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REVIEWS

CITICOLINE SUPPLEMENTATION IN ISCHAEMIC STROKE: MOLECULAR BIOMARKERS FOR EVALUATION OF THERAPEUTIC RESPONSE

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ABSTRACT

INTRODUCTION: Ischaemic stroke (IS) is a complex neurological disorder involving multifactorial molecular and cellular responses that lead to neuronal damage and functional deficits. Despite progress in diagnostics and treatment, supplementary neuroprotective approaches and sufficiently informative biomarkers to assess therapeutic outcomes remain a challenge for scientists and clinicians. Citicoline (cytidine-5′-diphosphocholine) has gained attention as a multiple-action agent with membrane-stabilising, anti-inflammatory, antioxidant, and neuroregenerative effects.

AIM: This review aims to provide a comprehensive integration of available data on citicoline supplementation in IS patients, as well as molecular biomarkers associated with therapeutic response.

METHODS: A literature search was conducted in PubMed, ScienceDirect, and ResearchGate using predefined keywords related to citicoline, ischaemic stroke, neuroprotection, and molecular biomarkers.

RESULTS: The review summarises evidence regarding biomarkers associated with citicoline responsiveness, including SIRT1, BDNF, glutathione (GSH), malondialdehyde (MDA), cardiolipin, arachidonic acid, 8-isoprostaglandin F2α, and trimethylamine-N-oxide (TMAO), and their relevance to monitoring therapeutic outcomes

CONCLUSION: The integration of citicoline-therapy-sensitive biomarkers can be considered reliable within the framework of precision medicine.

Keywords: *citicoline, ischaemic stroke, biomarkers, neuroprotection, oxidative stress*

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INTRODUCTION

Ischaemic stroke (IS) remains a leading cause of mortality and long-term disability worldwide, driven by a complex pathophysiological cascade involving neuronal, glial, vascular, and immune components (1,2). The process initiated by cerebral ischaemia is characterised by a rapid depletion of oxygen and glucose, leading to energy failure, ionic imbalance, and excitotoxic neuronal injury. This cascade

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progresses through oxidative stress, inflammation, blood–brain barrier (BBB) disruption, and apoptosis (3). The therapeutic approaches for IS primarily aim to restore blood flow and neuronal function (4). In this context, neuroprotective agents capable of modulating the ischaemic cascade have gained considerable attention.

Cytidine 5′-diphosphocholine (CDP-choline) or citicoline is an essential endogenous intermediate in the phospholipid biosynthesis pathway, particularly in the production of phosphatidylcholine, a vital structural component of cell membranes, including neurons. For the first time, CDP-choline was obtained experimentally through crystallisation from yeast (5). In the 1970s, when the idea of its use as a pharmaceutical substance emerged, the name citicoline became widely adopted in scientific literature. The first scientific evidence of the neuroprotective effects of citicoline, administered as a pharmaceutical, was reported by Manaka et al. (1974), and since then, hundreds of studies have been published, based on clinical research and various experimental models (6). After being absorbed into the body, citicoline releases its two main components, cytidine and choline, which are distributed throughout the body, including crossing the BBB to reach the central nervous system (7). It is believed that its role in stimulating the synthesis of brain phospholipids underpins many of its beneficial effects (8). Additionally, it has been found that, when administered orally, citicoline is absorbed almost completely, with approximately the same bioavailability as when administered parenterally, and is well tolerated (8).

In addition of above mentioned, the identification of molecular biomarkers has become a key area of research aimed at improving diagnosis, prognosis, and therapeutic monitoring. Biomarkers provide measurable indicators of biological processes and therapeutic responses, and their incorporation into clinical practice may enable personalised treatment strategies (9).

This review summarises current knowledge on citicoline supplementation in IS, focusing on molecular biomarkers that indicate therapeutic response and the underlying biological mechanisms.

METHODS

A literature search was conducted on PubMed, ScienceDirect, and ResearchGate, covering publications up to March 2026. The following keywords were used in various combinations: “citicoline”, “history”, “ischaemic stroke”, “therapeutic potential”, “mechanism of action”, “molecular biomarkers”, “neuroprotective potential”, “supplement therapy”. Original research articles, clinical and experimental studies, systematic reviews, and meta-analyses focusing on citicoline, IS, and molecular biomarkers were considered eligible. Publications not directly related to IS, citicoline supplementation, or biomarker assessment were excluded. Priority was given to peer-reviewed studies providing mechanistic, clinical, or translational evidence relevant to therapeutic response monitoring.

RESULTS AND DISCUSSION

Molecular Mechanisms of Citicoline Action

Citicoline exerts its effects through several interconnected molecular mechanisms that directly target key components of the ischaemic cascade.

Lipid Metabolism

Citicoline is a precursor in the biosynthesis of phosphatidylcholine, a major component of neuronal membranes. During ischaemia, activation of cytosolic Ca^{2+}-dependent phospholipase A2 (cPLA2) leads to degradation of membrane phospholipids and the release of free fatty acids (FFA) into the serum and cerebrospinal fluid, including arachidonic acid (AA), which is metabolised into products that contribute to increased oxidative damage (10,11,12).

In this context, administration of citicoline enhances phosphatidylcholine synthesis and inhibits phospholipid degradation, thereby stabilising neuronal membranes (13). It also stabilises mitochondrial membranes by preventing the loss of cardiolipin, a key phospholipid essential for mitochondrial function (14). These effects not only preserve structural integrity but also reduce the availability of substrates for lipid peroxidation and inflammation.

Oxidative Stress and Antioxidant Effect of Citicoline

Oxidative stress plays a major role in the pathogenesis of stroke, and in the neurodegeneration it induces (15). Reperfusion after ischaemia is a crucial

factor in exacerbating oxidative damage at both the cellular and tissue levels.

Citicoline exerts its neuroprotective effects by mitigating oxidative stress through multiple mechanisms. One possible mechanism underlying the neuroprotective effect of citicoline is its positive influence on reduced glutathione (GSH) levels (16,17). Citicoline, similar to plant-derived polyphenols, has been found to maintain high levels of GSH, on the one hand by protecting it from oxidation and, on the other, by stimulating its synthesis (18,19). Reduced glutathione is one of the most important endogenous antioxidants, involved in the neutralisation of free radicals and peroxides, both directly via a non-enzymatic mechanism and as a cofactor of the enzyme glutathione peroxidase (GPx) (20). It has been suggested that oxidising choline to betaine might increase the levels of reduced cellular glutathione (18). Choline released from citicoline can be metabolised into betaine, which serves as a methyl donor in the conversion of homocysteine to methionine. Further, methionine is metabolised to S-adenosyl-l-methionine, which contributes to the sulfur pool required for the synthesis of glutathione (18).

Anti-Inflammatory and Neuroprotective Mechanisms

Inflammation is a key component of the pathogenesis of IS. The inflammatory response is a complex cascade of molecular events and interactions among pro- and anti-inflammatory mediators, including vasoactive peptides and amines, pro-inflammatory cytokines, eicosanoids, and acute-phase proteins, aimed at preventing tissue damage and restoring function. Ischaemic brain injury stimulates local production of tumour necrosis factor alpha (TNF-α) and interleukin beta (IL-1β) by microglial cells (9,21). Elevated levels of these pro-inflammatory cytokines are associated with BBB damage, reactive oxygen species (ROS) production, and upregulation of adhesion molecules. Furthermore, inflammatory signalling in stroke stimulates the expression of inducible isoforms of the pro-inflammatory enzymes cyclooxygenase (COX-2) and nitric oxide synthase (iNOS). COX-2 produces toxic prostanoids and superoxide radicals, which exacerbate post-ischaemic inflammation (22).

Neuroinflammation is a central driver of secondary injury in IS. Citicoline exerts anti-inflammatory effects through multiple mechanisms, including inhibition of nuclear factor kappa B (NF-κB), a key transcription factor that regulates inflammatory gene expression (19). Through this mechanism, citicoline reduces the production of pro-inflammatory cytokines such as TNF-α, IL-1β, and interleukin 6 (IL-6), while modulating microglial activation and limiting leukocyte infiltration across the BBB. Recently, increased expression of the NAD+-dependent deacetylase sirtuin1 (SIRT1) has been observed following citicoline treatment (23). The anti-inflammatory effect of SIRT1 is mediated by deacetylating NF-κB (24). Increased *SIRT1* expression has been associated with ischaemic tolerance, helping to prevent stroke recurrence (25).

Biomarkers for Assessing Therapeutic Response to Citicoline

The molecular pathogenesis of IS involves a complex interplay of excitotoxicity, oxidative stress, inflammation, and apoptotic signalling. Therefore, identifying biomarkers to assess stroke severity and monitor successful therapeutic outcomes remains a major challenge. In addition, a high percentage of IS patients have severe comorbidities, such as diabetes, hypertension, and cardiac rhythm problems. Such patients undergo various therapies, making it significantly more difficult to assess the response to a specific one. Table 1 summarises data on some biomarkers whose levels may be indicative of IS, as well as data on the effect of citicoline therapy on these biomarkers.

The degree of oxidative stress can be assessed using biomarkers that are both products of free radical oxidation and antioxidants. Markers of oxidative damage to membranes include malondialdehyde (MDA), the end product of membrane lipid peroxidation, and isoprostanes, such as 8-iso-prostaglandin F_2-alpha (8-iPGF$_{2\alpha}$) (26). Isoprostanes are a family of prostaglandin-like molecules formed non-enzymatically via ROS-mediated peroxidation of AA, independently of cyclooxygenase activity. Clinical studies have demonstrated increased circulating levels of 8-iPGF$_{2\alpha}$ in the plasma of IS patients (23). Levels of 8-iPGF$_{2\alpha}$ are considered the gold standard for measuring cumulative oxidative damage provoked

by various factors, including neuronal stress (27). Formed endogenously, 8-iPGF$_{2\alpha}$ can be measured in various biological samples, including urine, blood plasma/serum, exhaled breath condensate, amniotic fluid, saliva, and tissue material (28,29).

Recently, a formulation combining citicoline with antioxidants showed significant potential to reduce MDA, 8-iso-PGF$_{2\alpha}$, and AA in healthy controls (Table 1). The same study reported that the effect of supplementation on serum AA levels can vary by comorbidity (e.g., diabetes) but did not reduce 8-iso-PGF$_{2\alpha}$ levels in IS patients (23).

However, citicoline generally helps restore cellular and mitochondrial membranes. Administration of 500 mg/kg citicoline in experimental stroke significantly restored phosphatidylcholine (PC) and cardiolipin (CL) levels (Table 1) in experimental animals (18). Cardiolipin is a phospholipid specific to the inner mitochondrial membrane and plays an important role in maintaining neuronal homeostasis. Due to its unsaturated fatty acid content, CL can undergo lipid peroxidation under oxidative stress. Alterations in CL structure or a decrease in its total levels can lead to mitochondrial dysfunction (30). It has been reported that citicoline may stabilise mitochondrial integrity by inhibiting mitochondrial PLA2 activity and preventing cardiolipin loss (31,32).

One mechanism underlying the neuroprotective effects of citicoline is the activation of *SIRT1* gene expression (Table 1). The activity of the enzyme is associated with the regulation of many cellular functions, including maintaining metabolic homeostasis, extending lifespan, and reducing the risk of neurodegenerative diseases (33). Citicoline treatment has been shown to increase SIRT1 levels in cultured neurons, circulating blood mononuclear cells, and in the brain (34). A similar positive effect on *SIRT1* expression has been demonstrated for plant polyphenols, which is considered one mechanism by which they help protect cells from oxidative stress and inflammation. Recently, a six-month intervention study using a supplement combining citicoline with aronia and green tea extracts reported that *SIRT1* expression was significantly upregulated in IS patients, with the most notable increase observed in those with diabetes comorbidity. A possible synergistic effect of the ingredients was suggested (23).

In addition to the above, citicoline was reported to regulate neurotrophic factors, a group of secreted proteins that play roles in the development, growth, survival, and differentiation of the central nervous system, as well as in maintaining its homeostasis (35). Brain-derived neurotrophic factor (BDNF) is the most abundant of these factors and is widely distributed throughout the nervous system (36). Furthermore, BDNF is considered an early indicator of nerve regeneration (37). Deficiency of BDNF is associated with severe pathophysiological symptoms in IS patients, underscoring its central role in ischaemia (38). BDNF-enriched exosomes derived from neural stem cells demonstrate therapeutic potential for the treatment of IS by effectively attenuating cerebral ischaemia-induced injury (39). Citicoline treatment significantly increases BDNF expression, thereby enhancing neuronal survival and regeneration as an innate defence mechanism in response to neuronal injury (Table 1).

Additionally, GSH plasma levels are generally decreased in IS, serving as a marker of increased oxidative stress and stroke severity (40). Citicoline treatment resulted in a significant increase in total glutathione levels (Table 1), an increase in glutathione reductase activity, and a decrease in the glutathione oxidation ratio following transient cerebral ischaemia in adult rodents (18). Citicoline treatment restores the glutathione redox ratio diminished in staurosporine-treated human neuroblastoma cells (41).

Malondialdehyde is a marker of oxidative stress and lipid peroxidation in the brain (Table 1). Redox status is typically assessed by measuring serum MDA levels. Elevated serum MDA levels indicate increased lipid peroxidation and inflammation and serve as a reliable, non-invasive biomarker (42,43). The capacity of citicoline to reduce MDA concentrations has been documented in both clinical intervention studies and experimental models of neurodegenerative disorders and brain injury (17). Inhibiting membrane lipid peroxidation is particularly important for preserving neuronal integrity under pronounced oxidative stress, such as that seen in cerebral ischaemia (44). In an intervention study, MDA levels were significantly reduced after six months of citicoline supplementation in IS patients and controls, with participants stratified by sex and lifestyle characteristics (45).

Table 1. Biomarkers indicative of IS and of response to citicoline therapy.

Biomarker	Biological Role	Changes in IS (Ref.)	Effects of Citicoline Supplementation (Ref.)	Type of the Study
AA	Polyunsaturated fatty acid, released from membrane phospholipids; precursor of eicosanoids and isoprostanes	Elevated (10,23)	Decreased (23)	Astrocyte cell culture (10) Human intervention study (23)
8-iPGF$_{2\alpha}$	A marker of oxidative stress and lipid peroxidation, derived from AA	Significantly increased (23)	Decrease in healthy controls (23); Varies depending on the patients comorbidity (23);	Human intervention study (23)
CL	Major phospholipid in the inner mitochondrial membrane	Decreased due to reperfusion injury (18,47)	Restored levels due to PLA_2 inhibition (18,32)	Experimental animal model (18) Human intervention study (23) Clinical trials (47)
PC	Major structural phospholipid in cell membranes, essential for membrane integrity and signallinWg.	Decreased due to PLA_2 activation (18,48)	Donates substrates for PC synthesis, restores membrane integrity (13,18)	Experimental animal model (18) Human clinical study (13,48)
GSH	Cellular antioxidant neutralising hydrogen peroxide and lipid peroxides.	Decreased plasma levels (40,49)	Increased total GSH levels, increased glutathione reductase activity, decrease of glutathione oxidation ratio (18)	Experimental animal model (18) Human clinical study (40,49)
MDA	A marker of oxidative stress and lipid peroxidation in the brain	Significantly elevated levels (42)	Significantly decreased (45)	Human clinical study (42) Human intervention study (45)
SIRT1	Regulation of genes related to neuroinflammation and oxidative stress	Controversial: Decreased (50); Increased (51)	Increased markedly in IS patients with diabetes comorbidity (23)	Human intervention study (23) Human clinical study (50) Cell culture study (51)
BDNF	Role in the development, growth, cell survival, and differentiation of the central nervous system	Decreased levels (35,52)	Increases *BDNF* expression (37)	Experimental animal model (37) Human clinical study (35,52)
TMAO	Metabolite derived from gut microbiota metabolism of choline; linked to vascular and inflammatory processes	Significantly increased (46)	Indirect effect due to altered choline metabolism (53)	Cell culture studies (53) Experimental animal models (46,53) Human clinical studies (46,53) Human intervention studies (46)

Emerging evidence highlights trimethylamine-N-oxide (TMAO) as a potentially important biomarker linking gut microbiota metabolism with IS pathophysiology (Table 1). TMAO is generated through hepatic oxidation of trimethylamine, a metabolite produced by intestinal microbiota from dietary choline, phosphatidylcholine, and L-carnitine. Elevated circulating TMAO levels have been associated with endothelial dysfunction, platelet hyperreactivity, vascular inflammation, and atherosclerotic progression, all of which contribute to increased IS risk. Clinical studies and meta-analyses demonstrate that patients with acute IS exhibit significantly higher plasma TMAO concentrations compared to controls (44,46). Since citicoline is a source of choline, concerns have emerged about its potential influence on TMAO production (7). Current evidence remains limited and partially indirect. Experimental and metabolomic studies suggest that choline-containing compounds may increase substrate availability for gut microbial trimethylamine production. However, available data indicate that citicoline administration does not necessarily result in clinically significant elevations of TMAO, possibly due to differences in intestinal metabolism, hepatic conversion efficiency, and concurrent modulation of oxidative and inflammatory pathways. Recent analyses suggest that citicoline-associated neuroprotective benefits may outweigh potential metabolic concerns, although further controlled clinical studies evaluating longitudinal TMAO dynamics during citicoline supplementation in IS patients are still required (7,46).

In summary, the observed variability in clinical outcomes may reflect differences in biomarker profiles among patients. For example, individuals with higher baseline inflammatory or oxidative stress markers may derive greater benefit from citicoline therapy. Importantly, the absence of universally effective neuroprotective agents in stroke highlights the need for biomarker-guided patient selection. Citicoline multimodal mechanism of action makes it a suitable candidate for such personalised approaches.

