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Reference to a dataset:

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Engle, E. K., Cash, T. F., & Jarry, J. L. (2009). The Body Image Behaviours Inventory-3: Development and validation of the Body Image Compulsive Actions and Body Image Avoidance Scales. Poster session presentation at the meeting of the Association for Behavioural and Cognitive Therapies, New York, NY.

Reference to software:

Coon, E., Berndt, M., Jan, A., Svyatsky, D., Atchley, A., Kikinzon, E., Harp, D., Manzini, G., Shelef, E., Lipnikov, K., Garimella, R., Xu, C., Moulton, D., Karra, S., Painter, S., Jafarov, E., & Molins, S. (2020). March 25. Advanced Terrestrial Simulator (ATS) v0.88 (Version 0.88). Zenodo. https://doi.org/10.5281/zenodo.3727209.

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FROM THE EDITOR

International Journal of Plant Based Pharmaceuticals (IJPBP) is a peer-reviewed open access journal for original research articles, review articles and short communications related to all aspects of plant based pharmaceuticals and pharmaceutical analysis. IJPBP was launched in 2021, and published biannually.

IJPBP welcomes submissions from a diverse range of disciplines and geographic regions, reflecting its commitment to a truly global perspective on plant-based pharmaceuticals. Current areas of interest include, but are not limited to, the following topics:

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IJPBP encourages contributions that explore new frontiers in plant-based pharmaceutical sciences and that demonstrate the global relevance and applicability of their findings. Submissions highlighting cross-disciplinary approaches or international collaborations are particularly welcome.

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Editor-in-Chief
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REVIEW

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Echinacea purpurea: An overview of mechanism, efficacy, and safety in pediatric upper respiratory infections and otitis media

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ABSTRACT

Pediatric upper respiratory infections (URIs) and otitis media (OM) significantly impact the health of children globally. *Echinacea purpurea*, known for its immunomodulatory, anti-inflammatory, and antimicrobial properties, has been historically used to treat various ailments, suggesting its potential as an adjunctive treatment in pediatric respiratory conditions. This narrative review synthesizes literature from January 2000 to December 2023 on the efficacy and safety of *E. purpurea* in treating pediatric URIs, including OM. It focuses on clinical trials and empirical studies that explore the mechanisms of action, such as the modulation of cytokine production, inhibition of NF-κB signaling, and antimicrobial effects. The analysis reveals mixed outcomes regarding the efficacy of *E. purpurea* in pediatric populations, attributed partly to variability in study designs and lack of standardized treatment protocols. While some studies report reduced severity and duration of respiratory symptoms, others indicate minimal or no significant difference compared to placebo. The review also highlights the need for specifically designed products that cater to the unique physiological and metabolic needs of children. Rigorous, well-designed clinical trials are crucial for establishing clear guidelines on the use of *E. purpurea* in pediatric respiratory care, ensuring its safe and effective application in improving health outcomes for children.

1. Introduction

Upper respiratory infections (URIs) represent a significant health concern in pediatric populations, manifesting as a leading cause of acute illness and a primary reason for visits to healthcare providers worldwide. The incidence of URIs is notably elevated among preschool-aged children owing to their evolving immune systems and heightened exposure to pathogens in daycare or educational environments (Jin et al., 2021; Kusel et al., 2007; Ostrzyżek-Przeździecka et al., 2023). These infections, characterized by symptoms such as cough, fever, and sore throat, can significantly impact a child's well-being and development (Ogal et al., 2021; Ren et al., 2019). Common causative agents among pediatric cases include various viruses such as rhinovirus, influenza virus, respiratory syncytial virus (RSV), adenovirus, and coronaviruses. Additionally, bacterial pathogens like *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* may contribute to URIs, particularly in instances of secondary bacterial in-

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fection (Bellussi et al., 2019; van Doorn & Yu, 2020). URIs, including nasopharyngitis, pharyngitis, and tonsillitis, and complications such as otitis media (OM), account for 87.5% of all respiratory tract infections (Nguyen-Van-Tam et al., 2022). OM, a common complication of URIs, often manifests following these infections, illustrating a direct clinical linkage (Durmaz et al., 2021; Principi & Esposito, 2020). OM is predominantly caused by bacteria like *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis*, with viruses also playing a role (Folino et al., 2022; Zahid et al., 2024).

OM is bifurcated into acute OM, with distinct symptoms, and OM with effusion, marked by fluid retention without symptoms (Jamal et al., 2022; Spoială et al., 2021). Notably, acute OM affects a significant portion of children with viral URIs. The management of OM often involves extensive antibiotic therapy and surgical interventions on the ear, constituting a significant component of pediatric healthcare expenditures (Spoială et al., 2021). These conditions significantly contribute to the burden of illness, healthcare utilization, and economic costs. Despite advancements in medical management, treating pediatric URI and OM remains a clinical challenge, prompting the exploration of alternative and complementary therapeutic approaches.

In the treatment and prevention of URIs and OM, a broad spectrum of drugs are employed. For URIs, antiviral medications such as oseltamivir and zanamivir are commonly used to treat influenza, while antibiotics like amoxicillin and azithromycin are prescribed for bacterial infections (Nitsch-Osuch et al., 2016). In cases of OM, amoxicillin is typically the first-line treatment, with alternatives such as amoxicillin-clavulanate, cefdinir, and cefuroxime used when resistance or allergies are a concern (Dawit et al., 2021). In addition to pharmaceutical interventions, several species of plants, including *Echinacea purpurea*, have been explored for their therapeutic benefits.

E. purpurea, commonly known as purple coneflower, is a flowering plant native to North America with a long history of traditional medicinal use (Figure 1). The plant has been used for centuries by indigenous peoples and later adopted by European settlers for its purported therapeutic properties, particularly in the treatment of respiratory infections, wounds, and immune-related conditions. *E. purpurea* is rich in bioactive compounds, including alkamides, polysaccharides, flavonoids, and phenolic acids, which are believed to contribute to its immunomodulatory, anti-inflammatory, and antimicrobial effects (Balčiūnaitė-Murzienė et al., 2021; Burlou-Nagy et al., 2022; Daley, 2019).

Figure 1. *E. purpurea* plant parts
(A. root, B. stem, C. flower, D. Fruit)

With the rise in pediatric URIs, scientists have increased the discovery of herbal medicines to improve treatment effectiveness. Currently, *E. purpurea* is widely used across various global regions as an over-the-counter remedy for URIs and OM due to its reported immunomodulatory properties. The rationale for studying *E. purpurea* in pediatric URI and OM lies in its immune-modulating, anti-inflammatory, and antimicrobial properties, which may offer potential benefits in reducing symptom severity, duration of illness, and recurrence rates in affected children.

