominant. An oral trial vaccine containing killed *Vibrio* conferred more resistance than parenteral vaccine consisting toxoids. A synergistic immune response consisting of both toxins and bacterial antigens showed remarkable immune response than single component [21].

It is likely that the primary immune response might be a main determinant of protection against cholera infection since it is essentially a disease of the small intestine. Moreover infants under breastfeeding are less likely to be affected due to the presence of the secretory antibodies present in the milk [19, 21]. Secretory IgA, IgM and IgG are found in the intestinal mucosa. These antibodies confer protective immunity in the presence and absence of complementary proteins. The actual protection is due to the secretory IgA and other intestinal antibodies, and it is the vibriocidal antibodies in the serum which serves as the marker for IgA antibodies to direct against the same antigens [12, 21]. *V. cholerae* is a motile bacterium which is one of the major factors in the pathogenicity. The flagellar antibodies could prevent the invasion of the bacteria by immobilizing them; as well the somatic (O) and flagellar (H)

antibodies can cause clumping and lead to arrest of motion of cells. At the same time, the antitoxins can bind with the specific toxins at the epithelial surface and prevent the binding of the toxin [2, 12].

4.3. Vaccines

The observation that natural infection confers long-lasting immunity has led to the development of cholera vaccines which can elicit protective immunity. The first attempts of vaccine development started in 1960, which focused on whole-cell vaccine preparation with parenteral administration. Ninety percent immunity was obtained, but it was not long-lasting as the antibody baseline titer waned in an year [21]. An attempt has also been made by converting cholera toxin to toxoid in the presence of glutaraldehyde and formalin, but it had elicited a very low level of protection as toxoid is a poor antigen [12].

In recent years, the importance of intestinal immune response has paved a different direction in vaccine development, i.e., a shift from parenteral to oral vaccines. Parenteral administration of antigens elicits the stimulation of secretory antibodies (IgA, IgG, and IgM), but the antibodies have a short life span and a poor memory [21]. In controversy to it, oral administration of antigens elicits immune response of a longer shelf life and good memory proving to be a good vaccine. There is not much contribution from serum antibody in cholera infection as *V. cholera* does not invade erythrocytes in the process of infection but only colonizes the intestinal mucosal surfaces. Therefore to stimulate the production of secretory antibodies, administration of oral vaccine plays a vital role when compared to parenteral vaccines [12, 21].

The FDA has approved a single-dose live oral vaccine for cholera called Vaxchora (lyophilized CVD 103-HgR) for the age group 18–64 years old [21]. Though it is not recommended in the usual immunization category, it is administered for travelers who are moving to a region actively spread with the toxigenic vibrios. As per the current scenario, there is no cholera vaccine which provides 100% protection, and vaccination is not recommended as a prophylactic measure in preventing cholera by the WHO.

The vaccines under trial consist of live attenuated whole-cell vaccines and killed bacterial suspensions, both of which are administered orally.

5. Laboratory investigations

5.1. Isolation and biochemical identification of the bacteria

Based on the somatic O antigens, currently there are about 130 serogroups of *V. cholerae*. However only O1 serogroup is involved in epidemic and pandemic cholera [12]. Isolation and identification of *V. cholerae* serogroup O1 or O139 from the stool sample of infected individuals is the gold standard in the diagnosis of the disease. The ideal transport medium is Cary Blair media; however, Alkaline Peptone Water (APW) medium is also

recommended (pH, 8.5) [1, 12]. The culture media are blood agar and MacConkey agar, the selective media being the thiosulfate-citrate-bile salts agar (TCBS). The rapid and presumptive diagnosis of cholera is the most important factor in the diagnosis, and hence the stool sample as soon as received is examined for a wet-mount preparation to look for the darting motility exhibited solely by *Vibrio* species [9, 12, 23]. Thus a presumptive identification or probability of a cholera infection is made by which further treatment is carried out.

Overnight growth (18–24 hours) of *V. cholerae* on TCBS produces large (2–4 mm in diameter) yellow-colored colonies, slightly flattened with opaque centers and translucent peripheries. The yellow color is due to the fermentation of sucrose in TCBS medium. Non-sucrose-fermenting vibrios such as *V. parahaemolyticus* produce green to blue-green colonies. On MacConkey agar, the colonies are late lactose-fermenting colonies, initially being non-lactose fermenters [23]. Yellow colonies on TCBS agar are depicted in **Figure 2**.