Limitations and Future Directions

Despite substantial advances in biomarker research in IS, several important limitations continue to restrict the translation of these findings into routine clinical practice. One major challenge is the considerable heterogeneity of IS aetiology, clinical presentation, comorbidities, and treatment regimens, all of which may significantly influence biomarker expression and therapeutic response. In addition, many biomarkers demonstrate substantial temporal variability, requiring precise timing of sample collection and repeated measurements for reliable interpretation. Another critical limitation is the lack of standardised laboratory assays and validated reference ranges across different clinical centres. Certain highly promising biomarkers, including cardiolipin and specific lipid peroxidation metabolites, currently require sophisticated analytical platforms such as liquid chromatography-mass spectrometry (LC-MS/MS), which remain expensive, technically demanding, and not widely available in standard hospital laboratories (30,54,55,56). These methodological limitations considerably hinder their implementation in routine stroke diagnostics and therapeutic monitoring. Furthermore, many currently available studies are limited by relatively small patient cohorts, short follow-up periods, and insufficient integration of molecular biomarkers with neuroimaging and clinical outcome data.

Future research should therefore focus on large-scale multicentre prospective studies designed to validate biomarker panels with high diagnostic and prognostic accuracy. Integrating molecular biomarkers with imaging findings, genomic profiling, and artificial intelligence-based analytical approaches may substantially improve personalised therapeutic decision-making in IS. In addition, the development of cost-effective, standardised, and clinically accessible assays for advanced biomarkers such as cardiolipin, isoprostanes, and transcriptomic signatures could significantly facilitate the incorporation of precision medicine approaches into everyday neurological practice.

CONCLUSION

The integration of biomarker profiling with citicoline therapy is a promising step towards precision medicine in IS. However, further validation and standardisation are needed to translate these advances into routine clinical practice. Future research should prioritise large-scale, multicentre studies that integrate clinical, imaging, and molecular data. In addition, applying artificial intelligence and ma-

chine learning to biomarker analysis may help identify clinically relevant patterns and improve therapeutic decision-making.

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REVIEWS

FACTORS GOVERNING THE ANTIBACTERIAL ACTIVITY OF METAL-BASED NANOPARTICLES: PHYSICOCHEMICAL PROPERTIES, BIOLOGICAL ENVIRONMENT, AND EXPERIMENTAL CONDITIONS

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ABSTRACT

INTRODUCTION: The antibacterial activity of metal-based nanoparticles (MBNPs) varies considerably even among materials with the same elemental composition. Differences in particle size, morphology, surface properties, aggregation, synthesis, and exposure conditions can alter nanoparticle–bacteria interactions and complicate comparisons between studies.

AIM: This review explores the principal physicochemical and experimental factors that determine the antibacterial performance of MBNPs and considers how these variables influence the interpretation and reproducibility of antibacterial studies.

MATERIALS AND METHODS: A narrative literature review was conducted using Scopus, Web of Science, and PubMed. Studies addressing the relationship between MBNP characteristics or experimental conditions and antibacterial activity were considered.

RESULTS AND CONCLUSION: Antibacterial performance is determined by a combination of particle-related, biological, and experimental variables rather than nanoparticle composition alone. Smaller particle dimensions can increase available surface area and modify dissolution and surface reactivity, while morphology and surface chemistry influence bacterial contact and interfacial processes. Surface coatings can either restrict or enhance biological interactions, and aggregation may substantially change the effective particle size during antibacterial testing. Concentration, dissolution and metal-ion release, pH, ionic strength, proteins, organic matter, and light exposure further modify nanoparticle behavior. Differences in bacterial strain, growth state, inoculum, and assay methodology introduce additional variability and may contribute to apparently contradictory findings between studies. The antibacterial activity of MBNPs should be interpreted as the outcome of an interacting set of material and experimental variables rather than as a fixed property of a particular metal or metal oxide. Improved physicochemical characterization and more transparent reporting of microbiological test conditions are necessary for meaningful comparison between studies. Establishing reproducible relationships between nanoparticle characteristics and antibacterial performance will be important for the rational development of MBNP-based antimicrobial systems.

Keywords: *aggregation, antibacterial activity, antibacterial testing, metal-based nanoparticles, metal-ion release, nanoparticle functionalization, particle size, surface charge*

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1. INTRODUCTION

Metal-based nanoparticles (MBNPs) continue to attract attention as antibacterial materials because their nanoscale dimensions provide physicochemical characteristics that differ from those of the corresponding bulk materials (1–3). Depending on their composition, they may interact with bacterial surfaces, release biologically active metal ions, promote reactive oxygen species (ROS) formation, or interfere with essential cellular processes (1,4,5). Antibacterial activity has consequently been reported for several MBNPs against both Gram-positive and Gram-negative bacteria (2,3,5).

However, antibacterial activity does not imply that nanoparticles of the same chemical composition will necessarily behave the same way (1,5–7). Studies report considerable differences between apparently similar nanomaterials (6,7). A silver nanoparticle, for example, cannot be defined biologically by its silver content alone. Its dimensions, morphology, surface coating, charge, aggregation state, and dissolution behavior all contribute to the form in which the particle encounters a bacterial cell. The same principle applies to other MBNPs (1,5).

The aforementioned variables are primarily governed by the experimental procedure for MBNP synthesis (e.g., choice of reductant, reductant-to-salt ratio, etc.), are also interconnected (8). For example, a decrease in particle size changes the surface-area-to-volume ratio but may simultaneously affect dissolution and aggregation (5–7). A surface coating can improve colloidal stability while limiting direct contact between the inorganic surface and bacterial envelope (6,7). The surrounding medium may alter surface charge, promote aggregation, or lead to adsorption of proteins and other biomolecules. Consequently, a physicochemical characteristic examined in isolation may not fully explain the antibacterial response (5–8).

Variation also originates from the biological and experimental aspects of nanoparticle–bacterium interactions. Bacterial species and strains differ in cell-envelope organization and physiological characteristics, while inoculum density, growth phase, exposure time, culture medium, and antibacterial assay can influence the measured outcome. A nanoparticle preparation that appears highly active under one set of experimental conditions may therefore show a different response when the test system changes. This becomes particularly important when results obtained in simplified laboratory media are used to predict performance in biological or environmental settings (5,7,9,10).

Understanding the sources of variability matters for more than comparing published studies. Identifying relationships between nanoparticle characteristics and antibacterial performance can support rational material design, while recognizing experimental sources of variation can improve reproducibility. A review focused specifically on these determinants may therefore help distinguish the intrinsic contribution of the nanomaterial from effects introduced by its preparation, the surrounding environment, the bacterial target, or the testing procedure (6–10).

2. AIM

The aim of this review is to examine the key factors governing the antibacterial activity of MBNPs. Particular attention is given to particle size and morphology, surface charge and functionalization, concentration, aggregation and colloidal stability, dissolution and metal-ion release, synthesis method, and environmental conditions. The study also considers biological and methodological variables that influence the measured antibacterial response, with the broader objective of identifying factors to consider when comparing MBNP antibacterial studies and designing future antimicrobial nanoparticle systems.

3. MATERIALS AND METHODS

A narrative literature review was conducted to identify studies examining factors that influence MBNP antibacterial activity. Literature searches were performed in Scopus, Web of Science, and PubMed using combinations of terms related to "metal nanoparticles," "metal oxide nanoparticles," "antibacterial activity," "particle size," "shape," "morphology," "surface charge," "surface coating", "functionalization", "aggregation", "dissolution", "metal ion release", "synthesis," "culture medium," and "antibacterial assay."

Priority was given to peer-reviewed studies published in the last five years (from 2022 to 2026) examining relationships between nanoparticle charac-

teristics or experimental conditions and antibacterial outcomes, together with recent reviews providing broader interpretations of these relationships. Earlier studies of particular relevance to the topics discussed were also considered where appropriate. Studies concerning silver, gold, copper/copper oxide, zinc oxide, titanium dioxide, iron/iron oxide, and related antibacterial nanomaterials were considered when relevant to the factor under discussion. As this work was designed as a narrative rather than a systematic review, study selection was based on relevance to the scope and objectives of the review, and a formal systematic-review screening and study-selection protocol was not applied.

The literature was organized according to particle-related, environmental, biological, and methodological determinants of antibacterial activity. Because the objective was to provide a narrative synthesis rather than a quantitative comparison or meta-analysis, findings were interpreted according to reported trends and mechanistic relationships rather than pooled effect estimates.

AI-assisted technology (ChatGPT OpenAI, GPT-5.5) was used solely for language polishing and figure generation. All scientific content, interpretations, and conclusions were conceived, verified, and approved by the authors.

4. RESULTS

4.1. Factors Affecting Antibacterial Activity

The antibacterial performance of MBNPs cannot be predicted from elemental composition alone. Nanoparticles prepared from the same material may behave differently when their dimensions, morphology, surface properties, or state of dispersion differ. These characteristics influence the surface presented to bacterial cells, the stability of the particles in suspension, their interaction with the surround-

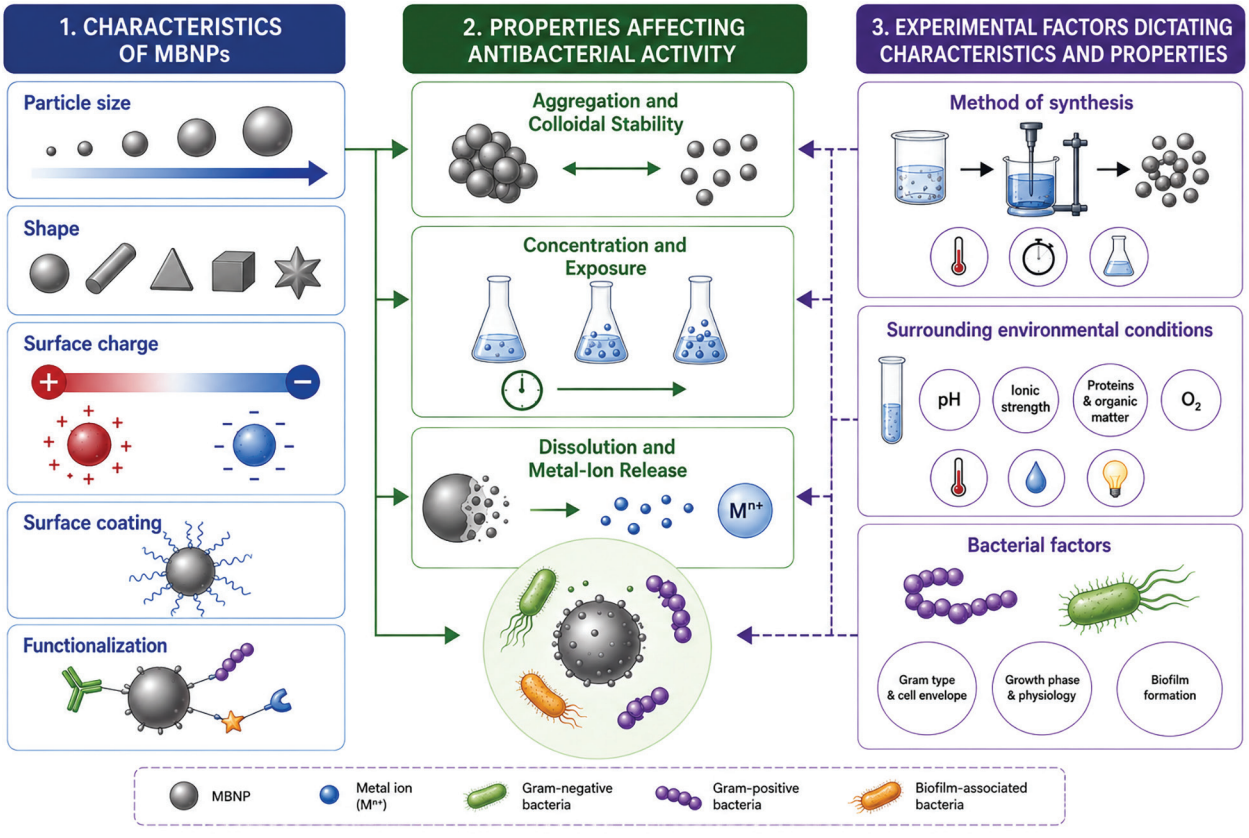


Fig. 1. Factors affecting antibacterial activity of MBNPs—physicochemical characteristics, properties and experimental factors. This figure was created with the assistance of ChatGPT (OpenAI, GPT-5.5). Artificial intelligence was used solely to generate the graphical illustration based on detailed author instructions. The scientific content, figure design, labels, workflow, and all technical information were conceived, verified, edited, and approved by the authors, who assume full responsibility for the final figure.

ing medium, and, where applicable, dissolution and ROS generation. In this respect, key physicochemical characteristics of MBNPs are herein related to processes and circumstances determining their physical state and biological reactivity, and ultimately, to their properties directing the antibacterial activity (1,5–7) (Fig. 1).

4.1.1. Physicochemical Characteristics of MBNPs Affecting Antimicrobial Activity

Particle Size

Particle size strongly affects antibacterial activity because smaller nanoparticles generally provide a higher surface-area-to-volume ratio. Consequently, more atoms are exposed at the particle surface, where interactions with the surrounding medium and bacterial cells occur. Depending on the material, this may favor dissolution and ion release, surface reactions, ROS formation, or direct interaction with the bacterial envelope. Smaller particles may also establish closer or more extensive contact with bacterial surfaces (5–7,9,11).

However, the relationship between size and antibacterial activity is not linear. Very small particles may aggregate, particularly in media containing salts, proteins, or other biological components, thereby reducing the effective surface area available for interaction. Surface coating and dissolution behavior can further modify the size-dependent response. Thus, nominal particle diameter alone is insufficient to predict antibacterial performance (6,7,9,11).

Shape

Nanoparticles can occur as spheres, rods, triangles, plates, cubes, stars, flower-like structures, and numerous other morphologies. Shape affects more than the physical appearance of the particle because different geometries expose different crystallographic facets and alter the proportion of surface atoms available for interaction. As a result, nanoparticles of the same chemical composition but different morphologies may differ in surface reactivity, dissolution, ROS production, and their interactions with bacterial envelopes. This effect is highly material-dependent, and no single morphology can be considered universally superior for antibacterial purposes. However, several studies have demonstrated the stronger antibacterial activity of nanosized cubes, triangles, rods, or wires in comparison to spherical MBNPs, owing to increased surface-to-volume area, sharp edges that may mechanically damage bacterial cell walls (by triangular plates), and/or geometrically facilitated penetration in bacterial biofilms (in the case of rods and wires). Comparisons of particle shape require caution because altering the synthesis conditions used to control morphology often changes particle size and surface chemistry at the same time (6,12–14).

Surface Charge

Bacterial surfaces are generally negatively charged under physiological conditions. However, this electrostatic attraction is heavily modulated by the ionic strength of the surrounding medium and the specific lipopolysaccharide or teichoic acid composition of the target strain, increasing particle accumulation at the cell surface and potentially facilitating membrane disruption (7,15). Surface charge can also influence colloidal stability and nanoparticle interactions with proteins and other components of biological media (5,7,16).

A strongly positive surface is not necessarily desirable, however (16,17). The same interactions that favor contact with bacteria can increase nonspecific association with mammalian cell membranes and consequently affect cytocompatibility. Surface charge, therefore, needs to be considered alongside antibacterial selectivity rather than antibacterial potency alone (7,14,16,17).

Surface Coating and Functionalization

The surface of MBNPs is rarely chemically inert (6,7,18,19). Stabilizers, polymers, surfactants, biomolecules, or deliberately attached functional groups can change particle charge, hydrophobicity, colloidal stability, and interaction with biological surfaces (6,7,16,19). A coating may reduce aggregation and improve dispersion, but it can also restrict dissolution or physically separate the inorganic surface from the bacterial envelope. Conversely, functionalization with antibiotics, antimicrobial peptides, or bacterial-targeting ligands may enhance antibacterial activity or improve selectivity (6,7,18,19). For this reason, results obtained with coated or functionalized nanoparticles should not automatically be attributed to the metal or metal oxide core. The biological response reflects the properties of the complete nanoparticle system (6,7,16,19).

4.1.2. Properties of MBNPs Dependent on the Physicochemical Characteristics and Affecting Antimicrobial Activity

Aggregation and Colloidal Stability

Aggregation is closely connected to several of the characteristics described above. Nanoparticles characterized in water or immediately after synthesis may behave quite differently once introduced into culture medium, physiological fluids, or environmental samples. Increased ionic strength can screen surface charges and promote aggregation, while proteins and other biomolecules may adsorb onto the particle surface, forming a corona. Such changes alter the effective particle size and the surface presented to bacterial cells (7,9,16). Moreover, such events may complicate the process of determining the minimum inhibitory concentrations (MICs) using turbidity-based detection. For example, there have been documented cases of colloidal destruction and palpable opalescence when MBNP samples are introduced into inoculated bacterial culture media (20).

Concentration and Exposure

Nanoparticle concentration is one of the most immediate determinants of the antibacterial response (1,7,9,20). Increasing concentration generally increases the number of particles available for interaction with bacterial cells and, for dissolving materials, may increase the amount of biologically available metal ions; however, higher concentrations may simultaneously promote aggregation in high-ionic-strength media, potentially reducing the effective surface area available (1,9). Dose-dependent reductions in bacterial growth or viability are therefore commonly reported (1,7,20). The relationship is not necessarily proportional, however, because changes in aggregation, sedimentation, dissolution, and surface availability can occur as concentration increases (7,9,20).

Exposure time should be considered together with concentration. A high concentration applied for a short period and a lower concentration maintained for longer may not produce equivalent effects (1,5,20). Time is particularly relevant when antibacterial activity depends partly on gradual dissolution or accumulation of cellular damage. Reporting concentration without sufficient information on exposure duration therefore provides an incomplete description of the antibacterial challenge (1,5,9).

Increasing dose also raises an important question of selectivity. A concentration that produces strong bacterial inhibition may have limited practical value if it causes comparable damage to mammalian cells or non-target organisms. For biomedical applications in particular, antibacterial efficacy should therefore be considered in relation to the concentration range over which acceptable cytocompatibility is maintained (14,16,17,20).

Dissolution and Metal-Ion Release

For nanoparticles capable of dissolution, metal-ion release can substantially influence antibacterial performance. The nanoparticle in this case acts not only as a particulate material but also as a source of soluble metal species (21–25). The extent and rate of dissolution depend on the material composition, particle size, surface characteristics, and the chemistry of the surrounding medium (12,21,22,24,25). This relationship is particularly relevant to Ag-, Cu-, and Zn-containing nanoparticles, where released ions can interact with proteins, enzymes, membranes, and other cellular components (21–25). Smaller particles may dissolve differently from larger ones because of their greater specific surface area, illustrating how dissolution is linked to other physicochemical characteristics rather than functioning as an independent variable (12,22,25). Surface coatings may further restrict or modify contact between the particle and medium and consequently change ion availability (22,24,25).