2. Methodology

This study employed a narrative review methodology, as previously described (Snyder, 2019). This review synthesized research findings from studies published from January 2000 to December 2023, providing an overview of the efficacy and safety of *E. purpurea* in pediatric respiratory conditions. The review specifically focused on the exploration of mechanisms of action, such as immunomodulatory, anti-inflammatory, and antimicrobial effects. A structured approach was used to search the literature, employing keywords like '*E. purpurea*', 'pediatric respiratory infections', 'children URI treatment', and '*Echinacea* safety in children', using AND/OR operators to refine the search results. Inclusion criteria were studies that investigated the clinical efficacy and safety of *E. purpurea* in children, with a focus on randomized controlled trials, observational studies, and empirical research. Exclusion criteria included studies lacking sufficient data on outcomes or those focusing on adult populations. Data extraction targeted information on the age range of participants, treatment duration, outcomes measured (i.e. efficacy and safety), and key findings.

3. Empirical use of *E. purpurea*

3.1. Historical values of E. purpurea

E. purpurea, belonging to the Asteraceae family, has been historically valued in indigenous medicine for its healing properties and has gained popularity in modern complementary medicine, particularly for immune system support and as a remedy for colds and respiratory infections. Indigenous peoples of North America, such as the Plains Indians, historically used *E. purpurea* for its medicinal properties to treat various ailments, including respiratory infections, wounds, and snake bites (Aarland et al., 2017; Oláh et al., 2017; Pires et al., 2016). European settlers adopted the use of *E. purpurea* from Native American traditions, leading to its incorporation into Western herbal medicine practices in the late 19th century. *E. purpurea* has been prepared for use as a topical treatment for skin and wound inflammation and is currently licensed in Europe for treating infections of the upper respiratory tract and for wound healing (Burlou-Nagy et al., 2022; Kilani-Jaziri et al., 2017; Ogal et al., 2021; Sharifi-Rad et al., 2018; Thomsen et al., 2018). In modern clinical practice, *E. purpurea* is utilized for its immunomodulatory, anti-inflammatory, and antimicrobial properties, making it a popular over-the-counter remedy for URIs and OM. The bioactive compounds in *E. purpurea*, such as alkamides, polysaccharides, flavonoids, and phenolic acids, are believed to contribute to its therapeutic effects (Balčiūnaitė-Murzienė et al., 2021; Burlou-Nagy et al., 2022; Daley, 2019).

3.2. Phytochemical profile of E. purpurea

The plant is rich in phytochemicals such as alkamides, glycoproteins, polysaccharides, and flavonoids, which are believed to contribute to its therapeutic effects (Truong et al., 2023). These compounds are thought to enhance immune function, offering anti-inflammatory, antiviral, and antioxidant benefits. Potential mechanisms of *E. purpurea* include modulation of cytokine production and enhancement of leukocyte activity, which may help mitigate the symptoms and duration of URI. Its widespread use in alternative medicine prompts the need for continued research, into its efficacy and safety, particularly in vulnerable populations such as children.

E. purpurea contains a complex mixture of bioactive compounds, including alkamides, caffeic acid derivatives, polysaccharides, flavonoids, and essential oils (Daley, 2019; Dosoky et al., 2023; Xu et al., 2021). The main chemical composition of *E. purpurea*, as reported by previous studies (Burlou-Nagy et al., 2022), is outlined in Table 1. These chemical constituents are of significant importance in both botanical and pharmaceutical research, as they underlie the potential therapeutic properties associated with *E. purpurea*. The chemical composition of *E. purpurea* is characterized by a wide range of bioactive compounds distributed across different plant parts. The root, with its alkamides and glycoproteins, appears to be a particularly valuable source of bioactive compounds (Petrova et al., 2023). Additionally, the presence of polysaccharides, caffeic acid derivatives, and volatile oils in various plant parts prompts the complexity of the chemical.

Table 1. Summary of chemical components and their pharmacological effects in some parts of *E. purpurea*

Chemical components	Plant part	Pharmacological effects	References
Alkamides	Root, aerial part	Immunomodulatory, anti-inflammatory, antimicrobial, anticancer, antiviral, antifungal, analgesic, antioxidant, antidiabetic	(Aarland et al., 2017; Balciunaite et al., 2020; Burlou-Nagy et al., 2023; Manayi et al., 2015; Miller & Yu, 2004; Petkova et al., 2023)
Polysaccharide: Pectin-like polysaccharide	Root, aerial part	Immunomodulatory, anti-inflammatory, antidiabetic, antioxidant, antimicrobial, anticancer, antifungal, antiviral, gastrointestinal protective, hypoglycemic, hepatoprotective	(Burlou-Nagy et al., 2023; Hou et al., 2020; Jiang et al., 2021; Manayi et al., 2015; Petrova et al., 2023; Ren et al., 2023; Xu et al., 2021)
Caffeic acid derivatives: Chicoric acid, caftari acid	Root, aerial part	Immunomodulatory, anti-inflammatory, antioxidant, antimicrobial, anti-tumoral, antiosteoporotic, neuroprotective action	(Adebimpe Ojo et al., 2024; Ávila-Gálvez et al., 2024; Manayi et al., 2015; Miller & Yu, 2004; Petrova et al., 2023; Tsai et al., 2012)
Glycoprotein	Root	Immunomodulatory	(Adebimpe Ojo et al., 2024; Balciunaite et al., 2020)
Flavonoids: Luteolin, rutosid, nicotiflorin, rutin	Leaves	Anti-inflammatory, antiallergic, antioxidant, antiviral	(Burlou-Nagy et al., 2023; Kurkin et al., 2011; Lee et al., 2010; Maleki et al., 2019; Manayi et al., 2015)
Alkaloids: Tussilagin, isotussilagin	Root, aerial part	Gastrointestinal-protective, anti-tumoral, hepatoprotective	(Aarland et al., 2017; Burlou-Nagy et al., 2023; Manayi et al., 2015; Miller & Yu, 2004; Petrova et al., 2023; Xu et al., 2021)
Volatile constituents: germacrene D, α-phellandrene, β-caryophyllene, γ-curcumene, α-pinene, δ-cadinene, α-cadinol	Root, leaves, flower	Antimicrobial, analgesic, anti-inflammatory, immunomodulatory	(Dosoky et al., 2023; Nyalambisa et al., 2017)

4. Potential mechanisms of action of *E. purpurea* on treating URI and OM

Research suggests that the bioactive constituents of *E. purpurea* exert immunomodulatory, anti-inflammatory, and antimicrobial effects, which contribute to its therapeutic properties in treating respiratory infections (Dobrange et al., 2019). The potential mechanisms of action and the rationale for studying *E. purpurea* in the context of URIs and OM include:

4.1. Immune-modulating

E. purpurea contains a diverse array of bioactive compounds, including alkamides, caffeic acid derivatives, polysaccharides, and glycoproteins, which are believed to underlie its immunostimulatory properties (Awortwe et al., 2021; Balciunaite et al., 2020; de Oliveira et al., 2021; Ren et al., 2023; Vieira et al., 2023). These ingredients have been shown to have the ability to activate both cellular and humoral immunity by increasing the production and activation of white blood cells, lymphocytes, monocytes, and cytokines (Declerck et al., 2021; Khalaf et al., 2019).