Table 1 is taken from Laboratory Methods for the Diagnosis of *V. cholerae*, Centers for Disease Control and Prevention [12].

5.2. Serologic identification

As discussed previously, there are more than 130 serogroups of *V. cholerae*, among which O1 serogroup is the causative agent of most of the epidemics and pandemics. Serologic identification of *V. cholera* O1 serogroup is carried out by agglutination test with the specific antisera. This is the most rapid method and specific method of identifying *V. cholerae* O1 [16].

Isolates of O1 serogroup is further divided into three serotypes, namely, Ogawa, Inaba and Hikojima (rare). Strains that are positive with polyvalent O1 antisera are further tested for agglutination with Ogawa and Inaba antisera. In some instances, there may be strains that agglutinate very strongly and equally with both Ogawa and Inaba antisera. If ever such rare reactions are suspected, those strains should be referred to a reference laboratory for further confirmation and may be referred to as "possible serotype Hikojima" [9, 12].

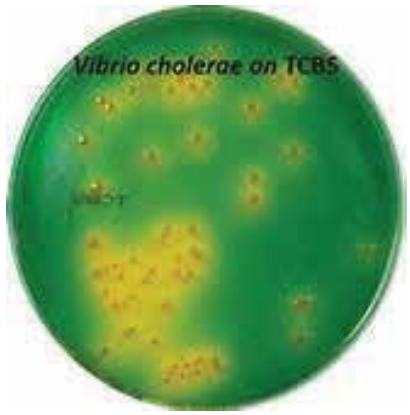

Figure 2. Colony morphology of *V. cholerae* on TCBS—yellow color colonies.

Biochemical tests	Reaction
Oxidase	Positive
String test	Positive
Kligler's iron agar	K/A, no gas, no H_2S
Triple sugar iron agar	A/A, no gas, no H_2S
Glucose (acid production)	Positive
Glucose (gas production)	Negative
Sucrose (acid production)	Positive
Lysine	Positive
Arginine	Negative
Ornithine	Positive
Voges-Proskauer	Variable*

*Most isolates of *V. cholerae* serotype O1 biotype El Tor are positive in the Voges-Proskauer test, whereas biotype classical strains are negative.

Table 1. Biochemical characteristics of isolates of ***V. cholerae*** O1.

Table 2 is taken from Laboratory Methods for the Diagnosis of *V. cholerae*, Centers for Disease Control and Prevention [12].

5.3. Tests to distinguish biotypes of *V. cholerae* O1

The typing of *V. cholerae* O1 is not necessary for the identification and diagnostic point of view but is necessary for the epidemic surveillance in helping to identify the source of infection, particularly when cholera is prevalent in a particular geographical area [12]. There are two biotypes—classical and El Tor, among which El Tor biotype is predominant throughout the world and classical is rare except in certain regions of Bangladesh.

Table 3 is taken from Laboratory Methods for the Diagnosis of *V. cholerae*, Centers for Disease Control and Prevention [12].

5.4. Toxin assays

The virulence of the O1 serogroup is marked by the production of CT toxin. The CT molecule comprises one A (active) subunit and five B (binding) subunits. The B subunit attaches to

Serotype	Major O factors present	Agglutination in absorbed sera	
		Ogawa	Inaba
Ogawa	A, B	+	–
Inaba	A, C	–	+
Hikojima	A, B, C	+	+

Table 2. Identifying characteristics of serotypes of ***V. cholerae*** serogroup.

Property	Reaction	
	Classical	El Tor
Voges-Proskauer (modified with 1% NaCl)	−	+
Zone around polymyxin B (50 U)	+	−
Agglutination of chick erythrocytes	−	+
Lysis by bacteriophage		
Classical IV	+	−
El Tor V	−	+
Hemolysis	−	+

Table 3. Differentiation of classical and El tor biotypes of ***V. cholerae*** serogroup O1.

the GM1 ganglioside receptors on epithelial cells of the intestinal mucosa during the process of infection. After this process, cleavage occurs between the subunits so as to facilitate the entry of A component into the cell. The A1 component is responsible for the stimulation of adenylate cyclase which in turn produces cyclic AMP (cAMP) [3, 12]. Increased intracellular production of cAMP leads to disruption of electrolytes across the cell membrane leading to fluid secretion into the small intestine. When the fluid entering the intestine is greater than its reabsorption capability, it leads to diarrhea [12, 23]. CT toxin is similar to *Escherichia coli* (*E. coli)* heat labile toxin (LT) both antigenically and in mechanism. So, most of the toxin-detecting assays are common for CT and LT. The mechanism of action of CT is well explained in "Mechanism of pathogenesis" and illustrated in **Figure 2**.