4.1.3. Experimental Factors Dictating the Characteristics and Properties of MBNPs

Method of Synthesis

Chemical, physical, and biological or green synthesis methods can produce nanoparticles with different sizes, shapes, surface coatings, and aggregation behavior. The synthesis route can therefore influence antibacterial activity indirectly through the physicochemical characteristics of the final product. Residual reactants, reducing agents, stabilizers, and other compounds remaining after synthesis may also affect the measured biological response if purification is incomplete (8,26–28).

Biological synthesis using plant extracts, microorganisms, or biomolecules has received consid-

erable interest because biological components can act as reducing and stabilizing agents (8,26,29). Such approaches may reduce the need for some conventional chemical reagents and can produce nanoparticles carrying biologically derived surface molecules (26,29). At the same time, the composition of biological extracts is inherently complex and may vary with source and preparation conditions. Batch-to-batch reproducibility, identification of surface-bound compounds, purification, and precise control of particle properties therefore remain important considerations (8,20,26,29). It should be noted that biologically-based synthetic approaches most commonly yield spherical MBNPs (29).

Green synthetic approaches for MBNPs often result in richer surface functionalization than chemically produced MBNPs. The so-obtained "crown" on the surface often consists of bioactive compounds; therefore, in addition to being responsible for the reduction of metal ions to metal nanoparticles and their stabilization in colloidal dispersions, it may also contribute to the antimicrobial potency of the particles (8,26,29,30).

Comparing synthesis routes requires more than identifying a material as chemically, physically, or biologically produced. The properties of the resulting nanoparticles and the composition of their surfaces need to be considered. Otherwise, differences attributed to the synthesis method may actually reflect differences in particle size, morphology, surface-bound compounds, or residual components of the preparation (8,26,27,29,30).

Surrounding Environmental Conditions

Ionic strength, pH, temperature, organic matter, light exposure, and the composition of the surrounding medium can influence nanoparticle aggregation, dissolution, surface charge, and ROS production (9,25,31,32). Their importance varies by nanoparticle composition. Acidic conditions, for example, may alter the dissolution of some metal oxides, whereas light exposure is particularly relevant for photocatalytic materials such as TiO_2 and ZnO (9,25,31). Proteins and other organic molecules can adsorb onto nanoparticle surfaces, further modifying their biological identity and interactions with bacteria (32).

Once nanoparticles enter a complex biological medium, the surface initially characterized after synthesis may no longer be the surface presented to bacterial cells (16,32,33). Adsorption of proteins and other biomolecules can alter particle charge, colloidal stability, recognition, and interaction with cellular surfaces (7,16,32,33). The medium composition is therefore not merely a background experimental variable but can become part of the nanoparticle system itself (32,33).

Light exposure deserves separate consideration for photocatalytic materials (25,31,34,35). TiO_2 and, under appropriate conditions, ZnO can show altered ROS production following irradiation (25,31,34). Antibacterial results obtained under defined light conditions should therefore not be extrapolated directly to settings in which the intensity or wavelength of exposure differs substantially (31,34,35).

Bacterial Factors

Variation in antibacterial activity cannot be explained solely from the nanoparticle side of the interaction (10,15,36,37). The bacterial target also contributes substantially to the measured response. Differences in cell-envelope structure between Gram-positive and Gram-negative bacteria can influence nanoparticle adsorption, access to the cytoplasmic membrane, and susceptibility to surface-mediated damage (15,36,37). These structural differences are important, but they do not establish a universal rule whereby one bacterial group is consistently more susceptible to MBNPs than the other (10,36,37).

Variation can also occur between species and even between strains of the same species (14,36–39). Surface composition, stress-response systems, metal homeostasis, antioxidant defenses, efflux mechanisms, and other physiological characteristics may alter the response to nanoparticle exposure (14,15,38,39). The resistance phenotype of a strain should likewise be clearly reported, particularly when investigating multidrug-resistant clinical isolates (14,38,39).

The physiological state of the bacteria further affects susceptibility. Cells in different growth phases do not necessarily respond identically to antimicrobial stress, and bacteria within biofilms differ markedly from their planktonic counterparts (40–42). Biofilm-associated cells are embedded in an ex-

Table 1. Representative MBNP studies and key determinants of activity.

MBNP type	Shape	Size	Surface Charge	Surface modification	Target Bacteria	Key MIC Values or Antibacterial Outcomes	Reference
ZnO NPs	**Granular/mixed shape**	Mean size of about 48–52 nm (SEM); manufacturer-reported average size of 67 nm	**Negative**; numerical ζ-potential not reported	Not reported; commercial ZnO NPs	*S. aureus*	ZnO NP toxicity in Mueller–Hinton broth was mediated predominantly by dissolved Zn^{2+}; medium composition strongly influenced the antibacterial response.	(9)
ZnO NPs	Not reported	22, 35, and 66 nm (TEM)	Not reported	None	*E. coli*, *P. aeruginosa*, *S. aureus*	Antibacterial activity was size-dependent, with smaller ZnO NPs showing greater antibacterial activity than larger particles.	(11)
AgNPs	Spheres, platelets, cubes, and rods	Spheres glucose synthesis 40–70 nm (SEM); 45 nm (DCS); 108 nm (DLS); diameter: 145 nm, height: 48 nm (AFM); Spheres microwave synthesis and 120–180 nm (SEM); 123 nm (DCS); 128 (DLS); Platelets 20–60 nm; 30 nm (DCS); 48 nm (DLS); diameter: 80 nm, height: 16 nm (AFM); Cubes 140–180 nm; 167 nm (DCS); 211 nm (DLS); Rods 50–100 nm diameter, 1000–20000 nm length (SEM); 558 nm (DLS)	Spheres: −6 and −3 mV; Platelets: −7 mV; Cubes: −11 mV; Rods: −20 mV	PVP-stabilized	*S. aureus*	Antibacterial effects varied with particle size and shape despite comparable surface functionalization; activity was associated with Ag dissolution and specific surface area.	(12)
AgNPs	Spherical (AgNS) and truncated-octahedral (AgNOct)	AgNS average diameter–195 nm; AgNOct average diameter–194 nm (SEM)	Positive; numerical ζ-potential not reported	None	*E. coli*, *E. faecium*	Antibacterial activity differed according to particle shape; AgNOct showed stronger antibacterial effects than AgNS under the tested conditions.	(13)

AgNPs–chlorhexidine conjugate	Not reported	Unconjugated Ag NPs: 92.34 nm (DLS)	Ag NPs: −50.01 mV; after chlorhexidine conjugation: +44.59 mV	Green-tea-derived surface coating; chlorhexidine conjugation	*E. coli*, *S. aureus*	AgNP–chlorhexidine conjugate MIC: Ag 5.5 µg/mL + chlorhexidine 8.8 µg/mL against both bacteria. AgNPs alone had an MIC of 175 µg/mL against *E. coli*, while no MIC against *S. aureus* was established within the investigated range.	(20)
ZnO NPs	Not reported	< 100 nm	Not reported	Not reported; commercial ZnO NPs	*P. aeruginosa*, *Salmonella* spp., *K. pneumoniae*, *E. coli*, *S. aureus*, *E. faecalis*, *S. saprophyticus*, *S. pyogenes*	MIC range: 15.625–125 µg/mL; MBC range: 62.5–250 µg/mL; inhibition zones 16–21 mm. Gram-positive isolates were generally more susceptible.	(28)
Pt, Ag, and Au NPs with HSA protein corona	Spherical	PtNPs: 10 ± 2 nm; PtNP–HSA: 30.5 ± 3 nm; AgNPs: 9 ± 3 nm; AgNP–HSA: 18.0 ± 3 nm; AuNPs: 10 ± 2 nm; AuNP–HSA: 21.6 ± 3 nm (DLS)	Not reported	HSA corona	*E. coli*	Formation of an HSA protein corona modified nanoparticle–bacterium interactions and altered antibacterial behavior.	(33)
Cu-doped / Ag-decorated TiO_2 NPs	Irregularly shaped	TiO_2-A: 20.2 ± 5.7 nm (TEM), 19.81 ± 4.55 nm (SEM), 74.47 ± 23.9 nm (DLS); TiO_2-A:Cu-1: 21.9 ± 5.4 nm (TEM), 23.93 ± 6.2 nm (SEM), 33.55 ± 11.4 nm (DLS); TiO_2-A:Cu-2: 22.20 ± 5.52 nm (SEM), 57.41 ± 24.27 nm (DLS); TiO_2-A:Cu-3: 22.05 ± 4.85 nm (SEM), 357.9 ± 109.9 nm (DLS); TiO_2-B: 82.1 ± 18.2 nm (TEM), 89.4 ± 13.3 nm (SEM), 165.1 ± 96.76 nm (DLS); TiO_2-B:Ag-1: 140.4 ± 37.0 nm (TEM), 127.1 ± 44.2 nm (SEM), 167.3 ± 30.1 nm (DLS); TiO_2-B:Ag-2: 131.4 ± 24.0 nm (SEM), 154.3 ± 94.4 nm (DLS); TiO_2-B:Ag-3: 142.2 ± 31.7 nm (SEM), 142.8 ± 67.58 nm (DLS	TiO_2-A: −13.2 ± 5.4 mV; TiO_2-A:Cu-1: −18.4 ± 4.24 mV; TiO_2-A:Cu-2: −22.8 ± 5.31 mV; TiO_2-A:Cu-3: −24.8 ± 6.56 mV; TiO_2-B: +25.3 ± 6.38 mV; TiO_2-B:Ag-1: −10.4 ± 5.73 mV; TiO_2-B:Ag-2: −54.9 ± 13.6 mV; TiO_2-B:Ag-3: −67.7 ± 11.1 mV	Cu doping or Ag decoration	*E. coli*, *S. aureus*	Modified TiO_2 NPs showed enhanced antibacterial activity, particularly under visible-light irradiation, associated with enhanced photocatalytic/ROS-generating activity.	(35)

AgNPs	Spherical	2 nm	Not reported	Not reported; commercially obtained AgNPs	Selected Gram-positive and Gram-negative pathogens	AgNPs exhibited antibacterial activity against both Gram-positive and Gram-negative bacteria, with differences in susceptibility among tested organisms.	(36)
C-phycocyanin-stabilized Ag NPs (Ag-Pc)	Hexagonal/ spherical	Classic AgNPs : 175.25 nm (DLS); 15–25 nm (TEM); Ag-Pc: 215.6 nm (DLS); 26–43 nm (TEM)	Classic Ag NPs: −20.4 mV Ag-Pc: −18.1 mV	C-phycocyanin stabilization	MRSA; carbapenem-resistant *P. aeruginosa*	Ag-Pc MIC: 7.4 µg/mL against both bacteria. MBC: 7.4 µg/mL against MRSA and 14.9 µg/mL against *P. aeruginosa*. Ag-Pc also showed greater antibiofilm activity than unmodified AgNPs.	(38)

Abbreviations: AFM—atomic force microscopy; AgNPs—silver nanoparticles; DCS—differential centrifugal sedimentation; DLS—dynamic light scattering; HSA—human serum albumin; MBC—minimum bactericidal concentration; MBNPs—metal-based nanoparticles; MIC—minimum inhibitory concentration; MRSA—methicillin-resistant S. aureus; PVP—polyvinylpyrrolidone; ROS—reactive oxygen species; SEM—scanning electron microscopy; TEM—transmission electron microscopy; TiO_2 NPs—titanium dioxide nanoparticles; ZnO NPs—zinc oxide nanoparticles.

tracellular matrix and display metabolic and physiological heterogeneity that can modulate nanoparticle access and antibacterial responses. Consequently, activity measured against planktonic cultures should not be used as a direct predictor of antibiofilm performance (10,38,40–42).

Representative experimental studies illustrating how major physicochemical, environmental, and biological determinants influence the antibacterial activity of MBNPs are summarized in Table 1. The reported antibacterial endpoints should be interpreted within the experimental conditions of each study rather than compared directly across studies, because nanoparticle characterization, bacterial strains, exposure conditions, and antibacterial assays differ considerably.

4.2. Interdependence of Factors

Although the factors discussed above are often investigated separately, their effects on antibacterial activity are closely interconnected (6,7,9,43,44). Particle size can influence dissolution and aggregation; surface coating can modify charge, colloidal stability, bacterial contact, and ion release; and the surrounding medium can alter these properties once the nanoparticle enters the test system (6,7,9,43). Concentration may likewise affect aggregation, while bacterial physiology and assay conditions determine how the resulting nanoparticle state is translated into a measurable antibacterial response. For this reason, attributing increased or decreased antibacterial activity to a single characteristic requires caution (6,7,43,44). A synthesis modification intended to change particle morphology may simultaneously alter size and surface chemistry (6,8). Similarly, two nanoparticle preparations with the same primary particle diameter may have very different effective sizes once dispersed in bacterial culture medium. Relationships between structure and antibacterial activity are therefore most informative when several physicochemical characteristics are measured together and under conditions relevant to the antibacterial experiment (7,9,43,44).

4.3. Implications for Characterization and Reporting

The variability described throughout this review highlights the need to characterize the material actually used in antibacterial testing rather than

relying only on nominal composition or manufacturer specifications (43–45). At minimum, studies should provide sufficient information on particle dimensions, morphology, surface properties, concentration, and coating or functionalization. Where relevant, aggregation state and dissolution behavior in the exposure medium should also be considered (6,7,43–45).

Microbiological conditions require comparable attention. Bacterial strain, inoculum, culture medium, exposure time, assay method, and endpoint should be clearly reported (43–45). For biofilm experiments, the stage and method of biofilm formation are also important (40–42,44). Such information does not eliminate biological variability, but it allows differences between studies to be interpreted more meaningfully (43–45).

A further consideration is the relationship between antibacterial activity and safety (14,20,46). For systems intended for biomedical use, reporting strong bacterial inhibition without evaluating the response of relevant mammalian cells provides only part of the information needed to assess potential usefulness (16,20,45,46). The concentration associated with antibacterial activity should therefore be considered in the context of the intended application and corresponding biological exposure (14,20,45,46).

5. DISCUSSION

The literature indicates that antibacterial activity is an emergent property of the complete nanoparticle–environment–bacterium system rather than a fixed characteristic of a particular metal or metal oxide. This helps explain why studies using nanoparticles of apparently similar composition can reach different conclusions. Nominal composition provides only the starting point; the form in which the material is presented to bacteria determines the interactions that can subsequently occur (5,7,9,32,43,44).

Among particle-related characteristics, size receives particularly extensive attention, but its influence highlights the difficulty of assigning activity to a single variable. A decrease in particle diameter increases specific surface area, yet the resulting biological effect may involve altered dissolution, surface reactivity, bacterial contact, or aggregation (6,7,11,12). Similar interdependence applies to morphology and surface functionalization (6,7,12,16). A coating introduced to stabilize a nanoparticle may simultaneously change its charge, interaction with the bacterial envelope, and accessibility of the inorganic surface. Consequently, simple relationships such as "smaller particles are more antibacterial" are useful as general observations but insufficient as predictive rules (6,7,11,16).

The nanoparticle state during the antibacterial experiment is equally important. Characterization performed immediately after synthesis or in water does not necessarily describe the material present in a bacterial culture medium or biological fluid (7,9,32,33). Ionic strength can promote aggregation, proteins can adsorb to the surface, and dissolving nanoparticles may progressively generate soluble metal species (9,21,32,33). The antibacterial system therefore changes with time and with the composition of the surrounding medium (9,32,33,43). This distinction is particularly relevant when considering laboratory findings for biomedical or environmental applications (32,43–45).

Variation on the bacterial side further complicates interpretation (10,15,36,38). Differences between Gram-positive and Gram-negative envelopes can influence initial particle interactions, but Gram classification alone cannot predict bacterial susceptibility (15,36). Species, strain, physiological state, and biofilm growth can all modify the response (10,38,40,42). The use of a single laboratory strain may therefore be useful for controlled comparison but provides limited information about the range of activity expected against clinically or environmentally relevant bacterial populations (14,36,38,43).

Methodological differences are another important source of apparent disagreement. Diffusion-based, broth-based, time-kill, and biofilm assays do not measure identical outcomes, and nanoparticle-specific behavior can interact with the method itself (10,43,44). Limited diffusion through agar, sedimentation in liquid culture, optical interference, or gradual ion release can influence the result independently of bacterial susceptibility. Direct comparison of antibacterial values across studies is therefore most meaningful when the nanoparticle characterization and microbiological methodology are sufficiently similar (9,20,43–45).

These observations have practical implications for designing antibacterial nanomaterials (7,14,16,17). Optimization should not focus on maximizing a single characteristic such as small size, positive charge, or high concentration (7,16,17,20). Instead, the objective is to identify combinations of properties that provide sufficient antibacterial activity while retaining stability, selectivity, and an acceptable safety profile under the intended conditions of use. This is especially important in biomedical applications, where enhanced interaction with bacterial membranes may also increase interaction with mammalian cells (14,16,17,20,45).

Future work would benefit from experimental designs that deliberately examine interactions between variables rather than modifying one nominal parameter without characterizing the accompanying changes (7,43–45). Physicochemical measurements performed in the actual exposure medium, assessment of ion release where relevant, and transparent reporting of bacterial and assay conditions would improve interpretation (9,21,43–45). Studies incorporating clinically relevant strains, established biofilms, and parallel cytocompatibility assessment would further help determine whether physicochemical advantages observed in simplified systems remain useful under more realistic conditions (14,20,38,40,42,43).

The field therefore faces a shift from demonstrating antibacterial activity toward understanding and controlling it. Establishing reproducible relationships between material characteristics, exposure conditions, and biological response will be necessary to design MBNPs rationally rather than evaluate them largely on a formulation-by-formulation basis (7,43–45).