Previous studies have shown that *E. purpurea* can modulate interferon signaling and silence endogenous retroviral sequences through DNA hypermethylation in monocytes, highlighting its potential to boost innate and adaptive immunity, crucial for combating respiratory infections in pediatric populations (Declerck et al., 2021). Clinical evidence supports this potential. Cohen et al. (2004) found that *E. purpurea* reduced the incidence and duration of URIs in children aged 1 to 5 years, while Weber et al. (2005) reported a decreased risk of subsequent URIs in children using *E. purpurea*. Additionally, Wahl et al. (2008) observed that *E. purpurea*

could reduce the frequency of OM episodes over six months, indicating its role in mitigating complications associated with URIs and OM in pediatric patients. A recent study found that *E. purpurea* significantly reduced the incidence and duration of URTIs and their complications, including otitis media, in children aged 4-12 (Ogal et al., 2021). These findings suggest the importance of further research to establish optimal dosage and administration guidelines for *E. purpurea* in treating pediatric respiratory infections.

E. purpurea has been shown to enhance immune function through multiple pathways, including the activation of white blood cells, lymphocytes, and cytokines. Recent studies, have demonstrated that *E. purpurea* can modulate interferon signaling and silencing of endogenous retroviral sequences through DNA hypermethylation in monocytes (Declerck et al., 2021). These mechanisms highlight the potential of *E. purpurea* in boosting innate and adaptive immunity, which is crucial for combating respiratory infections in pediatric populations

An increasing amount of research indicates that *E. purpurea* possesses immunostimulatory characteristics. *E. purpurea* boosts the immune system through three mechanisms: enhancing phagocytosis, stimulating fibroblasts, and promoting respiratory movement. These actions enhance the motility of white blood cells. Additionally, *E. purpurea* enhances immune function by increasing the quantity, functionality, and mobility of various immune cells, such as neutrophils, polymorphonuclear leukocytes, and natural killer (NK) cells, thereby augmenting innate immunity and exerting anti-inflammatory effects (Khattab et al., 2019; Paulovičová et al., 2022). For instance, *E. purpurea* root extract was found to enhance the immune system by reducing the frequency and function of regulatory T cells (Kim et al., 2012). In a separate study, oral administration of an *E. purpurea* extract boosted natural killer cell activity in mice by elevating the levels of MHC II, CD4 T cells, and Th1 cytokines (Park et al., 2021). Similarly, an ethanolic extract of the aerial parts was observed to regulate cytokine responses in human T-cells (Fonseca et al., 2014). The study conducted by Dosoky et al. (2023) demonstrated that the essential oils of *E. purpurea* and its major components exhibited microbicidal properties, while also exerting immunomodulatory effects on neutrophil activation.

Additionally, alkamides have shown efficacy against the cannabinoid receptor type 2 (CB2), suggesting a role in immune regulation through structural similarities with endogenous ligands (Woelkart & Bauer, 2007). The immunomodulatory effects of alkamides are driven by several signaling pathways. These include adenosine cyclic monophosphate (cAMP), p38 mitogen-activated protein kinase (p38/MAPK), and c-Jun N-terminal kinase (JNK). Other pathways involved are nuclear factor kappa light chain of activated B cells (NF-κB) and activating transcription factor cAMP response element binding protein 1 (ATF-2/CREB-1). These mechanisms occur in primary human macrophages and monocytes (Hohmann et al., 2011). A study by Declerck et al. (2021) revealed that phytochemicals derived from *E. purpurea* enhance antiviral innate immunity by regulating tonic interferon (IFN) levels, modulating pattern recognition and chemokine gene expression, and silencing human endogenous retroviruses (HERVs) through DNA repeat hypermethylation in monocytes. Moreover, polysaccharide extracts from *E. purpurea* roots and aerial parts have been reported to modulate the expression of insulin-like growth factor receptor (IGF1R) and certain genes related to immune cell function or activation. Conversely, aboveground parts of the plant, excluding flowers, have been observed to affect immune cell function negatively (Wang et al., 2006). The immunomodulatory properties of *E. purpurea* are particularly pertinent to pediatric URIs and OM, as effective immune responses are crucial for combating pathogens and minimizing tissue damage (Manayi et al., 2015). By enhancing both innate and adaptive immune defenses, *E. purpurea* may alleviate respiratory symptoms, prevent secondary infections, and expedite recovery in pediatric patients.

Furthermore, *E. purpurea* extracts have been found to stimulate mucosal immunity and reinforce the epithelial barrier function in the respiratory mucosa (Meeran et al., 2021). Through activation of immune cells such as dendritic cells and lymphocytes, *E. purpurea* enhances the secretion of mucosal immunoglobulins, strengthening the body's defense against respiratory pathogens (Balčiūnaitė-Murzienė et al., 2021; Declerck et al., 2021; Kim et al., 2021). Additionally, *E. purpurea* upregulates the expression of tight junction proteins and mucins in the respiratory epithelium, thereby maintaining the integrity of the epithelial barrier and preventing pathogen infiltration (Schoop, 2020). Mechanism of action for immune-modulating effects of *E. purpurea*, that may decrease the symptoms of URI and OM was proposed in Figure 2.