Before testing for the toxin, the isolate should be confirmed as *V. cholera* O1 strain. The non-O1 serogroups also produce CT or other toxins similar to heat labile enterotoxin or Shiga toxin, but these toxins are not of diagnostic value as they have not yet been associated with epidemic disease [12].

5.4.1. Bioassays

5.4.1.1. Animal methods

The first cholera enterotoxin assay was the adult rabbit ileal loop method in the 1950s in which the enterotoxin was injected into the ligated segments of the intestine (ileal loops) of experimental animals such as rabbits, pigs, dogs and calves, and this caused accumulation of fluid. This mechanism is routinely used for the study of mechanism of CT, heat labile toxin of *E. coli* and other toxins. A cell-free supernatant is injected into each of the ileal loop after the exteriorization and ligation of the rabbit's small intestine after which the abdomen is closed for 18 hours. The quantity of the accumulated fluid due to the stimulation of the toxin is measured by measuring the loops once the intestine is removed [12]. The disadvantage of this model is that it is time-consuming, cumbersome and difficult to standardize.

Another model was developed in 1955, the infant rabbit infection model which was also used for the determination of different toxins. Here, a 7-day-old infant rabbit is infected

with the test culture either by gastric intubation or by intraluminal injection. A positive assay results in watery diarrhea of the rabbit and eventual death due to dehydration. The small intestine is removed, and the loops are measured to quantify the excess fluid accumulation [8]. The drawback of this procedure is the requirement of one animal per test sample.

The vascular permeability factor assay is a dermal test wherein the activity of CT or LT is determined by the neutralization of standardized amount of specific antisera against CT. Here 20–30 supernatants can be injected per rabbit. A cell-free supernatant is injected intradermally into the rabbit, following which Evans blue dye is injected intravenously. The increased capillary permeability mediated by CT leads to perfusion of the dye in the skin causing a blue coloration with localized induration at the site of injection. The diameter of discoloration and induration is measured with a negative control [1, 12].

5.4.1.2. Tissue culture methods

Tissue culture methods are widely used in laboratories nowadays for the toxin assay. It requires skilled professionals and equipment in the laboratory with existing tissue culture facilities. It is a very sensitive assay with high reproducibility. In addition to detecting toxins, these assays are also useful in detecting toxin-neutralizing antibodies.

The Chinese hamster ovary (CHO) and the Y1 mouse adrenal (Y1) cell lines are used for the assay of CT and LT, although Vero monkey kidney cells are also used. The toxin if present in the cell-free supernatant increases the intracellular production of cAMP in the cell lines. This can be made out by the morphological changes in the cell lines (Y1 cells round and CHO cells elongate) [7, 12].

5.4.2. Immunoassays

5.4.2.1. Elisa

As CT and LT are immunogenic to both humans and animals, many immunogenic assays have been developed to detect the presence of toxins in vitro. As discussed earlier, the GM1 ganglioside is the natural receptor for CT. In ELISA method, this GM1 ganglioside receptor is purified and is used for the preparation of ganglioside-capture enzyme-linked immunosorbent assay. The cell-free supernatant in which the toxicity is to be tested is added to the microtiter wells coated with GM1 ganglioside. CT antiserum is then added on the plate to detect the binding of the toxin with the bound receptor on the plate, followed by the addition of enzyme-conjugated antiglobulin antibody [12].

5.4.2.2. Latex agglutination

In this assay, highly purified specific anti-CT is bound to latex particles. A commercial version of kit is available in the market which allows easy detection of cholera toxins in the laboratory.