6. CONCLUSION

The antibacterial activity of MBNPs is determined by more than their chemical composition. Particle size and morphology, surface properties, aggregation, concentration, dissolution, and synthesis all contribute to the form and reactivity of the material presented to bacterial cells. These characteristics are further modified by the surrounding medium and interact with bacterial and experimental variables, making antibacterial performance difficult to predict from any single parameter.

A better understanding of these relationships is needed to explain differences between published studies and to guide the rational design of antibacterial nanomaterials. Future investigations should combine thorough physicochemical characterization with clearly defined microbiological conditions and, where possible, characterize nanoparticle behavior in the medium in which antibacterial activity is actually measured. For biomedical applications, antibacterial efficacy should also be evaluated together with cytocompatibility rather than as an isolated endpoint. Greater consistency in characterization and reporting would make comparisons between studies more meaningful and help identify which combinations of nanoparticle properties are genuinely advantageous for antibacterial use.

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REVIEWS

METAL-BASED NANOPARTICLES AS ANTIBACTERIAL AGENTS: MECHANISMS, ACTIVITY, AND THERAPEUTIC POTENTIAL

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ABSTRACT

INTRODUCTION: Metal-based nanoparticles (MBNPs) have attracted considerable interest as antibacterial materials because they can act on bacterial cells through multiple mechanisms and can be tailored for diverse antimicrobial applications.

AIM: This narrative review examined the antibacterial activity of major MBNP classes, emphasizing their mechanisms of action, activity against bacterial biofilms, potential applications, and current limitations.

MATERIALS AND METHODS: Relevant literature on silver, gold, copper and copper oxide, zinc oxide, titanium dioxide, and iron-based nanoparticles was reviewed. Particular attention was given to their activity against Gram-positive and Gram-negative bacteria, the mechanisms involved in bacterial damage, and their reported effects on bacterial biofilms.

RESULTS AND CONCLUSION: The reviewed studies demonstrate antibacterial activity across all major MBNP classes, although the nature and extent of the effects differ among nanomaterials. Several mechanisms are involved, including disruption of the bacterial envelope, release of metal ions, generation of reactive oxygen species, interference with proteins and enzymes, DNA damage, and disruption of cellular metabolism. The microbicidal potency of some MBNPs is fortified through functionalized, photocatalytic, or magnetically responsive antimicrobial systems (e.g., iron-based MBNPs). Some types of MBNPs have additionally shown activity against bacterial biofilms. Metal-based nanoparticles provide several routes for antibacterial action and offer possibilities that extend beyond direct bacterial killing. Their future value may lie particularly in antimicrobial coatings, delivery systems, combination approaches, and other applications that leverage both their antibacterial and material-specific properties. Pharmaceutical development of MBNP therapeutic systems should consider antibacterial efficacy along with biological safety and performance under conditions relevant to the intended route of application.

Keywords: *antibacterial activity, antimicrobial resistance, bacterial biofilm, metal nanoparticles, nanomedicine, reactive oxygen species*

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1. INTRODUCTION

Bacterial infections remain one of the most serious causes of morbidity and mortality worldwide (1). At the same time, the increasing prevalence of antimicrobial-resistant bacteria has reduced the effectiveness of many conventional antibiotics (2,3). A particularly serious problem arises in infections caused by multidrug-resistant organisms and those involving persistent bacterial biofilms (3,4). That is

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why numerous studies aim to develop antibacterial agents that can either replace conventional antibiotics or improve their effectiveness (3–5).

Nanotechnology offers some of the most promising "weapons" against antimicrobial resistance. Metal-based nanoparticles (MBNPs), for instance, attract great interest due to their inherent antimicrobial properties (6,7). Their nanoscale particle size and corresponding large surface area additionally aid interactions with bacterial cells. Metal-based nanoparticles have been shown to exert antibacterial effects through multiple pathways simultaneously, including damage to membranes and intracellular targets, initiation of metal-ion release, and induction of oxidative stress (6,7). Their efficacy against a wide range of Gram-positive and Gram-negative bacteria has been demonstrated, with several types of nanocarriers showing potential against bacterial biofilms (6,8). However, the activity of MBNPs depends heavily on the method of synthesis used and the surface functionalization, physicochemical properties, and concentration thus obtained, as well as on the environmental conditions (6,9).

2. AIM

This narrative review examines the antibacterial activity of MBNPs, focusing on their mechanisms of action, antibiofilm effects, potential applications, and current limitations. Particular attention is given to the major classes of MBNPs and the differences in their antibacterial properties.

3. MATERIALS AND METHODS

A narrative literature search was conducted using PubMed, Scopus, Web of Science, and ScienceDirect. The search included publications available up to July 2026. Combinations of terms such as "metal-based nanoparticles," "metal nanoparticles," "metal oxide nanoparticles," "antibacterial activity," "mechanism of action," "antimicrobial resistance," and "bacterial biofilm" were used, together with the names of the individual nanomaterials discussed in this review. Relevant original studies and review articles published in English were considered. Publications were selected based on their relevance to antibacterial mechanisms, antibiofilm effects, potential applications, and safety.

AI-assisted technology (ChatGPT OpenAI, GPT-5.5) was used for data analysis, improvement of the manuscript's readability and language, and generation of graphical illustrations.

4. RESULTS

4.1. Classification of MBNPs

Metal-based nanoparticles can broadly be divided into two groups: metal nanoparticles and metal oxide nanoparticles. The nanoparticles in the first group contain the corresponding metal in nanoscale form, including silver (Ag), gold (Au), copper (Cu), iron (Fe), and others. The metal oxide nanoparticles include nanoscale ZnO, CuO, TiO_2, Fe_3O_4, γ-Fe_2O_3, MgO, and others (6,7,10). Not exclusively, metal nanoparticles may acquire surface oxidation and thus a hybrid (of both groups) nature and properties—a consideration that should be taken into account with respect to potential time-dependent shift in the particles' functionality (11,12).

4.2. Antibacterial Mechanisms of MNPs

The antibacterial activity of MBNPs is complex and cannot generally be attributed to a single mechanism. Several processes can occur simultaneously, with their contribution varying according to the type of nanoparticle, its physicochemical properties, and the bacterial species involved. Membrane damage, metal-ion release, oxidative stress, interference with proteins and nucleic acids, and disruption of cellular metabolism are among the mechanisms most often associated with their antibacterial effects (6,7,13) (Fig. 1).

Interaction with the Bacterial Cell Envelope

The bacterial cell envelope is one of the first structures contacted by nanoparticles. Electrostatic interactions between nanoparticles and bacterial surfaces can promote nanoparticle adsorption. This close contact can disrupt cell-envelope organization and, in some cases, lead to membrane defects and increased permeability (6,7,10). Loss of membrane integrity may then cause leakage of intracellular components and disrupt normal exchange of substances between the cell and its environment. The response is not necessarily the same in all bacteria. Differences in the architecture and composition of Gram-positive and Gram-negative cell envelopes can influence their interaction with nanoparticles and, consequently, their susceptibility to membrane dam-

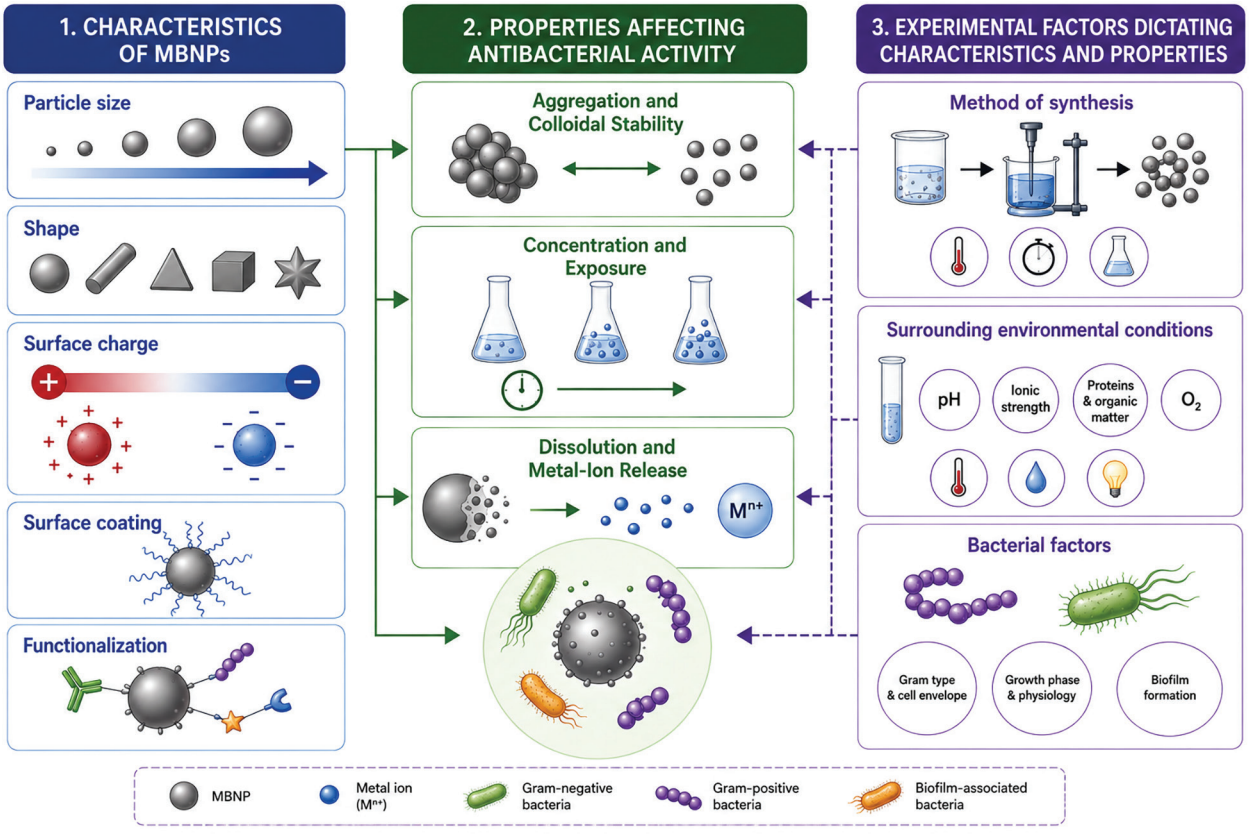


Fig. 1. Mechanisms of antimicrobial action inherent to MBNPs. This figure was created with the assistance of ChatGPT (OpenAI, GPT-5.5). Artificial intelligence was used solely to generate the graphical illustration based on detailed author instructions. The scientific content, figure design, labels, workflow, and all technical information were conceived, verified, edited, and approved by the authors, who assume full responsibility for the final figure.

age (6,10). In this regard, many studies, particularly those involving silver nanoparticles, report greater susceptibility of Gram-negative bacteria. This difference is attributed primarily to the substantially thicker peptidoglycan layer of Gram-positive bacteria and the negatively charged lipopolysaccharide-rich surface of Gram-negative bacteria (relevant to particles' sorption) (6,14).

Release of Metal Ions

For many MBNPs, antibacterial activity is also related to the release of metal ions. Once liberated into the surrounding medium, these ions can reach cellular targets and interact with proteins, enzymes, nucleic acids, and membrane components (6,7,13). Interactions with thiol-containing proteins are particularly important for some metals and may interfere with normal enzymatic function. As these effects accumulate, essential metabolic processes become impaired and bacterial viability decreases. The importance of this mechanism varies considerably between MBNPs, since ion release depends on their chemical composition, dissolution behavior, and the conditions of the surrounding medium (6,7,13). Importantly, this mechanism allows remote antimicrobial efficacy independent of size- and surface-related events associated with increased toxicity, such as whole-particle absorption or uptake into the bloodstream or deeper tissue layers (15).

Generation of Reactive Oxygen Species

One of the most frequently proposed mechanisms of MBNP-mediated bacterial killing is the generation of reactive oxygen species (ROS), including superoxide anions, hydroxyl radicals, and hydrogen peroxide (6,7,13). At sufficiently high levels, ROS overwhelm bacterial antioxidant defenses, leading to oxidative stress. The resulting damage is not confined to a single cellular target: membrane lipids may undergo oxidation, proteins may lose their normal

function, and oxidative lesions may develop in nucleic acids (6,7,13). ZnO, Ag, and TiO_2 nanoparticles are among the materials for which ROS generation has frequently been linked to antibacterial activity. Nevertheless, the contribution of oxidative stress differs among nanoparticle types and is strongly affected by experimental conditions (6,13,16).

Protein and Enzyme Damage

Proteins represent another major target of metal-based antibacterial action. Released metal ions, and in some cases the nanoparticles themselves, can interact with functional groups in proteins and alter their structure or activity (6,7,17). Inhibition of individual enzymes may then affect much broader cellular processes. Respiration, ATP production, nutrient utilization, cell-wall synthesis, and DNA replication are all dependent on functional protein systems and can therefore be disrupted. Rather than acting through the inhibition of a single enzyme, MBNPs may affect several protein-dependent pathways at the same time, contributing to progressive loss of bacterial function (6,16,17).

DNA Damage

Damage to bacterial genetic material may occur as a secondary consequence of oxidative stress or through interactions involving metal ions and other cellular components (6,13,17,18). Reactive oxygen species can induce oxidative modifications of nucleobases and DNA strand breaks, while disruption of proteins involved in DNA maintenance may further compromise genomic integrity. For some MBNPs, direct or indirect interference with replication and transcription has also been described (6,13,17–19). When such damage exceeds the bacterial cell's repair capacity, normal proliferation is impaired, and cell death may follow (13,17,19). Although nucleic acids may be impaired through multiple of the above mechanisms, DNA susceptibility to MBNP-related damage is closely linked to the particles' ability to enter the nucleus directly, and thus to their size and surface functionality. Therefore, increased genotoxicity is associated with MBNPs of smaller dimensions (< 10 nm) (18,19).

ATP Depletion and Metabolic Disruption

The antibacterial response can also involve a broader collapse of bacterial energy metabolism. Damage to the cytoplasmic membrane may disturb the proton motive force and interfere with membrane-associated electron transport, reducing the cell's capacity to generate ATP. This loss of energy has consequences throughout the cell. Active transport becomes less efficient, membrane homeostasis is more difficult to maintain, and energy-dependent processes such as protein synthesis and DNA repair are compromised. Metabolic disruption may therefore reinforce the damage initiated by membrane injury, oxidative stress, or enzyme inhibition (6,13,17,19,20).

Biofilm Disruption

The activity of MBNPs against bacterial biofilms is especially relevant because bacteria growing within a biofilm generally show greater tolerance to antimicrobial treatment than planktonic cells. Bacterial biofilms are multicellular communities surrounded by an extracellular polymeric substance matrix (EPS) composed of polysaccharides, proteins, extracellular DNA, and other components. The matrix alone does not explain their increased tolerance; limited antimicrobial penetration, altered metabolic activity, physiological heterogeneity, and other biofilm-associated adaptations may all contribute (6–8,19,21).

Metal-based nanoparticles can affect biofilms at different stages of their development. Depending on their surface properties and size, they may interact with components of the EPS matrix and penetrate the biofilm to varying degrees (6,8,19). Once in contact with embedded bacteria, the same antibacterial processes described above, including membrane injury, metal-ion release, ROS generation, and metabolic disruption, may remain active. Some nanoparticles can also interfere with bacterial attachment or the early development of the biofilm. Thus, their antibiofilm effect may involve both inhibition of biofilm formation and damage to established biofilm communities (8,19).

Although the antibacterial mechanisms described above are shared by several MNPs, their relative importance differs considerably among nanomaterials. Table 1 summarizes the principal mechanisms associated with the major MNP classes discussed in the following sections, together with char-

Table 1. Overview of the principal antibacterial mechanisms and distinguishing characteristics of selected metal and metal oxide nanoparticles.

Type of Nanoparticles (NPs)	Main Antibacterial Mechanisms	Distinctive Feature(s)	Important Limitation(s)/ Consideration(s)	Reference
AgNPs	Membrane disruption, Ag^+ release, ROS generation, protein and DNA damage	Broad antibacterial activity; readily functionalized	Activity strongly depends on particle properties and Ag^+ release. Generally distinguished by higher toxicity (particularly neurotoxicity) due to intrinsic oxidative decomposition.	(19,20,22)
AuNPs	Surface-mediated interactions; effects on membrane and metabolism depending on functionalization	Highly tunable surface; useful as a carrier and multifunctional platform	Intrinsic antibacterial activity is variable and often strongly dependent on surface modification; inert and less toxic than AgNPs.	(23)
Cu/CuO NPs	Cu^{2+} release, membrane damage, ROS generation, metabolic disruption	Relatively low cost and broad antibacterial activity	Dissolution and activity are strongly affected by the surrounding medium.	(24)
ZnO NPs	ROS generation, Zn^{2+} release, membrane damage	Antibacterial and photocatalytic properties	Activity depends on particle size, dissolution, aggregation, and exposure conditions.	(25)
TiO_2 NPs	Primarily photocatalytic ROS generation and subsequent oxidative damage	Light-activated antibacterial activity; suitable for antimicrobial surfaces	Conventional TiO_2 generally requires suitable irradiation for strong photocatalytic activity.	(26)
Fe_3O_4/γ-Fe_2O_3 NPs	Surface interactions, ROS-related effects, iron-mediated processes	Magnetic manipulation, targeting, recovery, and use in multifunctional systems	Intrinsic antibacterial potency is variable and strongly influenced by surface modification.	(27)

acteristics that distinguish their potential antibacterial applications.

4.3. Silver Nanoparticles

Silver nanoparticles (AgNPs) are among the most widely investigated MBNPs for antibacterial applications. Their interest comes largely from their broad antibacterial spectrum and the fact that their activity is not restricted to a single cellular target. Susceptible Gram-negative bacteria include *Escherichia coli*, *Pseudomonas aeruginosa, Klebsiella pneumoniae*, and members of the *Salmonella* and *Enterobacter* genera, although the degree of susceptibility varies considerably between strains and nanoparticle preparations (19,28).