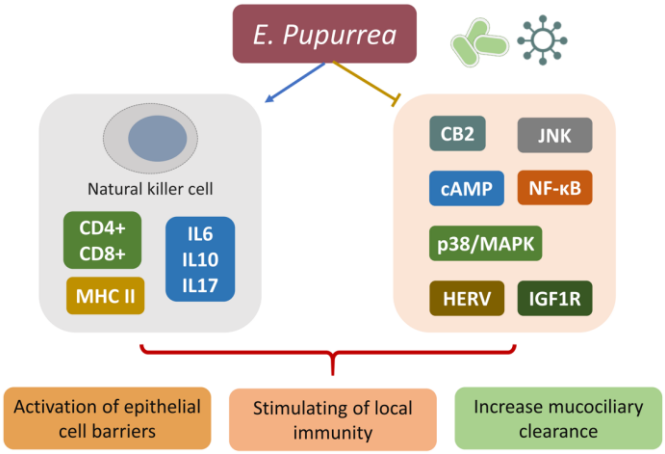

Figure 2. Proposed mechanism of action for immune-modulating effects of *E. purpurea*

4.2. Anti-inflammatory

Inflammation plays a crucial role in sustaining and safeguarding human health (Furman et al., 2019). However, excessive inflammation plays a central role in the pathogenesis of pediatric URIs and otitis media, contributing to tissue damage, symptom severity, and disease progression. Many studies have shown that alkamides, sesquiterpenes, polysaccharides, and caffeic acid derivatives are present in roots and aerial parts of *E. purpurea* exhibits anti-inflammatory effects (Burlou-Nagy et al., 2022; Cheng et al., 2020; Hou et al., 2020; Jiang et al., 2021; Kakouri et al., 2024; Li et al., 2020; Zhang et al., 2020).

The anti-inflammatory mechanism of the active compounds in *E. purpurea* is believed to be through inhibition of nuclear factor-kappa B (NF-κB) signaling, suppression of prostaglandin synthesis, and modulation of cyclooxygenase-2 (COX-2) expression. Studies have shown that alkylamides present in *E. purpurea* exhibit their anti-inflammatory effects by inhibiting the phosphorylation of p38, ERK 1/2, STAT 3, and/or NF-κB signaling pathways, and/or by reducing the expression of cyclooxygenase 2 (Vieira et al., 2023; Vieira et al., 2022). Other research conducted by Cheng et al. (2020) indicated that sesquiterpenes derived from *E. purpurea* demonstrate potent anti-inflammatory properties by inhibiting nitric oxide (NO) production in lipopolysaccharide (LPS)-induced RAW246.7 macrophages through the NF-κB signaling pathway.

Polysaccharides, predominant constituents found in medicinal plants, hold significant relevance in various essential biological functions, notably exhibiting anti-inflammatory properties (Batista et al., 2020; Huang et al., 2019; Sun et al., 2019; Wang et al., 2019). Research conducted by Zhang et al. (2020) demonstrated that *E. purpurea* mitigates lung injury induced by lipopolysaccharide (LPS) through the inhibition of inflammation, apoptosis, and the activation of the Toll-like receptor 4 (TLR4)/NF-κB signaling pathway. Multiple research investigations have indicated that among the phenolic compounds present in *E. purpurea*, caffeic acid derivatives, notably chicoric, caftaric, and chlorogenic acids, serve as principal bioactive components with anti-inflammatory properties. These compounds operate by mechanisms that decrease the synthesis of inflammatory mediators such as cytokines, reactive oxygen species (ROS), nitric oxide, TNF-α, and IL-1β (Vieira et al., 2023; Vieira et al., 2022).

By attenuating the release of pro-inflammatory cytokines and chemokines, *E. purpurea* may mitigate inflammation-induced mucosal injury, nasal congestion, and ear pain in pediatric patients with URI and OM. Mechanism of action for anti-inflammatory effects of *E. purpurea* may reduce the symptoms of URI and OM was proposed in Figure 3.

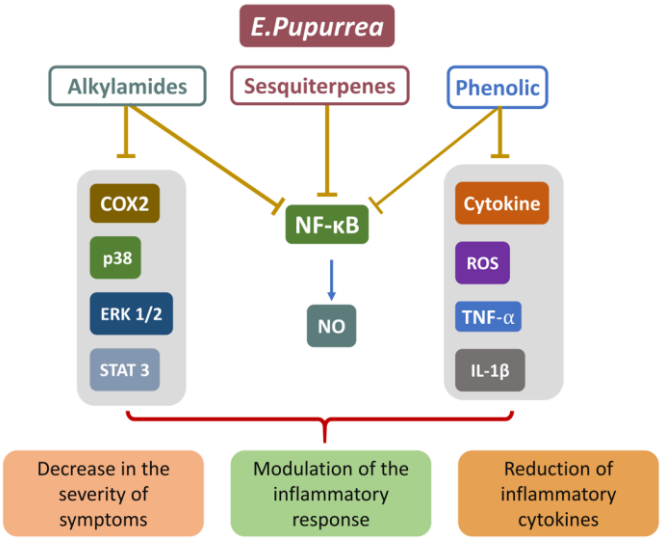

Figure 3. Proposed mechanism of action for anti-inflammatory effects of *E. purpurea*

4.3. Antiviral and antimicrobial

Conventional treatments for pediatric URIs and OM primarily focus on symptomatic relief and, in the case of bacterial infections, antibiotic therapy. However, concerns regarding antibiotic overuse, antimicrobial resistance, and adverse effects suggest the need for alternative, non-pharmacological interventions that are safe, effective, and well-tolerated in children (Basa & Sovtić, 2022; Marchisio et al., 2019).

E. purpurea represents a promising complementary therapy with the potential to reduce reliance on antibiotics, mitigate treatment-related adverse effects, and provide additional options for managing pediatric respiratory infections in a holistic and integrative manner. In addition to its immunomodulatory and anti-inflammatory properties, *E. purpurea* exhibits direct antimicrobial activity against both viral and bacterial pathogens implicated in pediatric respiratory infections. The chemical constituents responsible for the antimicrobial properties of *E. purpurea* encompass alkamides (Burlou-Nagy et al., 2022; Dobrange et al., 2019; Petkova et al., 2023), essential oils (Askari et al., 2020; Dosoky et al., 2023), polysaccharides, and caffeic acid derivatives. Alkylamides and caffeic acid derivatives found in *E. purpurea* extracts have been shown to inhibit viral replication and attachment, particularly against respiratory syncytial virus (RSV), influenza virus, and rhinovirus. Moreover, *E. purpurea* constituents possess antibacterial effects by disrupting bacterial cell membrane integrity, inhibiting biofilm formation, and interfering with bacterial quorum sensing, thus potentially reducing the risk of secondary bacterial complications in pediatric URI and OM. Additionally, in vitro experiments have shown direct antiviral and antibacterial activity of *E. purpurea* constituents against common pathogens implicated in pediatric URIs and otitis

media. The mechanism of action for the antibacterial effects of *E. purpurea*, possibly related to URI and OM was proposed in Figure 4.