5.4.3. DNA-based assays

5.4.3.1. Polymerase chain reaction (PCR)

PCR is a molecular approach based on specific DNA sequences. In PCR, DNA polymerase enzyme is used to amplify certain specific sequences of DNA (amplicons) which can be detected on an agarose gel or using specific DNA probes [23]. The toxigenicity of *V. cholerae* can be detected by using PCR and primers that specifically amplify only CT genes. The advantage of PCR is that it does not require a pure culture or even viable strains. The toxigenicity of the organism can be detected from the infected stool samples, contaminated water or food samples, thus determining the presence of *Vibrio* even without culturing it. The PCR method also has the advantage of detecting CT toxins that is produced in very low levels by certain strains, by identifying ctx genes possessed by them [12, 23]. The technical problems associated with radioisotopic labels have been solved by non-radioactive DNA labels such as biotin and digoxigenin. Apart from the fact that DNA-based assays require skilled professionals and sophisticated laboratories, these methods provide reliable, specific and rapid results unlike the conventional methods.

Table 4 is taken from Laboratory Methods for the Diagnosis of *Vibrio cholerae*, Centers for Disease Control and Prevention [12].

Assay	Sensitivity (per ml)	Type of assay	Specific target of assay	Sample tested
Rabbit ileal loop	30 ng	Bioassay	Stimulation of fluid accumulation	Culture supernatant
Infant rabbit assay	250–500 ng	Bioassay	Stimulation of fluid accumulation	Broth culture/supernatant
Rabbit skin test	0.1–3.5 ng	Bioassay	Permeability factor	Culture supernatant
Y1 mouse adrenal cells	10 pg	Bioassay	Accumulation of cAMP	Culture supernatant
Chinese hamster ovary cells	10 pg	Bioassay	Accumulation of cAMP	Culture supernatant
GM1-ELISA	10 pg	Immune	B subunit	Culture supernatant
Coagglutination	50 ng	Immune	B subunit	Culture supernatant
Reverse passive latex agglutination	1–2 ng	Immune	B subunit	Culture supernatant
DNA probe	Detects Ctx gene	Genetic	Ctx gene	DNA (colony blot)

Table 4. Commonly used assays for the detection of cholera toxin.

6. Conclusion

V. cholerae is one among the most successful emerging and reemerging pathogens that has both human and environmental components in its life cycle. The epidemic and pandemic occurrence of *V. cholerae* especially *V. cholerae* O139 has been a turning point in the history of the most dreaded diarrhoeal disease—cholera. The conventional methods of screening and identification of these Gram-negative, motile bacilli are not sufficient enough for the rapid diagnosis of cholera. Although molecular methods have evolved for the identification of most of the serotypes and biotypes of *V. cholerae,* the most important factor to control the epidemic and pandemic of the dreaded, fatal, life-threatening disease is to sanitize the food and water environment as the most important prophylactic measure. India, Bangladesh and Sri Lanka have been victims of cholera epidemics, and now it is affecting a multitude of countries. Being developing countries, the concerned governments along with medical professionals should come up with certain safety measures and evoke the necessity of sanitization and cleanliness of the food and water systems to all people. Along with the above-said preventive measures, researchers and medical professionals are on the verge of producing an effective vaccine which will enable to boost the immune system of the individuals. As mentioned in the chapter, the antigenic variation and shift of the organism are the hindrances in producing an effective vaccine. If people are immunized broadly, herd immunity results, with a decrease in the extent of contamination in the environment.

6.1. Future perspectives of the chapter

The Centers for Disease Control and Prevention (CDC) is always one step ahead in investigating any cholera outbreaks across the globe. They have trained professionals and sophisticated laboratory protocols for identification of toxigenic vibrios and emergence of any new antigenically modified strain as the Haiti outbreak. The WHO in collaboration with the CDC also provides information to the public and the health officials regarding the necessity of proper sanitization and preventive control measures to overcome the dreaded disease. The antigenic variation of the *V. cholerae* strains is one of the major limitations and hindrances in developing a 100% protective vaccination against it. The successful accomplishment of an effective vaccine will pave the way for a cholera-free globe so that the developed and the developing countries will not be able to face any more epidemics like the Haiti's and the West Bengal O139.

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Bacterial pathogens have been becoming the main problem in hospital and community-acquired infections. It is hard to treat the strains that are resistant to antibiotics, due to the causing recurrent and untreatable infections. In recent years, the combination treatments and the novel technologies have been preferred to overcome the emergence of antibacterial resistance of pathogens. In this book, examples of pathogenesis by clinical cases, control by antibiotics and bioactive antimicrobials, control by novel technologies with the collection of up-to-date researches and reviews are presented. This book can be useful for researchers interested in antibacterials, bioactive compounds, and novel technologies.

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