The interaction of AgNPs with Gram-negative bacteria is strongly influenced by the organization of their cell envelope. Their relatively thin peptidoglycan layer lies beneath an outer membrane containing lipopolysaccharides and porins, both of which can influence nanoparticle interaction with the bacterial surface (29,30). Following contact, AgNPs may accumulate at the envelope, disturb membrane organization, and increase permeability. This initial damage can be followed by leakage of cellular contents and loss of normal membrane function. However, greater activity against Gram-negative bacteria should not be regarded as a general rule, since susceptibility also depends on particle size, surface charge, coat-

ing, concentration, and the bacterial strain being examined (29–31).

Silver nanoparticles have also been proven to be active against Gram-positive bacteria, including *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), *Bacillus subtilis*, and *Enterococcus faecalis* (19,28,29). The thicker peptidoglycan layer characteristic of Gram-positive bacteria can restrict nanoparticle penetration, but it does not prevent antibacterial activity. This is particularly relevant for resistant organisms such as MRSA, for which the antibacterial and antibiofilm effects of AgNPs have been repeatedly investigated (19,29,31,32).

As with other MBNPs, the effect of silver is the result of several processes occurring together. Silver nanoparticles can directly damage the bacterial envelope, while Ag^+ released from their surface provides an additional route of toxicity. Once available, silver ions can interact with proteins and enzymes, interfering with normal cellular metabolism. Reactive oxygen species generation adds another level of damage, affecting membranes, proteins, and nucleic acids. Interference with DNA replication and protein synthesis has also been described. The relative importance of these mechanisms is not fixed and changes with the physicochemical characteristics of the AgNPs and the conditions under which they are used (19,20,29,33).

4.4. Gold Nanoparticles

Gold nanoparticles (AuNPs) differ from many other MBNPs because elemental gold is relatively chemically inert and does not rely on extensive metal-ion release for its antibacterial effect. Their biological activity is therefore particularly sensitive to particle characteristics, including size, shape, surface charge, coating, and functionalization. This partly explains why the antibacterial performance reported for AuNPs varies considerably between studies (23,29,34).

Antibacterial effects have been described against both Gram-negative and Gram-positive species, including *E. coli*, *P. aeruginosa*, *S. aureus*, and *Bacillus subtilis* (23,29,34). AuNPs have also been studied against oral pathogens, including *Porphyromonas gingivalis* (35). Depending on their surface properties, AuNPs can interact with the bacterial envelope and disturb membrane-associated processes. Effects on cellular metabolism, ATP production, and intracellular components have also been reported, although the mechanisms are less uniform than those commonly described for AgNPs. Importantly, antibacterial activity observed with functionalized AuNPs may arise partly (or predominantly) from the molecules attached to their surface rather than from the gold core itself (23,29,34).

This ability to modify the particle surface is one of the main reasons for the continuing interest in AuNPs. Antimicrobial peptides, antibiotics, antibodies, aptamers, and other bioactive or targeting molecules can be conjugated to the gold surface. In such systems, the nanoparticle acts not only as an antimicrobial material but also as a carrier or scaffold that can bring the active compound into closer contact with bacterial cells (23,34,36). Surface functionalization can also be used to improve bacterial recognition and, in some cases, increase selectivity toward particular microorganisms. Gold nanoparticles are therefore particularly attractive as multifunctional antibacterial platforms rather than simply as direct bactericidal agents (23,29,36).

4.5. Copper and Copper Oxide Nanoparticles

Copper has long been recognized for its antimicrobial properties, and its use in nanoparticle form has attracted increasing attention. An additional practical advantage is its relatively low cost compared with noble metals such as silver and gold, which may be important for large-scale antimicrobial applications. Both elemental copper nanoparticles (CuNPs) and copper oxide nanoparticles (CuO NPs) have been investigated, although their antibacterial behavior should not be assumed to be identical (37,38).

Both CuNPs and CuO NPs have shown activity against a broad range of bacteria, including both Gram-positive and Gram-negative species. Among the commonly studied organisms are *E. coli*, *P. aeruginosa*, *S. aureus*, and *Bacillus cereus*. Differences in susceptibility between Gram-positive and Gram-negative bacteria have been reported, but the results are not consistent enough to suggest that one group is generally more sensitive. Particle size, morphology, concentration, surface characteristics, and the conditions of the antibacterial assay can all influence the observed response (24,37,38).

The antibacterial action of copper-based nanoparticles involves several overlapping processes. Release of Cu^{2+} ions is particularly important. These ions can interact with cellular proteins and enzymes, disrupting essential metabolic reactions. Contact between nanoparticles and the bacterial surface may also compromise membrane integrity, leading to altered permeability and leakage of intracellular material. Oxidative stress provides another route of damage, as copper can participate in redox reactions that promote ROS formation. The resulting oxidative injury may affect membrane lipids, proteins, and DNA (24,37–39).

The balance between these mechanisms differs between CuNPs and CuO NPs and is also influenced by the surrounding environment. Dissolution and Cu^{2+} release, for example, depend on factors such as pH and medium composition (24,37–39). For this reason, antibacterial activity reported for one copper-based nanoparticle preparation cannot necessarily be extrapolated to another. Nevertheless, their broad antibacterial activity, combined with the comparatively low cost and availability of copper, makes CuNPs and CuO NPs attractive candidates for antimicrobial coatings, wound-related materials, and other biomedical applications (38,39).

4.6. Zinc Oxide Nanoparticles

Zinc oxide nanoparticles (ZnO NPs) have received considerable attention as antibacterial materials. Their relatively low cost and broad activity are important advantages, while the physicochemical and photocatalytic properties of ZnO offer several routes by which bacterial cells can be affected (25,40). Antibacterial effects have been reported against both Gram-positive and Gram-negative bacteria, including *E. coli*, *P. aeruginosa*, *K. pneumoniae, S. aureus*, MRSA, *Bacillus subtilis, E. faecalis, Proteus vulgaris*, and *Vibrio cholerae*. However, susceptibility varies between bacterial strains and ZnO NP preparations (25,40,41).

Several mechanisms appear to contribute to the ZnO NPs's antibacterial activity. Reactive oxygen species generation is among the most frequently described mechanisms and can cause oxidative damage to membrane lipids, proteins, and nucleic acids (25,40,41). Dissolution of ZnO and the resulting release of Zn^{2+} ions provide further contribution to antibacterial activity, particularly when sufficient concentrations of these ions become available in the surrounding medium. Direct contact between ZnO NPs and the bacterial surface can further disturb membrane organization and permeability. These processes are closely related rather than independent, and their relative importance varies with the particle properties and the conditions under which they are tested (25,40–42).

Particle size is particularly relevant to the antibacterial performance of most MBNPs, including ZnO NPs. A reduction in size increases the surface-area-to-volume ratio, providing a larger reactive surface for interaction with the surrounding medium and bacterial cells. Smaller ZnO NPs have therefore often shown stronger antibacterial effects, although particle size alone does not determine activity. Shape, surface charge, aggregation, concentration, Zn^{2+} dissolution, and the composition and pH of the exposure medium can substantially alter the response (25,40,42,43).

The role of light also warrants consideration, given ZnO's photocatalytic nature. Under suitable irradiation, photoexcitation can enhance ROS formation and increase antibacterial activity (25,40,43). ZnO NPs can nevertheless exhibit antibacterial effects under dark conditions, indicating that photocatalysis is only one part of their overall mode of action. This distinction matters when ZnO NPs are considered for biomedical applications, where particles may not be exposed to the irradiation conditions commonly used in photocatalytic experiments (25,40,42).

4.7. Titanium Dioxide Nanoparticles

Titanium dioxide nanoparticles (TiO_2 NPs) are best known for their photocatalytic activity, which also accounts for much of their antimicrobial potential. TiO_2 is a semiconductor, and when it absorbs light of sufficient energy, electron–hole pairs are generated at the particle surface. Their subsequent reactions with oxygen and water can generate ROS, including hydroxyl radicals and superoxide species. These highly reactive products can attack bacterial cells at several sites (26,44,45).

The bacterial envelope is usually among the first structures affected by TiO_2 NPs. Oxidative damage to membrane components can increase permeability and disturb normal membrane function, while con-

tinued exposure may lead to leakage of intracellular material. Oxidative stress can also damage proteins and nucleic acids. The antibacterial effect is therefore not limited to the surface of the bacterial cell, particularly when ROS production is sustained. Activity has been reported against a range of Gram-positive and Gram-negative bacteria (including *E. coli*, *P. aeruginosa*, *K. pneumoniae, S. aureus*, *Bacillus subtilis, Salmonella enterica*, and *Vibrio cholerae*), although its extent depends strongly on the properties of the TiO_2 NPs and the irradiation conditions (26,44,45).

This light dependence distinguishes TiO_2 from many other antibacterial nanomaterials. Conventional TiO_2 is activated mainly by ultraviolet radiation, which limits its usefulness in situations where UV exposure is unavailable or undesirable. Considerable attention has therefore been given to modifying TiO_2 through doping, surface modification, or combination with other materials to extend photocatalytic activity into the visible-light region. Such modifications can also influence charge separation and ROS generation, and consequently the antibacterial response (26,44–46).

Titanium dioxide nanoparticles are particularly attractive for applications in which the antimicrobial material remains at or near an illuminated surface. They have been incorporated into self-cleaning and antibacterial coatings and investigated for water treatment, environmental remediation, and surface disinfection. In these settings, photocatalytic activation offers the possibility of repeated antimicrobial action without relying solely on the release of a conventional antibacterial compound. At the same time, antibacterial results obtained under controlled irradiation should be interpreted with caution when considering applications in which light intensity, wavelength, or exposure time may differ substantially (26,44–46).

4.8. Iron and Iron Oxide Nanoparticles

Iron-based nanoparticles, particularly magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3), occupy a somewhat different position among antibacterial nanomaterials. Their antimicrobial effects are of interest, but much of their appeal comes from their magnetic behavior. This allows particles to be manipulated, concentrated, or separated using an external magnetic field, opening possibilities not readily available with non-magnetic nanoparticles (27,47,48).

Antibacterial activity has been reported for iron oxide nanoparticles against both Gram-positive and Gram-negative bacteria (including *E. coli*, *P. aeruginosa*, *K. pneumoniae, S. aureus*, *Staphylococcus epidermidis,* and *Staphylococcus mutans*) (49). Several mechanisms may be involved. Interaction with the bacterial surface can alter membrane integrity, while iron-dependent ROS formation may contribute to oxidative damage of lipids, proteins, and nucleic acids. Released iron ions can also take part in redox reactions and influence cellular processes. However, the magnitude of these effects depends heavily on the specific iron oxide formulation, and unmodified particles do not necessarily show strong antibacterial activity under all conditions (27,48,49).

Their magnetic properties become particularly useful when combined with other antibacterial strategies. Iron oxide nanoparticles can serve as carriers for antimicrobial compounds or be incorporated into composite systems containing other active materials. An external magnetic field may then be used to direct or concentrate the particles at a desired site (27,47,48). Magnetic separation offers another practical option: after interacting with microorganisms, appropriately designed particles can be recovered from a liquid medium, an approach that has attracted interest for water purification and environmental applications (27,47,49).

Surface modification is especially important for these nanoparticles. Coatings can improve colloidal stability, provide sites for attaching drugs or targeting molecules, and alter particle interactions with bacterial cells. At the same time, coating the iron oxide surface may change its intrinsic antibacterial behavior, making it difficult to discuss all magnetic iron oxide nanoparticles as a single antimicrobial material. Particle size, oxidation state, surface chemistry, aggregation, and the surrounding medium all influence the final response (27,47–49).

For this reason, the main advantage of Fe_3O_4 and γ-Fe_2O_3 nanoparticles may lie less in exceptionally high intrinsic antibacterial potency and more in the combination of antimicrobial activity with magnetic control. Their potential for magnetic targeting, recovery, and incorporation into multifunction-

al systems distinguishes them from many of the other MBNPs discussed above (27,47–49).

5. DISCUSSION

The literature considered in this review shows that metal-based nanoparticles comprise a diverse group of antibacterial materials rather than a single class with a common pattern of activity. Silver, copper-based, zinc oxide, gold, titanium dioxide, and iron oxide nanoparticles have all been associated with antibacterial effects, but the basis and extent of these effects differ among materials. This distinction is important when comparing their potential use (19,23–27). Strong antibacterial activity is a characteristic frequently associated with AgNPs and copper-based nanoparticles, whereas other materials offer properties that may be valuable for more specific applications (19,24,33,37–39). Gold nanoparticles, for example, are particularly suitable for surface functionalization and delivery of antimicrobial compounds, while the photocatalytic properties of TiO_2 and the magnetic behavior of iron oxide nanoparticles provide possibilities that extend beyond direct bacterial killing (23,26,27,34,36,44,47).

Despite these differences, a common feature of MBNP antibacterial activity is the involvement of several cellular targets (19,24–27,33). Damage to the bacterial envelope can occur together with metal-ion release, oxidative stress, interference with proteins and enzymes, disruption of energy metabolism, and damage to nucleic acids. These events should not be regarded as completely independent mechanisms (19,24,25,33,39,40). Membrane injury, for instance, may disturb cellular homeostasis and energy production, while ROS generated at or near the bacterial surface can subsequently affect proteins, lipids, and DNA (19,24–26,33,44). Similarly, released metal ions may interfere with several intracellular processes simultaneously. The antibacterial response is therefore better understood as the cumulative consequence of interconnected cellular disturbances rather than the result of a single molecular target (19,24–27,33,39).

These mechanisms contribute differently across MBNPs (19,23–27). Ion release is particularly relevant to materials such as Ag- and Cu-based nanoparticles, whereas ROS generation has a prominent role in the antibacterial action of several metal oxides (19,24,25,33,37–40). In the case of TiO_2, this mechanism is closely connected to photocatalytic activation and, therefore, to the irradiation conditions (26,44–46). Gold nanoparticles present a somewhat different situation because elemental gold is relatively inert, and the antibacterial properties of these systems may depend strongly on the molecules present at their surface (23,29,34,36). Iron oxide nanoparticles are also distinct in that their magnetic properties create opportunities for targeting, recovery, and incorporation into multifunctional antimicrobial systems (27,47–49). Consequently, the value of an MBNP should not be judged solely by the magnitude of its direct antibacterial effect (19,23–27). Its suitability also depends on what additional function the material is expected to provide (23,26,27,36,44,47).

Differences between Gram-positive and Gram-negative bacteria add another level of complexity (19,24,25,29). Their cell envelopes differ substantially in structure and composition, and this can influence the initial interaction of nanoparticles with the bacterial surface (19,29,31,37). Nevertheless, the studies considered here do not justify a general assumption that either Gram-positive or Gram-negative bacteria are consistently more susceptible to MBNPs. Activity has been demonstrated against organisms from both groups, and susceptibility ultimately depends on the particular nanoparticle–bacterium combination (19,24–27,29,40). This is especially relevant when discussing resistant pathogens such as MRSA or clinically important Gram-negative species including *P. aeruginosa* and *K. pneumoniae* (28,32,33,39–41).

Biofilm-associated bacteria present an additional challenge (19,21,32). Their behavior differs from that of planktonic cells because the extracellular matrix, altered metabolic states, and physiological heterogeneity within the biofilm contribute to antimicrobial tolerance (21,32,33). The MBNPs' ability to act through multiple antibacterial pathways is therefore particularly interesting in this setting. Nanoparticles may interact with the extracellular matrix and, depending on the system, reach bacteria located within the biofilm, where membrane injury, oxidative stress, and metal-ion-mediated effects can still occur (19,21,32,33,39). Some MBNPs may also interfere with bacterial attachment and early biofilm development (19,21,32,41). However, activity against planktonic bacteria should not be taken as evidence of equivalent efficacy against mature biofilms, and

the two situations need to be evaluated separately (21,32,33).

The broad antibacterial potential of MBNPs must also be considered together with their biological safety. The same properties responsible for damage to bacterial membranes, proteins, or nucleic acids may also affect mammalian cells at sufficiently high exposure levels. This is particularly important for biomedical applications involving direct or prolonged tissue contact. An antibacterial effect is therefore not sufficient on its own to establish that a nanoparticle system is suitable for therapeutic use. The effective antibacterial exposure must be considered alongside cytocompatibility and the intended route and duration of application. Environmental applications raise related concerns because released nanoparticles or metal ions may affect non-target organisms after their intended antimicrobial function has been completed (22,38–40,47,49).

Another important consideration is how MBNPs might ultimately be used. Their most realistic role may not be as free-standing substitutes for conventional antibiotics (19,23,33,38–40). Their versatility allows them to be incorporated into antimicrobial coatings, wound-related materials, delivery systems, and composite formulations or to serve as carriers for existing antimicrobial compounds (23,36,39,44,47,48). Functionalization may further introduce targeting or additional therapeutic functions, while magnetic and photocatalytic materials offer possibilities that conventional antimicrobial molecules do not provide. Combination approaches are particularly attractive because they leverage the properties of the nanomaterial without requiring the nanoparticle itself to confer the entire antibacterial effect (19,23,36,38,39,44–47).

Taken together, the reviewed evidence supports continued investigation of MBNPs as antibacterial materials, but their diversity makes broad generalizations difficult. The different nanoparticle classes should be considered based on the antibacterial mechanism and functional properties most relevant to the intended application, rather than treated as interchangeable alternatives. Future studies should place particular emphasis on clinically relevant bacterial strains, established biofilms, appropriate safety assessment, and models that better reflect the conditions of intended use. The central question is therefore moving beyond whether MBNPs can inhibit bacterial growth toward determining where their particular combination of antibacterial and material properties offers a meaningful advantage over existing antimicrobial strategies (19,21–27,33,38,40,44,47,49).

6. CONCLUSION

Metal-based nanoparticles represent a diverse group of materials with considerable potential for antibacterial applications. Although the nanoparticle classes discussed in this review differ in their properties and modes of action, their antibacterial effects commonly involve several interconnected processes, including disruption of the bacterial envelope, oxidative stress, metal-ion-mediated effects, and interference with essential cellular functions. The contribution of these mechanisms varies among materials, making it difficult to regard MBNPs as a single antibacterial platform.

Their potential also extends beyond direct bacterial killing. Ag-, Cu-, and ZnO-based nanoparticles have attracted particular interest for their antibacterial activity, whereas AuNPs, TiO_2, and iron oxide nanoparticles offer additional possibilities related to functionalization, photocatalysis, or magnetic manipulation. These characteristics support their use in antimicrobial coatings, delivery systems, wound-related materials, and combination approaches. Activity against biofilm-associated bacteria is another important area, although effects demonstrated against planktonic cells cannot be assumed to translate directly to established biofilms.