4.4. Reduce the severity and duration of symptoms

Shortening the duration of illness and reducing symptom burden can enhance the quality of life for pediatric patients and their caregivers, as well as potentially decrease healthcare resource utilization and absenteeism from school or daycare (Venekamp et al., 2020). Clinical and preclinical evidence suggests that *E. purpurea* may have therapeutic benefits in alleviating symptoms associated with URI and OM (Burlou-Nagy et al., 2022). *E. purpurea* holds promise in reducing the severity and duration of symptoms, thereby improving patient outcomes and reducing healthcare burden. The mechanism of action of *E. purpurea* affecting the immune system, anti-inflammatory and antibacterial effects, leading to the reduction of symptoms related to URI and OM was proposed in Figure 5.

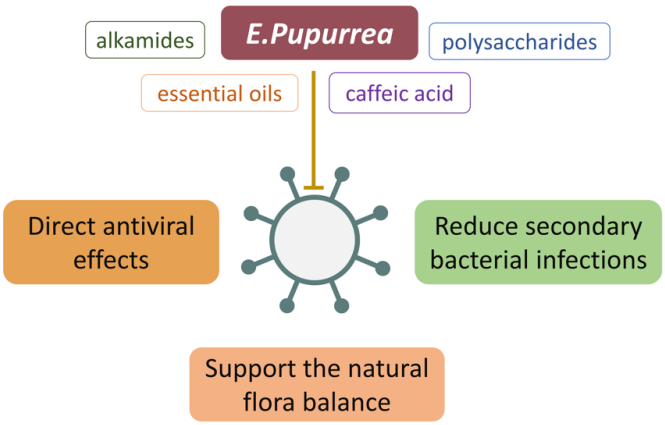

Figure 4. Proposed mechanism of action for antibacterial effects of *E. purpurea*

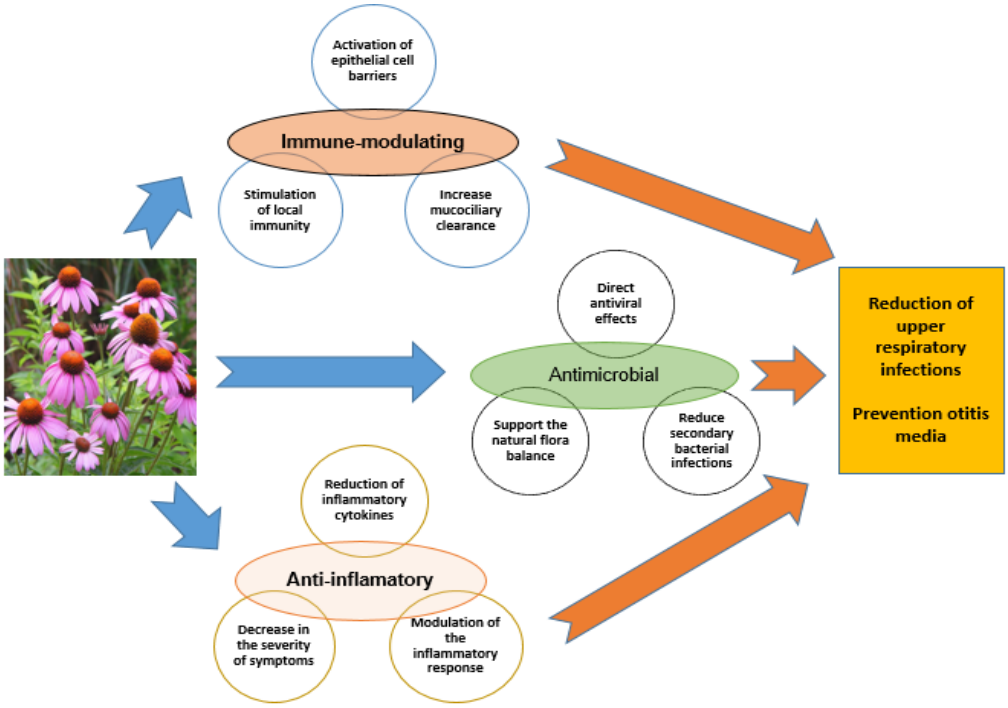

Figure 5. Mechanism of action of *E. purpurea* affecting the immune system, anti-inflammatory and antibacterial effects

Several studies have demonstrated the efficacy of *E. purpurea* in reducing the duration and severity of URI in pediatric populations (Aucoin et al., 2020; Weishaupt et al., 2020) (Table 2). By enhancing innate and adaptive immune responses, *E. purpurea* can help combat viral and bacterial pathogens, leading to faster recovery times and reduced symptom severity (Declerck et al., 2021; Weishaupt et al., 2020). Additionally, its anti-inflammatory properties may alleviate symptoms such as nasal congestion, sore throat, and coughing, further enhancing the patient's comfort and well-being (Meeran et al., 2021). Moreover, *E. purpurea* has shown potential in reducing the incidence and severity of otitis media, a common complication of URIs in children (Basa & Sovtić, 2022; Venekamp et al., 2020). By strengthening the immune system and reducing inflammation in the middle ear, *E. purpurea* may help prevent the progression of URIs to otitis media and mitigate its symptoms (Ogal et al., 2021). This can lead to fewer instances of ear pain, discharge, and hearing impairment, improving the overall quality of life for pediatric patients and their families (Wahl et al., 2008). Therefore, *E. purpurea* holds promise as a natural remedy for reducing the severity and duration of symptoms associated with

pediatric URIs and otitis media. Further research is needed to elucidate the optimal dosage, duration of treatment, and potential interactions with conventional therapies. However, its immunomodulatory and anti-inflammatory properties make it a promising candidate for integrative approaches to pediatric healthcare, offering potential benefits in symptom management and disease prevention.

Table 2. Overview of clinical trials evaluating effectiveness of *E. purpurea* in pediatric URI management

Age of participants	Treatment outcome	Adverse events	References
2-11 years	No significant difference in symptom severity or duration	Not specifically mentioned	(Taylor et al., 2003)
2-11 years	No significant difference in symptom severity or duration	Not specifically mentioned	(Barrett, 2004)
1-5 years	Reduced number of illness episodes, fever days, and treatment time	Rare, mild, and transient	(Cohen et al., 2004)
4-11 years	Symptoms improved on days 2-3; reduction in leukocyte count on day 8	One patient developed an allergic skin reaction	(Spasov et al., 2004)
2-11 years	Associated with a 28% decreased risk of subsequent URI	None reported	(Weber et al., 2005)

Table 3. Efficacy and safety of *E. purpurea* in pediatric otitis media treatment

Age range	Treatment outcome	Adverse events	References
1-10 years	No significant difference in treatment duration; reduced rate of biological characters usage in the *Echinacea* group	None reported	(Wustrow, 2004)
1-5 years	*E. purpurea* was associated with a borderline increased risk of having at least one episode of OM during the 6 months	Not specifically mentioned	(Wahl et al., 2008)

5. Clinical evidence on the efficacy of *E. purpurea* in children

The clinical efficacy of *E. purpurea* in treating pediatric URIs and OM has been explored in numerous studies with varying outcomes (Tables 2 & 3). Research indicates that *E. purpurea* may reduce the duration and severity of symptoms in some pediatric populations, though results are not universally conclusive. Studies often compare the effects of *E. purpurea* against placebos or standard care, focusing on outcomes like symptom relief, duration of illness, and incidence of secondary infections. While some trials report modest benefits, emphasizing shorter illness durations and reduced symptom severity (Cohen et al., 2004; Spasov et al., 2004; Weber et al., 2005), others find no significant difference between *Echinacea* treatment and control groups (Barrett, 2004; Taylor et al., 2003). Variability in study design, *Echinacea* preparations, dosages, and participant characteristics contribute to these mixed results. Despite these discrepancies, *E. purpurea* is generally well-tolerated with a safety profile comparable to placebo in most pediatric studies. The evidence suggests potential value in *E. purpurea* as an adjunctive treatment for URIs, warranting further research to define optimal use parameters. For example, Ogal et al. (2021) found that long-term use of *E. purpurea* significantly reduced the incidence and duration of URIs and their complications, including OM, in children aged 4-12 years, and also led to a substantial decrease in antibiotic prescriptions, highlighting its effectiveness in preventing URIs and reducing the need for antibiotics in pediatric patients.

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Research by Taylor et al. (2003) showed that *E. purpurea* is not effective in treating URI symptoms, and using the preparation increases the risk of rash in patients from 2 to 11 years old. A previous large-scale study (524 children) investigated the effectiveness of *E. purpurea* in improving treatment time, reducing the number of attacks, and increasing the safety of the product in treating URI (Weber et al., 2005). The results showed that *E. purpurea* has a positive effect in reducing the occurrence of subsequent URI in children. Recently, another study recommended the use of *E. purpurea* for long-term prevention of URI, helping to reduce URI complications and antibiotic use in children (Ogal et al., 2021).

Generally, the herb is considered safe for short-term use in children, with most studies reporting minimal side effects that are comparable to those observed with placebo treatments (Ogal et al., 2021; Weishaupt et al., 2020). Commonly noted adverse effects include rash, gastrointestinal discomfort, and allergic reactions, though these are typically mild and transient (Tables 2 & 3). Despite its good safety profile, there remains a need for caution, particularly in children with autoimmune conditions or those taking immunosuppressive medication, due to theoretical concerns about immune system stimulation. Rigorous long-term safety studies are lacking, suggesting the importance of further research to ensure the safe use of *E. purpurea* in pediatric care.

6. Safety profile and controversies

E. purpurea is generally regarded as safe for short-term use in adults and children when administered within recommended dosages (Wahl et al., 2008; Wustrow, 2004). Reported adverse effects are typically mild and transient, including gastrointestinal upset, allergic reactions, and rare cases of hepatotoxicity (Table 3). However, controversies surround the efficacy and safety of *E. purpurea* due to variability in product quality, lack of standardization among preparations, and conflicting findings from clinical trials (Weishaupt et al., 2020). Regulatory agencies such as the U.S. Food and Drug Administration (FDA) have issued warnings regarding unsubstantiated health claims and potential risks associated with *Echinacea* products, emphasizing the importance of evidence-based use and quality control measures. It is also important to note the theoretical concerns about immune system stimulation, particularly in children with autoimmune conditions or those taking immunosuppressive medication. Rigorous long-term safety studies are lacking, suggesting the importance of further research to ensure the safe use of E. purpurea in pediatric care.

7. Strategic approaches to *E. purpurea* use in pediatric respiratory conditions

Researching *E. purpurea* in pediatrics faces several challenges. Standardization is crucial due to variations in preparation methods affecting consistency and efficacy. Determining appropriate dosages for children is complex, requiring consideration of age, weight, and disease severity, with a need for age-appropriate formulations. Ethical and regulatory compliance is vital in pediatric research to ensure participants' safety and rights. Furthermore, potential interactions with conventional pediatric medications necessitate careful integration of *Echinacea* with standard treatments, highlighting the importance of monitoring for adverse effects and drug-herb interactions. These considerations are essential for advancing research and ensuring the safe use of *Echinacea* in pediatric populations.

Future clinical trials investigating *E. purpurea* for pediatric respiratory ailments should adhere to rigorous methodological standards. These include the use of randomized, double-blind study designs, the recruitment of adequate sample sizes, and the implementation of standardized treatment protocols to ensure the reliability and consistency of the results. Research should also examine its integration with conventional treatments, identifying synergistic benefits and optimal treatment strategies while assessing novel formulations for improved delivery and adherence. Importantly, longitudinal studies are necessary to ascertain the long-term safety and efficacy of *E. purpurea*, focusing on the potential for adverse effects and the importance of pharmacovigilance in monitoring its use in pediatric populations.

8. Study limitations

Primarily, the nature of a narrative review inherently involves selectivity in literature inclusion, which could introduce bias. Efforts were made to mitigate this through a structured approach to literature search and selection, yet the possibility of omitted studies relevant to the topic remains. This review emphasizes the need for a systematic review and meta-analysis to provide a more precise quantification of the effects of *E. purpurea* on pediatric upper respiratory tract infections and otitis media. Additionally, the search strategy was limited to specific databases, which may not have encompassed all relevant publications. Future studies could expand the range of databases searched to ensure a more exhaustive review of available literature. Lastly, the practical application of *E. purpurea* in treating pediatric URTIs and OM may require specifically designed products that cater to the unique physiological and metabolic needs of children. The current body of research lacks detailed exploration into optimal formulations and dosages suitable for pediatric use, pointing to a significant area for future investigation.

9. Conclusions

Pediatric URI and OM represent prevalent childhood illnesses that impose significant morbidity and healthcare burdens. *E. purpurea*, known for its immunomodulatory, anti-inflammatory, and antimicrobial properties, has garnered attention for its potential in managing these conditions. However, existing research on *E. purpurea* in pediatric respiratory infections presents mixed findings, suggesting the need for further investigation. Despite the inconclusive evidence, *E. purpurea* remains a popular herbal remedy for pediatric respiratory infections, highlighting the importance of evidence-based guidance for healthcare providers. In summary, while *E. purpurea* shows promise as a potential therapeutic agent for pediatric respiratory infections, further research is required to establish its role in clinical practice, ensure patient safety, and optimize respiratory health outcomes in children.