Further development of MBNP-based antibacterial systems will require careful consideration of safety alongside antimicrobial efficacy, particularly for applications involving direct contact with biological tissues. Rather than simply replacing conventional antibiotics, MBNPs may be most useful when their antibacterial effects are combined with their distinctive material properties or with existing antimicrobial treatments. Future research should therefore focus on identifying applications in which these features provide a clear advantage and on demonstrating their effectiveness and safety under conditions that reflect their intended use.

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ORIGINAL ARTICLES

EFFECT OF THE POLOXAMER 407–CARBOMER 940 RATIO ON THE THERMORESPONSIVE GELATION BEHAVIOR OF HYDROGELS

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ABSTRACT

INTRODUCTION: Poloxamer 407 is a thermoresponsive polymer capable of undergoing a temperature-induced sol–gel transition. Carbomer 940, a cross-linked polyacrylate, can modify the rheological and physicochemical properties of polymeric systems. Their combination may provide a means of tuning the thermal gelation behavior of in situ gelling formulations.

AIM: This study investigated the effect of varying the poloxamer 407-to-carbomer 940 ratio on the gelation characteristics of thermoresponsive hydrogels, with particular emphasis on gelation temperature onset (GTO), gelation temperature (GT), and gelation time (t_G).

MATERIALS AND METHODS: Hydrogels containing 5–30% (w/w) carbomer 940 dispersion, designated G05–G30, were prepared by progressively replacing poloxamer 407 solution. Formulations were characterized for visual appearance and pH. Gelation temperature onset was determined from viscosity–temperature profiles by rotational viscometry, GT was determined by the tilting method, and t_G was evaluated at 37 ± 0.5°C. Statistical comparisons were performed using analysis of variance with Tukey's post hoc test.

RESULTS AND CONCLUSION: Increasing carbomer dispersion content decreased pH from 7.07±0.04 to 5.08 ± 0.05 and progressively increased GTO from 25.31±0.88°C (G05) to 34.65±2.54°C (G25). The GT also increased from 25.43±0.06 to 34.60±0.36°C. G30 did not exhibit gelation up to 40°C. Gelation time increased markedly from 3.60±0.39 s for G05 to 142.24±5.67 s for G25. Increasing carbomer 940 incorporation, accompanied by decreasing poloxamer 407 content, substantially altered the thermoresponsive behavior of the hydrogels, resulting in higher gelation temperatures and a prolonged time required to attain the predetermined gelation state at 37°C. The polymer ratio therefore provides a simple approach for tuning the thermal gelation characteristics of poloxamer-based hydrogels.

Keywords: *carbomer 940, gelation temperature, gelation time, poloxamer 407, thermoresponsive hydrogels*

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INTRODUCTION

In situ gelling systems have attracted considerable interest in pharmaceutical formulation because they combine the ease of administration of a low-viscosity formulation with the favorable characteristics of gels. Such systems can improve formulation residence at the application site, facilitate localized drug delivery, and provide an opportunity to modulate drug release while avoiding the handling difficulties

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associated with preformed semi-solid dosage forms. Among the different mechanisms used to induce in situ gelation, temperature-responsive systems are particularly attractive because the sol–gel transition can be triggered by a change in temperature without requiring an external chemical or enzymatic stimulus (1–3).

Poloxamer 407 is one of the most exploited thermoresponsive polymers in the development of such systems. It is a non-ionic triblock copolymer composed of poly(ethylene oxide) and poly(propylene oxide) blocks. In an aqueous solution, poloxamer molecules undergo micellization and progressively pack into micelles as the temperature increases. This process results in a marked increase in viscosity and, at sufficiently high polymer concentrations, the formation of a gel network. However, the gelation temperature and the mechanical properties of the resulting gel depend strongly on the polymer concentration and the presence of other formulation components (4–7).

Carbomers are crosslinked polyacrylic acid polymers widely used in pharmaceutical formulations as viscosity-modifying, suspending, and gel-forming agents. Their ability to increase viscosity at relatively low concentrations enables them to alter the rheological and physicochemical properties of polymeric formulations. They also exhibit mucoadhesive properties owing to the presence of carboxylic acid groups. Their incorporation into poloxamer-based systems may therefore provide a means to modify the temperature-dependent behavior of the non-ionic polymer while potentially enhancing mucoadhesive interactions and prolonging the formulation's residence time at mucosal surfaces (7,8).

For a thermoresponsive pharmaceutical vehicle, the gelation temperature (GT) is an important formulation parameter because it determines whether the formulation remains sufficiently fluid during handling and administration while undergoing a predictable increase in viscosity and gelation after exposure to physiological temperature. Therefore, characterization of thermoresponsive systems should consider both the temperature-dependent change in viscosity and the formation of a sufficiently structured gel (8–11).

Although poloxamer 407 and carbomer are individually well-established pharmaceutical excipients, the effect of their relative proportions on the thermal behavior of combined hydrogel systems warrants further investigation (12). In particular, systematic assessment of the GT onset (GTO), the macroscopic GT, and the gelation time (t_G) may help clarify how modifying the polymer ratio tunes the formulation's thermal response. Such information is relevant to the development of thermoresponsive hydrogel platforms that may subsequently be adapted to different pharmaceutical applications depending on the desired administration conditions and performance requirements.

AIM

The present study investigated the effect of varying the relative proportions of poloxamer 407 and carbomer 940 on the physicochemical and thermoresponsive properties of the resulting hydrogels. Particular attention was given to the relationship between formulation composition and GTO, GT, and t_G. The study further aimed to assess whether variation in the polymer ratio can serve as a simple formulation approach to tune the temperature-dependent sol–gel transition of the hydrogel system, thereby providing a versatile platform for pharmaceutical applications.

MATERIAL AND METHODS

MATERIALS

Carbomer 940 was sourced from Thermo Fisher Scientific (Waltham, MA, USA), and poloxamer 407 (Kolliphor® P407) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

METHODS

Gel Preparation

The poloxamer solution was prepared by the cold method. The polymer (20%, w/w) was dispersed in distilled water and allowed to hydrate for 24 h at 4±1°C until fully dissolved (13). The carbomer dispersion was obtained by hydrating the polyacrylate (1%, w/w) in distilled water under stirring at 500 rpm at 40±1°C for an hour (7).

Blank formulations were prepared by dispersing the carbomer in the poloxamer solution under stirring at 700 rpm for 5 min at 4±1°C. Thus, six

samples with poloxamer solution-to-carbomer dispersion ratios ranging from 95:5 to 70:30 in 5% increments were obtained (Table 1).

Table 1. Composition of formulations G05–G30.

Formulation	G05	G10	G15	G20	G25	G30
Poloxamer solution, w/w%	95	90	85	80	75	70
Carbomer dispersion, w/w%	5	10	15	20	25	30

Gel Characterization

Visual Appearance

The color, odor, uniformity, and consistency of the formulations were evaluated visually.

pH

The pH value of each gel was determined using a portable pH meter (70 Vio, XS Instruments, Carpi, Italy). The measurements, performed in triplicate, were conducted after the formulations were conditioned to 4±1°C (14).

Gelation Temperature Onset, Gelation Temperature, and Gelation Time

The temperature dependence of gel viscosity was investigated using an IKA® Rotavisc lo-vi rotational viscometer (IKA®-Werke GmbH & Co. KG, Staufen, Germany). Measurements were performed up to a viscosity of 30,000 mPa·s using spindle No. 4 at a constant rotational speed of 20 rpm. Sample temperature was controlled using a thermostatic water bath and monitored up to 40°C. The GTO was determined as the x-coordinate of the intersection of two extrapolated linear regression lines fitted to the low- and high-viscosity regions of the viscosity–temperature curve. The regression regions were selected to achieve coefficients of determination (R^2)≥0.95 for both fitted lines, and the intersection of the resulting regression lines was taken as the GTO (Fig. 1) (15,16).

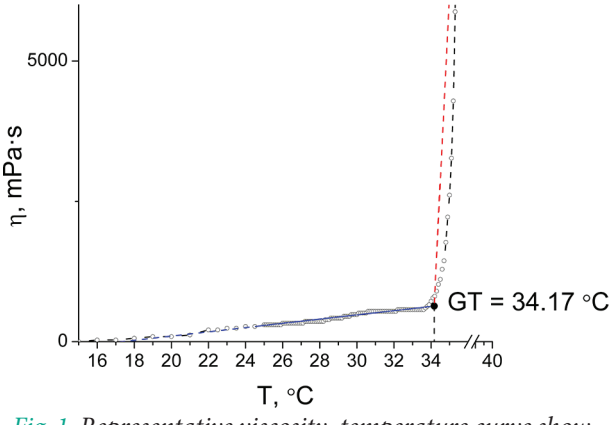


Fig. 1. Representative viscosity–temperature curve showing the determination of gelation temperature onset (GTO) by the intersection of linear regressions fitted to the low- and high-viscosity regions.

The GT was determined by the tilting method. Briefly, 3 mL of each hydrogel was placed in a vial, conditioned at 25°C, and heated at 0.1°C per minute in a thermostatic water bath. The vial was tilted and visually inspected for any flow before each temperature increment. Gelation temperature was defined as the lowest temperature at which no flow was observed for 30 s (17).

Gelation time was assessed separately at physiological temperature. After conditioning at 25°C, the 3 mL sample was transferred to a water bath maintained at 37±0.5°C. The time elapsed from transfer to the water bath until the formulation reached its predetermined GT was recorded as the t_G (18).

Statistical Processing

All measurements were performed in triplicate, and the resulting data were processed to report the mean ± standard deviation (SD). Analysis of variance was performed to compare the means, and Tukey's post hoc test was applied for multiple comparisons where significant differences ($p<0.05$) were detected. Differences between GTO and GT for each formulation were evaluated using a paired-samples *t*-test ($p<0.05$). Data processing was conducted with SPSS ver. 26 (IBM Corp., Armonk, NY, USA).

RESULTS

Visual Appearance

All developed vehicles were colorless and odorless. Formulation G05 was relatively transparent, whereas the other hydrogels showed varying degrees

of turbidity that increased with the content of carbomer dispersion (Fig. 2).

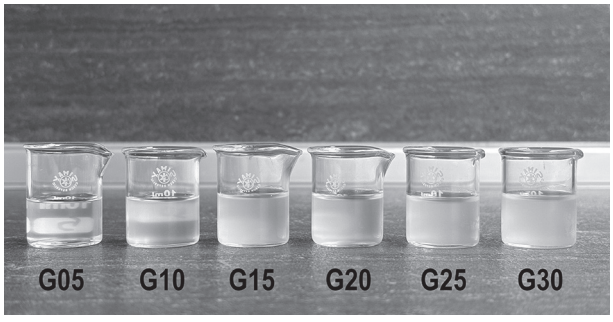


Fig. 2. Visual appearance of formulations G05–G30.

pH

The pH values ranged from 5.08 ± 0.05 to 7.07 ± 0.04, decreasing progressively as the carbomer dispersion content increased (Table 2). This trend is consistent with the acidic nature of polyacrylate and its increasing quantity in the formulations (19). Overall, the formulations remained within a moderately acidic to near-neutral pH range.

Gelation Temperature Onset, Gelation Temperature, and Gelation Time

Gelation behavior of the developed formulations demonstrates clear dependence on their composition (Table 2, Fig. 3). The GTO showed an overall increase from 25.31±0.88 for G05 to 34.65±2.54°C for G25. At the highest carbomer dispersion level investigated (G30), no gelation was detected up to 40 °C, indicating that the composition of G30 shifted the thermally induced transition beyond the investigated temperature range. Tukey's test indicated significantly higher GTO values for G20 and G25 compared with G05, whereas the intermediate formulations showed overlapping statistical groups.

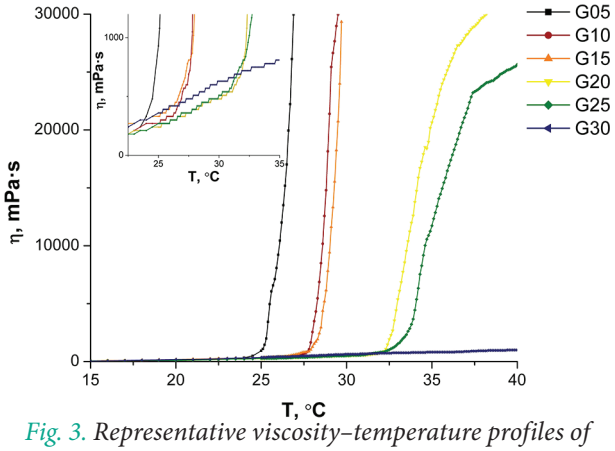


Fig. 3. Representative viscosity–temperature profiles of formulations G05–G30. The inset shows an enlarged view of the low-viscosity region to facilitate comparison of the gelation onset. Gelation temperature onsets (GTOs) were determined individually for each formulation as described in the Methods and are reported separately as mean ±SD (n=3) in Table 2.

A similar overall increase was observed for GT, which increased from 25.43±0.06 to 34.60±0.36°C (Table 2). Thus, increasing the relative proportion of carbomer dispersion, with a concomitant decrease in poloxamer 407 solution, shifted gelation toward higher temperatures.

The difference between GTO and GT is generally small, ranging from 0.05 to 1.23 °C. No statistically significant difference was observed for G05, G15, G20, or G25 ($p>0.05$); however, G10 showed a statistically significant difference between the two measurements ($p=0.045$). Despite this isolated difference, the generally close numerical correspondence

Table 2. pH values, gelation temperature onsets (GTO), gelation temperature (GT), and gelation time (t_G) of formulations G05–G30. Values are given as mean ± standard deviation (SD; n = 3).

	pH	GTO, °C	GT, °C	t_G, s
G05	7.07 ± 0.04	25.31 ± 0.88^{c}	25.43 ± 0.06^{d}	3.60 ± 0.39^{d}
G10	6.65 ± 0.02	28.36 ± 0.45^{b,c}	27.13 ± 0.12^{c}	17.78 ± 1.46^{c}
G15	6.38 ± 0.06	28.95 ± 0.94^{b,c}	27.77 ± 0.06^{c}	22.92 ± 1.23^{c}
G20	5.74 ± 0.06	32.21 ± 1.75^{a,b}	33.37 ± 0.65^{b}	101.47 ± 5.30^{b}
G25	5.37 ± 0.05	34.65 ± 2.54^{a}	34.60 ± 0.36^{a}	142.24 ± 5.67^{a}
G30	5.08 ± 0.05	n.d.	n.d.	n.d.

Means in a column with a common superscript letter ($^{a–d}$) do not differ ($p < 0.05$) as analyzed by Tukey's test; n.d.—not detected.

between GTO and GT across the formulation series indicates that the viscosity-based and macroscopic methods identified a broadly similar temperature range for gelation despite differences in the sample volumes investigated in each test (500 mL for GTO vs. 3 mL for GT; Table 2).

The progressive shift in GTO and GT with the increasing carbomer 940 content and simultaneously decreasing poloxamer 407 content was accompanied by marked prolongation of t_G—from only 3.60±0.39 s for G05 to 142.24±5.67 s for G25 (Table 2). The increase in t_G was particularly pronounced across the formulation series, indicating that changes in the poloxamer 407-to-carbomer 940 ratio substantially affected the time required for the formulations to attain their predetermined gelation state at 37°C.

DISCUSSION

The thermoresponsive behavior observed in this study showed that changes in polymer composition significantly affected the gelation of poloxamer 407-based systems. Thermogelation of poloxamer 407 is associated with temperature-induced dehydration of the polypropylene oxide blocks, followed by micellization and packing of the micelles into a three-dimensional structure (4,20,21). In the tested formulations, increasing the amount of carbomer 940 while decreasing the concentration of poloxamer 407 shifted gelation toward higher temperatures. The thermal behavior of these formulations therefore appears to depend on the balance between the two polymers rather than on carbomer 940 content alone. Since their concentrations were changed simultaneously, the contribution of each polymer to the observed shift cannot be distinguished from the present results. Similar changes in the gelation behavior of poloxamer 407 have been reported following the addition of other polymers or formulation components, indicating that its thermoresponsive properties are strongly influenced by the overall formulation composition (4,12,22).

The increase in t_G followed the shift in GT, with formulations that gelled at higher temperatures also requiring more time to reach the gelled state at 37°C. Since the samples were initially conditioned at 25°C, the measured t_G includes both the time needed to reach the formulation-specific GT and the subsequent development of the gel structure. Thus, t_G is better regarded as a practical measure of the time required for gelation under the experimental conditions rather than as a direct measure of gelation kinetics. This is relevant from a formulation perspective, as thermoresponsive systems should remain sufficiently fluid during administration and then form a gel after exposure to physiological temperature. The temperature at which this transition should occur depends on the intended route of administration and application (8,11). In the studied series, changing the balance between carbomer 940 and poloxamer 407 progressively shifted gelation to higher temperatures and increased the time needed to reach the gelled state. At the highest carbomer-to-poloxamer proportion (G30), the transition was no longer observed within the investigated temperature range, indicating a limit to this compositional adjustment. Similar changes in thermoresponsive behavior following the incorporation of secondary polymers have been reported for poloxamer 407-based systems (4,12,23).

Overall, these findings demonstrate that the relative proportions of poloxamer 407 and carbomer 940 provide a practical formulation variable for controlling the temperature range over which thermoresponsive gelation occurs. The present study establishes the thermal characteristics of the formulation series, while the textural consequences of the same compositional changes remain to be considered. Since GT alone does not capture the mechanical performance of the formed gel, subsequent evaluation of parameters such as hardness, cohesiveness, adhesiveness, and spreadability can provide complementary information about how the polymer ratio affects the final functional properties of the system (7).

CONCLUSION

The study established that varying the poloxamer 407/carbomer 940 ratio provides a practical means of tailoring the thermoresponsive behavior of hydrogels. Changes in polymer composition progressively shifted the GT toward higher values and prolonged the time required for the formulations to attain their predetermined gelation state at 37°C. G20 and G25 exhibited GTs approaching physiological temperature, whereas G30 did not produce a detectable gel up to 40°C. These findings identify the relative proportions of poloxamer 407 and carbomer 940 as important formulation variables and provide a ba-

sis for selecting compositions to meet the requirements of specific in situ gelling applications.

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ORIGINAL ARTICLES

ESSENTIAL OILS FROM *PINUS SYLVESTRIS, PINUS NIGRA* AND *PINUS PINASTER*: COMPOSITION, EXTRACTION, AND THERAPEUTIC POTENTIAL

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ABSTRACT

INTRODUCTION: Essential oils obtained from the most widespread representatives of the genus *Pinus* in the Republic of Bulgaria—*P. sylvestris* L., *P. nigra* Arn., and *P. pinaster* Sol.—are characterized by a similar aromatic profile; however, their chemical composition and physiological effects may differ significantly.

Their composition comprises more than 50 identified volatile compounds, varying both qualitatively and quantitatively. The observed therapeutic effects are determined not only by the diversity of their terpene composition but also by the extraction method employed. Understanding these relationships enables a more precise and scientifically grounded application of pine essential oils.

AIM: The aim of the present study is to present a laboratory method for the isolation of essential oils from needles and young shoots of *P. sylvestris* L., *P. nigra Arn*., and *P. pinaster* Sol., and to determine their composition using GC–MS analysis.

MATERIALS AND METHODS: For the purposes of this study, a steam distillation setup was employed, designed to simulate a real steam distillation system, along with needles and thin branches of *P. sylvestris*, *P. nigra*, and *P. pinaster*, and GC–MS instrumentation.

RESULTS: The proposed extraction method yielded essential oils with reproducible chromatographic profiles. GC–MS analysis revealed both common and species-specific constituents across the three *Pinus* samples.

CONCLUSION: The successful integration of extraction methodologies with comprehensive GC–MS analysis provides a robust foundation for future research on the sustainable utilization of pine-derived essential oils.

Keywords: *pinus, essential oils, composition, therapeutic potential*

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INTRODUCTION

Essential oils obtained from the most widespread representatives of the genus *Pinus* in the Republic of Bulgaria—*P. sylvestris L., P. nigra* Arn., and *P. pinaster Sol.*—are characterized by a similar aromatic profile; however, their chemical composition and physiological effects may differ significantly (1).

Their composition comprises more than 50 identified volatile compounds, varying both qualitatively and quantitatively. The observed therapeutic effects are determined not only by the diversity of their terpene composition but also by the extraction method employed. Understanding these relationships enables a more precise and scientifically grounded application of pine essential oils (2).

Essential oils extracted from the needles of coniferous tree species represent an important group of natural products with wide applications in medicine, pharmacy, cosmetics, and aromatherapy. Of particular interest are the oils derived from various *Pinus* species, due to their rich chemical composition and well-documented biological activities.

Accumulated scientific evidence indicates that pine needle essential oils exhibit a wide range of biological activities, making them a valuable subject of research both from a fundamental scientific perspective and for the development of new therapeutic and cosmetic products.

Phytochemical Profile and Pharmacological Classification of Terpenoid Compounds

The therapeutic efficacy of botanical extracts is dictated by the synergistic interaction of diverse terpenoid classes (3). These compounds are categorized by their molecular complexity—ranging from high-volatility monoterpenes to more structurally complex diterpenes—each contributing a specific set of biological activities.

1. Monoterpene Hydrocarbons: Respiratory and Stimulant Effects

The monoterpene hydrocarbon fraction (4) featuring α-pinene, β-pinene, limonene, myrcene, Δ-3-carene (Fig. 1), constitutes the primary volatile component of many essential oils. These molecules are rapidly absorbed and exert systemic effects (5).

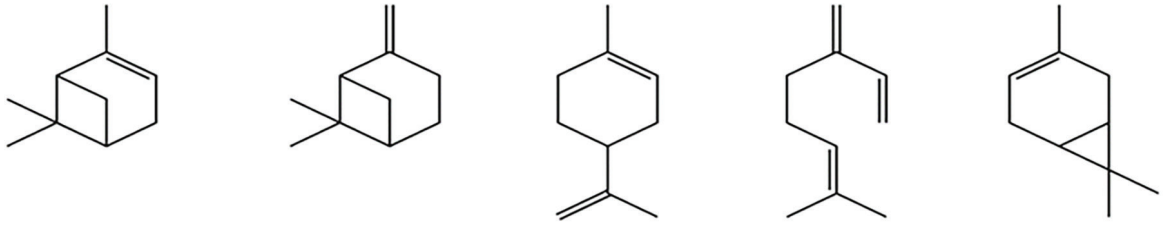


Fig. 1. 2D chemical structures of monoterpene hydrocarbons.

Essential oils from *Pinus sylvestris*, *Pinus nigra*, and *Pinus pinaster* are characterized by a high content of monoterpenes, among which α-pinene, β-pinene, and δ-3-carene are predominant, along with various oxygenated compounds such as borneol and terpineol. The relative proportions of these constituents vary considerably depending on species, geographical origin, environmental conditions, and extraction methods, leading to the formation of different chemotypes with distinct properties.

Respiratory modulation: These compounds act as potent bronchodilators and expectorants, facilitating the clearance of mucus and improving pulmonary airflow (6).

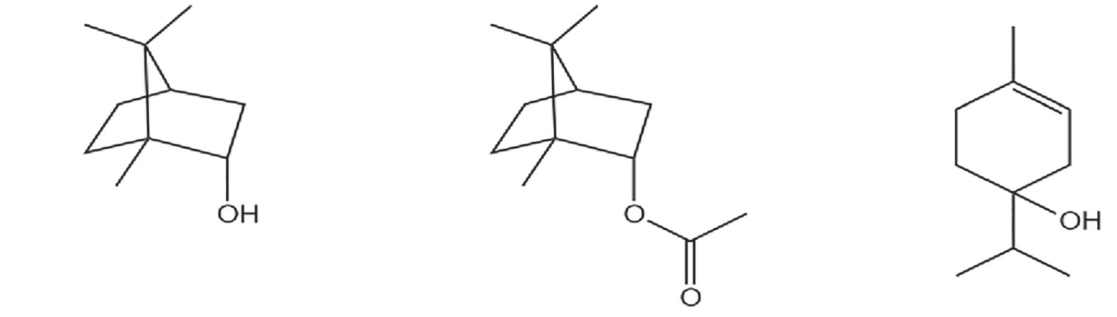
Antisepsis & inflammation: They exhibit intrinsic antiseptic properties and exert mild anti-inflammatory activity by inhibiting specific pro-inflammatory pathways (7).

Neurological impact: This class is associated with central nervous system (CNS) stimulation, enhancing cognitive alertness and metabolic focus (8).

2. Oxygenated Monoterpenes: Anti-Inflammatory and Regenerative Synergetics (9)

Oxygenated monoterpenes, including borneol, bornyl acetate, and terpinen-4-ol (Fig. 2), represent a more chemically stable group with enhanced bioavailability.

Analgesic & anti-inflammatory potency: These compounds offer strong anti-inflammatory activity, specifically targeting musculoskeletal inflammation.



BORNEOL BORNYL ACETATE TERPINEN-4-OL

Fig. 2. 2D chemical structures of oxygenated monoterpenes.

Synergistic modulation: These molecules serve to modulate the stimulatory effects of pinenes, preventing over-excitation of the nervous system while providing a grounding, anxiolytic effect.

Antimicrobial & dermal recovery: This fraction displays robust antimicrobial action and supports tissue regeneration, making it vital for cellular repair and immune defense.

3. Sesquiterpenes: Deep Tissue Analgesia and Chronic Pain Modulation

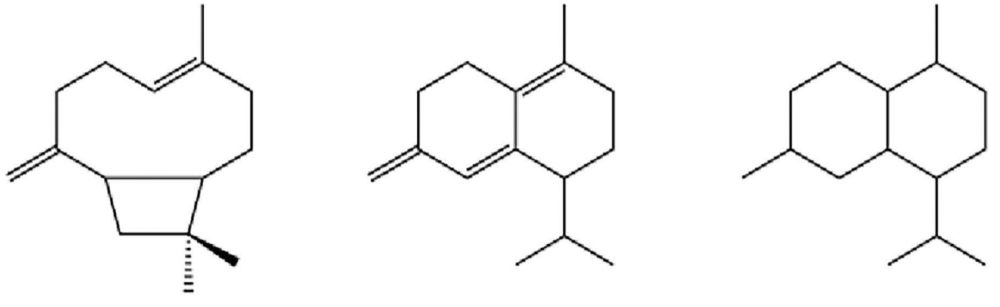
The sesquiterpene fraction, including β-caryophyllene, germacrene D and cadinenes (10) (Fig. 3), comprises higher-molecular-weight compounds characterized by a pronounced affinity for specific molecular targets, such as the CB2 receptor in the case of β-caryophyllene.

Homeostasis: They are particularly effective in managing chronic conditions, providing long-term nervous system stabilization and pain relief for joints and muscular tissues.

4. Diterpenes: Immunomodulation and Structural Support

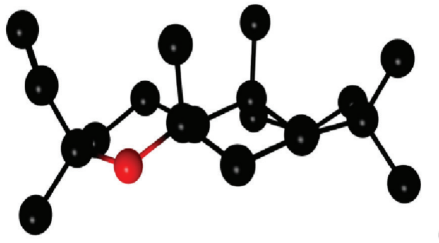
The diterpene fraction, exemplified by manool oxide (11) (Fig. 4), comprises higher-molecular-weight and less volatile terpenoids, often requiring specialized extraction techniques, such as supercritical CO_2 extraction (SFE), for efficient isolation.

Immunomodulatory activity: Diterpenes are recognized for their significant immunomodulatory



β-CARYOPHYLLENE GERMACRENE D CADINENES

Fig. 3. 2D chemical structures of sesquiterpenes.



MANOOL OXIDE

Fig. 4. 3D chemical structure of manool oxide.

properties, contributing to the regulation of the immune response.

Regenerative capacity: Through high-level regenerative pathways, they support structural cellular integrity and provide a foundational anti-inflammatory barrier against systemic stress.

A summarized relationship between the presence of specific chemical constituents, their primary therapeutic effects, and the symptoms they influence is presented in Table 1.

Relationship between Extraction Method, Chemical Composition, and Biological Activity

Essential oils from *Pinus sylvestris, Pinus pinaster,* and *Pinus nigra* exhibit different physiological effects determined not only by their specific terpene composition but also by the extraction method employed. (12).

Characteristics of Extraction Methods (13)

1. Steam Distillation

A widely used method for isolating volatile compounds, particularly essential oils, but associated with several limitations. High temperatures may cause thermal degradation (14) of thermolabile components, while the presence of water and steam can induce hydrolysis and chemical transformations. The simultaneous extraction of multiple compounds without precise control results in relatively low selectivity. Due to these limitations and its high energy consumption—especially during prolonged processes—this method is mainly suitable for volatile monoterpenes and water-insoluble compounds (15).

2. Vacuum Distillation (16)

A modification of conventional distillation in which reduced pressure allows operation at lower temperatures. Nevertheless, partial thermal stress remains unavoidable, and the selectivity is comparable to that of atmospheric distillation. Combined with

Table 1. Pharmacological profiles and therapeutic applications of select terpenoids.

Compound	Primary Biological Activity	Therapeutic Applications
α-Pinene	**Bronchodilation**	These compounds are employed in the treatment of respiratory pathologies (e.g., sinusitis and bronchitis), contributing to the reduction of airway resistance.
β-Pinene	**Antiseptic & Antimicrobial**	Exhibits broad-spectrum inhibitory effects against pathogens; utilized as an adjuvant in treating infectious etiologies.
Δ-3-Carene	**Desiccative Effect**	Induces mucosal drying; clinical utility in alleviating hypersecretion associated with rhinitis and rhinorrhea.
Limonene	**Immunomodulatory**	Enhances leukocyte response and cytokine regulation; indicated for fatigue syndromes and immune system optimization.
Bornyl acetate	**Sedative & Anxiolytic**	Modulates the central nervous system to induce relaxation; utilized in clinical protocols for stress reduction and sleep disorders.
β-Caryophyllene	**Anti-inflammatory & Analgesic**	Attenuates musculoskeletal inflammation via cannabinoid receptor interaction; effective in chronic pain management.
Germacrene D	**Homeostatic Stabilization**	Promotes physiological equilibrium; contributes to the long-term management of chronic metabolic and systemic imbalances.

higher technological complexity, these factors limit its broader application in essential oil extraction.

3. Supercritical CO_2 Extraction (17)

A modern and highly efficient method, although less effective for strongly polar compounds unless co-solvents (e.g., ethanol) are used. It is particularly suitable for extracting sesquiterpenes and diterpenes but requires sophisticated equipment and highly qualified personnel.

The influence of extraction methods on chemical composition and the resulting physiological effects is summarized in тhe following table provides a high-level scientific overview of how various extraction techniques influence the chemical composition, molecular integrity, and subsequent pharmacological efficacy of the resulting essential oils and extracts (18).

The dependence of essential oil composition on pine species is clearly expressed, as different species vary significantly in their dominant constituents and corresponding practical applications.

In the case of ***Pinus sylvestris*** *(Scots pine)*, the essential oil is primarily obtained by steam distillation. Its chemical composition is characterized by a high content of α-pinene (32–60%), β-pinene (5–22%), and limonene (7–12%). This combination of monoterpenes provides pronounced expectorant, antiseptic, and refreshing effects on the respiratory system. Consequently, Scots pine essential oil is widely used in the management of acute respiratory conditions, such as colds, bronchitis, and inflammation of the upper respiratory tract.

For ***Pinus pinaster*** *(Maritime pine)*, steam distillation is also the primary extraction method. The resulting oil contains an even higher proportion of α-pinene (71–85%) and β-pinene (11–20%), while limonene is present in lower amounts (1–7%). This composition accounts for its strongly stimulating and pronounced antiseptic properties. The oil is commonly used for air disinfection purposes and in formulations designed to provide invigorating and tonic effects.

In contrast, ***Pinus nigra*** *(Austrian pine)* essential oil is often obtained through vacuum distillation or carbon dioxide (CO_2) extraction. Its composition is dominated by sesquiterpenes such as β-caryophyllene and germacrene D, compounds known for their stronger anti-inflammatory and analgesic activities. As a result, Austrian pine oil is primarily applied in products intended for the relief of

Table 2. Comparative analysis of terpenoid extraction methodologies and phytochemical yields.

Extraction Methodology	Dominant Phytochemical Fractions	Degradation & Molecular Alterations	Pharmacological Profile & Bioavailability
Steam Distillation	Primarily low-molecular-weight **Monoterpenes (α-pinene, β-pinene, limonene, Δ-3-carene)**	Significant loss of **Sesquiterpenes**; susceptibility to **partial ester hydrolysis** due to steam exposure.	Rapid-onset **bronchodilatory** and **antiseptic** effects; optimized for acute respiratory relief.
Hydrodistillation	**Monoterpenes** with trace inclusions of heavier volatile fractions.	High risk of **thermal degradation** and aqueous hydrolysis of sensitive thermolabile compounds.	Attenuated therapeutic potency; **milder physiological response** compared to steam-based methods.
Vacuum Distillation (Reduced Pressure)	High retention of **Monoterpenes** and **Oxygenated Terpenoids** (esters/alcohols).	**Minimal oxidative stress** and thermal degradation due to significantly lower boiling points.	Synergistic **anti-inflammatory** and respiratory benefits; preserves the native „full-spectrum“ aromatic profile.
Supercritical CO_2 Extraction	Comprehensive profile: **Monoterpenes**, **Sesquiterpenes**, **Diterpenes**, and lipophilic **Resins**.	**Negligible**; non-thermal and non-aqueous process ensures maximum molecular structural integrity.	Potent **immunomodulatory**, **analgesic**, and systemic anti-inflammatory activity; high therapeutic density.

muscle and joint pain, as well as in conditions associated with inflammatory processes.

Thus, variations in chemical composition among pine species directly determine their specific therapeutic applications, highlighting the importance of botanical origin when selecting an essential oil for practical use (19).

The choice of extraction method depends on the physicochemical properties of the target compounds, their thermal stability, required selectivity, and economic constraints. The method acts as a selective factor determining which terpene compounds dominate the final product and, consequently, its physiological and therapeutic effects.

AIM

The aim of the present study is to present a laboratory method for the isolation of essential oils from needles and young shoots of *P. sylvestris L., P. nigra Arn.,* and *P. pinaster Sol.,* and to determine their composition using GC–MS analysis.

MATERIALS AND METHODS

For the purposes of this study, a steam distillation setup was employed, designed to simulate a real steam distillation system, along with needles and thin branches of *P. sylvestris*, *P. nigra*, and *P. pinaster*, and GC–MS instrumentation.

Steam distillation was carried out using a 10L steam generator connected to a 10L distillation flask, three condensers connected in series with silicone tubing for water circulation, and a distillate receiver (Fig. 5). The system produced 1 L of distillate in 41 minutes without plant material and in 37 minutes when steam was passed through the plant material.

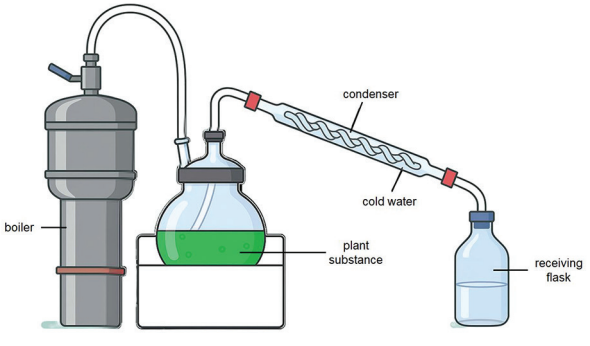


Fig. 5. Schematic representation of the steam distillation setup.

Needles and thin branches (350 g) of *Pinus pinaster*, *P. nigra*, and *P. sylvestris* were subjected to steam hydrodistillation. Each cycle lasted approximately 1 hour, yielding 2 L of floral water for each species. The floral waters were extracted twice with 20 mL of pentane. The organic phases were concentrated under a stream of argon (20 mL/min) and analyzed by GC–MS.