Acknowledgments

None.

Conflict of interest

The authors confirm that there are no known conflicts of interest.

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In this study, no method requiring the permission of the “Ethics Committee” was used.

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Supplementary File

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RESEARCH ARTICLE

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Exploring the therapeutic potential of silver nanocomposition of *Catharanthus roseus* leaves extract for antimicrobial and antiviral activities: A pilot study

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ABSTRACT

Silver nanoparticles (AgNPs) synthesized from natural sources offer promising solutions for combating microbial and viral infections. *Catharanthus roseus* (Periwinkle), renowned for its diverse pharmacological properties, provides a sustainable and eco-friendly method for producing AgNPs with significant antimicrobial and antiviral effects. This study explores the cytotoxic potential of AgNPs derived from *C. roseus* and their antibacterial, antifungal, and anti-HIV activities, highlighting the novelty of employing a green synthesis approach. AgNPs from *C. roseus* leaf extract (AgNP-CR) were synthesized and characterized using spectroscopic and microscopic techniques to determine their physicochemical properties. The antibacterial activity of AgNP-CR was assessed against clinically relevant bacterial strains, and antifungal activity was evaluated against common fungal pathogens. Additionally, anti-HIV activity was investigated through in vitro assays using HIV-infected cells. Results demonstrated significant antibacterial activity of AgNP-CR against both gram-positive and gram-negative bacteria. Furthermore, AgNP-CR exhibited antifungal activity against pathogenic *Aspergillus* species. Importantly, AgNP-CR showed promising anti-HIV activity by inhibiting viral replication and cytopathic effects in infected cells. Cytotoxicity assays were also conducted to ensure the safety profile of the nanoparticles. Overall, this pilot study underscores the therapeutic potential of AgNPs synthesized from *C. roseus* in addressing bacterial, fungal, and viral infections. Further research is warranted to elucidate their mechanisms of action and optimize formulations for clinical applications.

1. Introduction

Nanotechnology, the manipulation of matter on an atomic and molecular scale, has revolutionized various fields, including medicine, pharmacology, and anti-pathogenic drug delivery. In healthcare, nanoparticles have garnered considerable attention for their role in transforming treatment modalities (Davis et al., 2008). Particularly in cancer therapy, nanoparticles serve as potent tools, facilitating targeted drug delivery and enhancing therapeutic effectiveness while minimizing side effects (Gavas et al., 2021; Mundekkad & Cho, 2022). Amid the increasing threat of antimicrobial resistance and the persistent challenge posed by infectious diseases, the quest for novel therapeutic agents with broad-spectrum efficacy has intensified. Silver nanoparticles (AgNPs) have emerged as promising candidates due to their exceptional antimicrobial properties and potential antiviral activity (Bruna et al., 2021; Luceri et al., 2023; Naumenko et al., 2023; Ratan et al., 2021; Yin et al., 2020). Utilizing natural sources to synthesize AgNPs represents an environmentally sustainable approach that aligns with the principles of green chemistry (Osman et al., 2024; Sharma et al., 2009).

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In recent years, the synthesis of AgNPs using phytoextracts has garnered significant attention as a green and cost-effective approach. These nanoparticles inherit the bioactive properties of the plant extract, thus presenting a multifaceted strategy for combating microbial infections and viral diseases. Despite extensive research on the antimicrobial properties of AgNPs, there remains a notable gap in understanding the efficacy of plant-mediated synthesis methods, particularly those involving *Catharanthus roseus* (L.) G. Don, recognized for its diverse pharmacological properties. *C. roseus*, commonly known as Periwinkle, is distinguished by its rich phytochemical composition and has long been esteemed in traditional medicine for its medicinal virtues, containing bioactive compounds such as alkaloids, flavonoids, terpenoids, and phenolic molecules (Lee et al., 2020; Pham et al., 2020).

While previous studies have investigated the antibacterial and antifungal activities of AgNPs, research on their antiviral effects, particularly against HIV, remains limited. This study aims to address these gaps by utilizing a green synthesis approach with *C. roseus* leaf extract to produce AgNPs and evaluating their comprehensive antimicrobial and antiviral potential. This approach offers a sustainable and effective solution for combating a wide range of pathogens. The silver nanoparticles derived from *C. roseus* leaf extracts used in this research were prepared in our previous study (Joshi & Aithal, 2024). *C. roseus* leaves were selected due to their rich content of bioactive compounds known for various pharmacological properties (Bansal et al., 2023). The aqueous extract was chosen for its eco-friendly and sustainable nature, preserving water-soluble phytochemicals crucial for nanoparticle synthesis (Bhardwaj et al., 2020). Specific bacteria (both gram-positive and gram-negative) and fungi (*Aspergillus* species) were selected based on their clinical relevance and susceptibility to silver nanoparticles, facilitating a comprehensive assessment of the antibacterial and antifungal efficacy of the synthesized AgNP-CR composition. Furthermore, we aimed to evaluate the potential of AgNP-CR on TZM-bl cells as antiviral agents against HIV, a persistent global health threat. TZM-bl cells were chosen for their high susceptibility to HIV infection and their ability to express β-galactosidase and luciferase upon viral entry, enabling precise measurement of viral replication and cytopathic effects (Gaikwad et al., 2023). The cytotoxic concentration (CC_{50}) of AgNP-CR on TZM-bl cells was determined to be 0.08 mg/ml, whereas its anti-HIV-1 activity was observed at a much lower concentration of 0.02 mg/ml (EC_{50}), indicating a favorable safety profile and a promising therapeutic window for antiviral applications.

By exploring the multifunctional properties of the silver nanoparticle-based composition AgNP-CR, our study aims to contribute to the expanding knowledge in the fields of nanotechnology, antimicrobial research, and virology. The insights gained from this research hold promise for developing novel therapeutic strategies to tackle challenges associated with antimicrobial resistance and viral infections. Furthermore, the use of AgNP-CR highlights the significance of leveraging nature's pharmacopeia for innovative solutions in combating infectious diseases.