RESULTS AND DISCUSSION

The proposed extraction method yielded essential oils with reproducible chromatographic profiles. GC–MS analysis revealed both common and species-specific constituents across the three *Pinus* samples (Table 3).

The essential oil of *Pinus sylvestris* was characterized by the predominance of α-terpineol (18.72% of TIC), sclareol (15.26% of TIC), β-caryophyllene (7.15% of TIC), and verbenone (6.18% of TIC), indicating a composition rich in oxygenated monoterpenes. In contrast, *Pinus pinaster* exhibited elevated levels of (3E)-cembrene A (12.98% of TIC), neophytadiene (10.71% of TIC), and n-pentadecanal (8.27% of TIC), reflecting a profile dominated by diterpenoids and fatty acid derivatives. The essential oil of *Pinus nigra* contained primarily α-pinene (25.85% of TIC), δ-cadinene (12.83% of TIC), β-caryophyllene (10.85% of TIC), and γ-muurolene (7.30% of TIC), consistent with a resinous composition rich in sesquiterpenes.

Steam distillation enabled the isolation of the primary volatile fraction of the essential oils.

Subsequent liquid–liquid extraction with pentane facilitated the extraction of residual oil constituents remaining in the aqueous phase after distillation, likely due to partial solubility or incomplete phase separation. The use of pentane offers several advantages, including efficient recovery of nonpolar compounds, increased overall extraction yield, and facile removal prior to GC–MS analysis owing to its low boiling point.

However, this approach may also lead to the co-extraction of non-volatile or low-volatility lipophilic constituents, such as diterpenoids, waxes, and fatty acid derivatives, which are not typically considered part of the essential oil fraction. This may account for certain discrepancies observed between the experimental results and previously reported literature data.

Table 3. Comparative analysis of the chemical composition of essential oils obtained by steam distillation from P. sylvestris, P. nigra, and P. pinaster.

RT	RI	Name	% of TIC	RT	RI	Name	% of TIC	RT	RI	Name	% of TIC
17.48	1184	a-Terpineol	18.72	39.07	2210	(3E)-Cembrene A	12.98	9.24	933	α-Pinene	25.85
38.31	2244	Sclareol	15.26	42.26	2299	Neophytadiene	10.71	26.06	1522	β-Cadinene	12.83
23.58	1416	P-Caryophyllene	7.15	23.58	1416	n-Pentadecanal	8.27	23.58	1416	P-Caryophyllene	10.85
16.00	1145	cis-Verbenol	6.18	30.55	1708	n-Hexadecanol	5.94	25.15	1477	y-Muurolene	7.30
15.84	1134	trans-Sabinol	5.83	36.48	2085	2-keto-Manool oxide	5.45	25.93	1513	y-Cadinene	4.48
39.41	2213	Sandaracopimaral	4.93	38.39	2133	13-epi-Manool	5.11	24.98	1466	9-epi-(E)-Caryophyllene	4.07
17.82	1201	Verbenone	4.40	35.76	2020	Cembrene	4.77	15.84	1133	trans-Sabinol	3.96
36.64	2075	Dehydroabietan e	4.34	34.12	1938	Manool	4.46	17.48	1190	a-Terpineol	3.67
34.56	1950	Cembrene	4.23	35.57	1986	Limonene	4.16	29.26	1661	a-Cadinol	2.86
35.33	1971	Sandaracopimaradiene	3.86	33.95	1875	Manool oxide	2.69	16.00	1144	cis-Verbenol	2.81
25.13	1482	Germacrene D	3.29	39.60	2236	(2E.6E) - Farnesyl acetate	2.54	16.79	1171	a-Phellandren-8-ol	2.45
40.40	2238	Methyl pimarate	2.80	33.09	1815	Methyl pimarate	2.33	17.81	1202	Verbenone	2.17
12.39	1023	Eucalyptol	2.70	36.24	2063	Abieta-8 12-diene	2.21	25.56	1485	Germacrene D	1.95
16.81	1180	a-Phellandren-8-ol	2.42	33.58	1848	Methyl isopimarate	2.03	15.41	1104	Camphen-6-ol	1.67

Therefore, the chemical profiles obtained in the present study should be interpreted in the context of the combined extraction strategy involving both steam distillation and pentane extraction.

CONCLUSION

The results obtained from the applied distillation and extraction methods for essential oils from three *Pinus* species demonstrate their suitability for chemotaxonomic applications.

GC–MS analysis enabled the identification of distinct, species-specific terpene profiles, allowing for reliable differentiation among the investigated pine species.

Such findings are of considerable importance for biodiversity conservation and sustainable development, as they support the development of environmentally friendly alternatives to synthetic processes across various industrial applications.

The successful integration of extraction methodologies with comprehensive GC–MS analysis provides a robust foundation for future research on the sustainable utilization of pine-derived essential oils.

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ORIGINAL ARTICLES

ANTIOXIDANT CAPACITY OF *PELARGONIUM RADULA* AND *GERANIUM MACRORRHIZUM* EXTRACTS EVALUATED BY DPPH, TEAC, CUPRAC, AND FRAP ASSAYS

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ABSTRACT

INTRODUCTION: Oxidative stress is currently considered one of the main causes in the development of many chronic diseases, which has stimulated a growing interest in antioxidants, especially those derived from plants. Although antioxidant activity has been reported separately for the species *Pelargonium radula* and *Geranium macrorrhizum*, direct comparative studies between them are lacking.

AIM: The aim of this article is to evaluate the comparative in vitro antioxidant activity of extracts from *P. radula* and *G. macrorrhizum* using four complementary antioxidant assays.

MATERIALS AND METHODS: Aerial parts of both species, collected in Central Bulgaria, were extracted with 70% ethanol. Antioxidant activity was assessed using DPPH, TEAC, CUPRAC, and FRAP assays, and the results were expressed as μmol Trolox equivalents per gram of dry extract (μmol TE/g DE).

RESULTS: *G. macrorrhizum* consistently showed higher antioxidant activity than *P. radula* in all applied assays. Depending on the analytical method used, the antioxidant capacity of *G. macrorrhizum* was approximately 2.7–3.9 times higher than that of *P. radula*. The highest antioxidant capacities for both species were recorded by the CUPRAC assay, reaching 12,826.99 ± 747.65 μmol TE/g DE for *G. macrorrhizum* and 3,259.41 ± 145.31 μmol TE/g DE for *P. radula*. Similar trends were observed in DPPH, TEAC, and FRAP assays.

CONCLUSION: This study presents the first direct comparison of the antioxidant activity of extracts from *P. radula* and *G. macrorrhizum* under identical extraction and analysis conditions. The results show that *G. macrorrhizum* exhibits higher antioxidant activity and provide biological support for previously reported differences in the phytochemical composition of the two species. These results indicate that G. macrorrhizum is the more promising source of natural antioxidants among the species studied.

Keywords: *Geranium macrorrhizum, Pelargonium radula, antioxidant activity, Geraniaceae, phenolic compounds, oxidative stress*

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INTRODUCTION

Oxidative stress is a condition that arises from an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defenses. Excessive accumulation of ROS can cause oxidative damage to lipids, proteins, and nucleic acids, contributing to the onset and progression of numerous chronic diseases (including cardiovascular disease, diabetes mellitus, neurodegenerative diseases,

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and cancer) (1). Therefore, the identification of natural compounds that prevent or reduce oxidative stress remains an important area of pharmaceutical and biomedical research (2).

Medicinal plants are among the most valuable natural sources of antioxidants due to their high levels of phenolic compounds. Phenolic acids and flavonoids have attracted considerable scientific interest for their ability to donate electrons or hydrogen atoms, neutralize free radicals, and chelate transition-metal ions involved in oxidative reactions (3). Numerous studies have demonstrated that these secondary metabolites substantially contribute to the antioxidant properties of plant extracts and may exert beneficial effects against oxidative stress-related disorders (1,3).

The family *Geraniaceae* comprises a diverse group of species widely distributed across Europe, Asia, Africa, and the Mediterranean region. Members of the genera *Geranium* and *Pelargonium* have long been used in traditional medicine to treat inflammatory conditions, infections, gastrointestinal disorders, and wounds (4). Their biological activities have been linked to polyphenols, flavonoids, tannins, and other bioactive constituents with significant antioxidant potential (4,5).

Among the representatives of the family, *Geranium macrorrhizum L.* has been extensively investigated because of its rich phytochemical composition and broad spectrum of biological activities. Pronounced antioxidant activity has been reported for both extracts and essential oils from this species (6–8). Furthermore, the antioxidant potential of *G. macrorrhizum* has been demonstrated in an experimental model of gentamicin-induced nephrotoxicity, where treatment with the oil extract modulated oxidative stress parameters (9). In contrast, information on *Pelargonium radula* is comparatively limited. Nevertheless, previous studies have shown that aqueous and ethanolic extracts of *P. radula* exhibit considerable antioxidant activity and may exert protective effects under oxidative stress (10,11). Similar findings have also been reported for other representatives of the genus *Pelargonium* (12,13).

Because antioxidant activity involves multiple reaction mechanisms, complementary analytical methods are recommended to obtain a reliable and comprehensive assessment of antioxidant capacity (14,15). Assays based on different reaction principles may provide additional information on the radical-scavenging and reducing properties of plant-derived compounds (16).

A comparative phytochemical characterization of the aerial parts of *P. radula* and *G. macrorrhizum* cultivated in Bulgaria revealed substantial differences in the composition and abundance of phenolic acids and flavonoids (17). *G. macrorrhizum* contained higher levels of several compounds recognized for their antioxidant properties, including rosmarinic acid, *p*-coumaric acid, catechin, and epicatechin (17). These findings suggest that the two species may differ in their antioxidant performance. However, although antioxidant activity has been reported separately for both species, to the best of our knowledge, no study has directly compared the antioxidant activity of *P. radula* and *G. macrorrhizum* extracts using multiple complementary in vitro assays under identical experimental conditions. Such a comparison would provide valuable information on the relationship between phytochemical composition and antioxidant capacity and could help identify the more promising source of natural antioxidants.

AIM

The aim of the present study was to comparatively evaluate the in vitro antioxidant activity of *Pelargonium radula* and *Geranium macrorrhizum* extracts using four complementary antioxidant assays, namely 2,2-diphenyl-1-picrylhydrazyl (DPPH), Trolox Equivalent Antioxidant Capacity (TEAC), Ferric Reducing Antioxidant Power (FRAP), and Cupric Reducing Antioxidant Capacity (CUPRAC).

MATERIALS AND METHODS

Plant Material

The aerial parts of *P. radula* and *G. macrorrhizum* were collected during the flowering period in June 2025 from the Gabrovo region in Central Bulgaria (42°52′ N, 25°20′ E). Botanical identification was performed by Assist. Prof. Petya Boycheva (Department of Biology, Faculty of Pharmacy, Medical University of Varna, Bulgaria). After collection, the plant material was air-dried at room temperature in a shaded, well-ventilated environment until a constant weight was achieved. The dried material was then

milled to a particle size of approximately 0.5 mm and used to prepare the extract. Detailed information on plant collection and processing has been reported previously (17).

Preparation of Extracts

Dry powdered aerial parts (5.0 g) were extracted with 100 mL of 70% (v/v) ethanol under continuous stirring and reflux for 2 h. The extracts were then subjected to ultrasonic-assisted extraction for 30 min at room temperature and stored at 4°C for 48 h. After filtration, the solvent was removed under reduced pressure using a rotary evaporator (BUCHI R-300, Flawil, Switzerland) at 50°C and 97 mbar. The resulting dry extracts (DE) were stored at –10 °C until antioxidant evaluation. The extraction procedure followed a previously reported protocol (17).

Determination of Antioxidant Activity

The antioxidant capacity of the extracts was assessed using four complementary spectrophotometric microplate assays based on distinct antioxidant mechanisms: DPPH radical-scavenging assay, TEAC assay, FRAP assay, and CUPRAC assay. All analyses were conducted at the Laboratory of Cell Biosystems, Institute of Microbiology, Bulgarian Academy of Sciences, Plovdiv, Bulgaria, following a previously published protocol (18). Antioxidant activity was expressed as μmol Trolox equivalents per gram of dry extract (μmol TE/g DE). Each assay was performed in eight technical replicates.

Statistical Analysis

Antioxidant activity data are presented as mean values ± standard deviation (SD) from eight technical replicates per assay. Given the study's descriptive, comparative nature, and the absence of independent biological replicates, the results were evaluated descriptively and interpreted in terms of the observed differences in antioxidant capacity among the investigated extracts.

RESULTS

The antioxidant activity of the dry extracts of *P. radula* and *G. macrorrhizum* was evaluated using four complementary in vitro assays: DPPH, TEAC, CUPRAC, and FRAP. The results are shown in Table 1.

Across all assays, *G. macrorrhizum* consistently showed higher antioxidant activity than *P. radula* (Table 1). Depending on the analytical method used, the antioxidant capacity of *G. macrorrhizum* was approximately 2.7–3.9-fold greater than that of *P. radula*, indicating markedly stronger antioxidant potential.

Table 1. Antioxidant activity of Pelargonium radula and Geranium macrorrhizum dry extracts determined by different in vitro assays.

Assay (μmol TE*/g DE**)	*P. radula*	*G. macrorrhizum*
DPPH	1188.39 ± 139.73	3250.48 ± 191.29
TEAC	261.23 ± 52.99	877.45 ± 104.15
CUPRAC	3259.41 ± 145.31	12826.99 ± 747.65
FRAP	866.19 ± 71.38	2639.63 ± 265.98

TE—Trolox equivalents*
*DE**—dry extract*
Values are presented as mean ± SD (n = 8).

For both species, the highest antioxidant capacities were measured by the CUPRAC assay, whereas the lowest values were obtained by the TEAC assay. The most pronounced difference among the extracts was observed in the CUPRAC assay, where the antioxidant capacity of *G. macrorrhizum* was nearly four times that of *P. radula*. Similar trends were observed in the DPPH, TEAC, and FRAP assays, confirming the consistency of the results across different antioxidant reaction mechanisms.

Overall, the assays revealed a consistent pattern, with *G. macrorrhizum* showing superior radical-scavenging and reducing capacities compared to *P. radula*, regardless of the analytical approach.

DISCUSSION

The present study demonstrates that, regardless of the analytical approach, *G. macrorrhizum* consistently exhibits approximately 2.7–3.9 times greater antioxidant activity than *P. radula* across all assays tested. This was observed in all four assays performed, despite their different underlying reaction mechanisms. This reinforces the observed trend and suggests that the differences in antioxidant activity are not method-specific but rather correlate with the phytochemical composition of the extracts. The use of four complementary antioxidant assays is an additional strength of the investigation.

Antioxidant activity is a complex phenomenon involving multiple reaction pathways, and no single assay can comprehensively characterize the antioxidant potential of plant-derived samples (14,15). Differences in absolute values among the DPPH, TEAC, FRAP, and CUPRAC assays are expected because each method is based on distinct reaction principles and exhibits different sensitivities to individual classes of antioxidant compounds. Nevertheless, all four assays revealed the same comparative trend, thereby strengthening confidence in the observed results. The particularly high values obtained by the CUPRAC assay may reflect the strong reducing capacity of phenolic constituents present in the investigated extracts, as this method is considered highly responsive to a broad spectrum of plant-derived antioxidants (14,16).

To our knowledge, this is the first study to directly compare the antioxidant activity of extracts from *P. radula* and *G. macrorrhizum* obtained under identical extraction conditions and assessed using the same analytical methodology. Although antioxidant activity has been previously reported for both species, direct comparisons between published studies are often complicated by differences in geographical origin, extraction procedures, solvent systems, and analytical protocols. By minimizing these variables, the present study provides a more reliable assessment of the relative antioxidant potential of the two species.

The higher antioxidant activity of *G. macrorrhizum* may be linked to its greater content of phenolic constituents, which are known for strong antioxidant properties. Markedly higher concentrations of rosmarinic acid, p-coumaric acid, catechin, and epicatechin have previously been reported in *G. macrorrhizum* compared with *P. radula* (17). In addition, catechin and epicatechin were detected exclusively in *G. macrorrhizum*, whereas p-coumaric acid was not quantified in *P. radula* (17). Although the contribution of individual compounds was not assessed in the present study, the overall phenolic profile of *G. macrorrhizum* provides a plausible explanation for its consistently higher antioxidant activity.

Rosmarinic acid, for example, is widely recognized as one of the most potent naturally occurring phenolic antioxidants and exhibits pronounced radical-scavenging and reducing properties through multiple complementary mechanisms (19). Likewise, phenolic acids and flavonoids, including p-coumaric acid, catechin, and epicatechin, are well-established contributors to the antioxidant capacity of plant extracts (3). The higher abundance of these compounds in *G. macrorrhizum* is therefore consistent with the antioxidant profile observed in the present study.

A major strength of the present work is its direct correlation with our previous phytochemical study of the same plant materials (17), thus providing functional support for the biological relevance of the previously characterized phytochemical profiles of the two species.

Several limitations of the present study should be acknowledged. The study was limited to in vitro antioxidant assays and therefore does not allow direct conclusions about in vivo biological activity. Furthermore, the contribution of individual phytochemicals and potential synergistic interactions between extract constituents were not assessed. Future studies should focus on the isolation and characterization of the main antioxidant constituents, the evaluation of their individual and combined effects, and the evaluation of biological activity in more complex experimental systems.

CONCLUSION

The present study provides the first direct comparison of the in vitro antioxidant activity of *P. radula* and *G. macrorrhizum* extracts under identical extraction and analytical conditions. Both species exhibited measurable antioxidant capacity; however, *G. macrorrhizum* consistently showed superior activity across all assays, including DPPH, TEAC, CUPRAC, and FRAP. Concordant results from four complementary antioxidant methods strengthen the reliability of the observed differences between the extracts. When considered alongside previously reported phytochemical data, the present findings indicate that *G. macrorrhizum's* richer phenolic profile is associated with markedly higher antioxidant performance. These results expand the current knowledge of the antioxidant properties within the *Geraniaceae* family and indicate that *G. macrorrhizum* is the more promising source of natural antioxidants among the investigated species.

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