2. Materials and methods

2.1. Chemicals and equipment

The study utilized high-purity silver nitrate ($AgNO_3$) as the precursor for nanoparticle synthesis, sourced from HiMedia, India. Antimicrobial and antifungal assessments were conducted using nutrient agar and potato dextrose agar (Gibco, USA) media, along with sterile filter paper discs. Additional reagents included cell culture media and supplements such as DMEM (Gibco, USA), FBS (Moregate, Australia), HEPES (Gibco, USA), antibiotics (Sigma, USA), and other standard reagents essential for microbial and cell culture maintenance. Key equipment used included a UV-VIS spectrophotometer for nanoparticle characterization, an autoclave for sterilization, incubators for microbial growth, and microscopy tools for visual inspection. Antiviral studies were performed in Biosafety Cabinets (MicroFilt, India) for culture handling, CO_2 incubators (Thermo Fisher Scientific, USA) for maintaining cell cultures, and a Victor 4 Luminometer (Perkin Elmer, USA) for luminescence-based assays. Additionally, an ELISA plate reader (Thermo Fisher Scientific, USA) was utilized for p24 ELISA to quantify HIV-1 viral replication.

2.2. Preparation of C. roseus leaf extract

The collection of plant material was conducted following the requisite permissions and adhering strictly to international and institutional guidelines and legislation. Fresh and young leaves of *C. roseus* were collected following specific agronomical practices to ensure the quality and consistency of the plant material. The plants were cultivated under controlled conditions in the Botanical Garden of Dnyanopasak College, Parbhani, Maharashtra, India, using organic farming methods to avoid contamination with pesticides or synthetic fertilizers **(Figure S1-supplementary file)**. The leaves were harvested during the flowering stage in July to ensure optimal phytochemical content and were immediately processed to preserve their bioactive compounds. Voucher specimens were formally identified and deposited at the Western Regional Centre, Botanical Survey of India (BSI), Pune, India (authentication number SUPACARO2), by Prof. V. N. Naik, a distinguished taxonomist at Babasaheb Ambedkar Marathwada University, Maharashtra, India. We are grateful for his generous gift of the *C. roseus* leaf extract used in this study. Briefly, the collected leaves were thoroughly cleansed under tap water, followed by rinsing with distilled water to eliminate any dust particles. The cleaned leaves were then carefully shade-dried and pulverized to a fine consistency. The aqueous extract was prepared according to established protocols. Specifically, 1 g of the dried leaf powder was dissolved in 100ml of warm distilled water. This mixture was incubated at 60 °C on a rotary shaker for various time intervals: 15, 30, 45, 60, and 80 minutes. After the designated incubation period, the mixture was allowed to settle for 10 minutes to facilitate sedimentation. The supernatant was then meticulously filtered through Whatman filter paper no. 1 with a pore size of 25µm to obtain clear extracts. These filtered extracts were subsequently cooled and utilized as stock solutions for further investigations, as described in our previous study and elsewhere (Joshi & Aithal, 2024; Sulaiman et al., 2013).

2.3. Preparation and synthesis of silver nanocomposition – AgNP-CR

The silver nanocomposition of *C. roseus* extracts was prepared and characterized previously by our research group (Joshi & Aithal, 2024). Briefly, a 1mM aqueous solution of silver nitrate ($AgNO_3$) was meticulously prepared following the method outlined by Sulaiman et al. (2013), using distilled water at room temperature. Specifically, 0.169 g of silver nitrate was accurately weighed and dissolved in 1000 ml of distilled water, ensuring thorough mixing until a clear solution of $AgNO_3$ was achieved. Subsequently, the synthesis of silver nanoparticles (AgNPs) using extracts from periwinkle leaves was initiated. The particle size was determined using Scanning Electron Microscopy (SEM), and the size of the silver nanoparticles ranged from 23.56 nm to 361.2 nm. Extracts corresponding to different extraction times (10, 20, 30, 40, 50, 60, 70, and 80 minutes) were separately aliquoted into labeled screw-cap tubes. Then, 9 ml of the prepared $AgNO_3$ aqueous solution was added to each tube, creating a reaction mixture. The reaction tubes were carefully

shielded from light to prevent photoreactivation and were left to incubate at room temperature. After thirty minutes, a discernible color change was observed in the reaction solutions within the tubes, ranging from a pale-yellow hue to a deep brown coloration. These color changes served as indicators of the successful synthesis of silver nanoparticles. The observed results, including the color changes and reaction outcomes, were meticulously recorded. The synthesized colored solutions were then utilized for subsequent experimental analyses as per the methodology described earlier (Appidi et al., 2008; Joshi & Aithal, 2024).

2.4. Microorganisms

For the investigation of the antimicrobial potential of the prepared AgNP-CR, five microorganisms were selected. These included bacterial species, specifically *Escherichia coli* (MTCC 739/Y429) and *Bacillus subtilis* (MTCC 441/JCM), as well as fungal species, namely *Aspergillus niger* (MTCC 281/CEP) and *Aspergillus fumigatus* (MTCC 343/CEP). These microorganisms were obtained from the culture collection of the Microbiology Research Centre at DSM College, Parbhani, sourced from the Microbial Type Culture Collection (MTCC) in India, and were used to investigate the antimicrobial potential of the prepared silver nanoparticles (AgNPs). Additionally, one primary isolate of HIV-1 (VB028) was employed in this study, conducted at ICMR-National AIDS Research Institute, Pune.

2.5. Culture method

For the cultivation of bacterial species, *E. coli* and *B. subtilis*, and fungal species, *A. niger* and *A. fumigatus*, standard culture techniques were employed (Cavalieri, 2005). Bacterial strains were cultured on nutrient agar plates and incubated at 37 °C for 24 hours. Fungal strains were cultured on potato dextrose agar plates and incubated at 30 °C for 48 hours. Based on standard practices for antimicrobial assays, bacterial strains (*E. coli* and *B. subtilis*) were used at a concentration of approximately 1 x 10^5 CFU/ml, and fungal strains (*A. niger* and *A. fumigatus*) were used at a concentration of approximately 1 x 10^4 spores/ml. These concentrations ensure the reliability and reproducibility of the antimicrobial assessments. Additionally, the primary isolate of HIV-1VB028 (R5, Subtype C) was propagated in human peripheral blood mononuclear cells (PBMCs) using established protocols (Gaikwad et al., 2023). TZM-bl cells (a modified HeLa cell line) were procured from the National Institute of Health (NIH) HIV Reagent Program and maintained in DMEM (Gibco, USA) containing 10% FBS (Moregate, Australia) and supplemented with HEPES (Gibco, USA) and antibiotics (Sigma, USA) at 37 °C in a 5% CO_2 humidified chamber. The infection with HIV-1VB028 was allowed to proceed for a designated period under controlled laboratory conditions. The culture of bacterial and fungal species, as well as the antibacterial and/or antifungal studies, were conducted at the Department of Microbiology, Dyanopasak Shikshan Mandal, Parbhani. The anti-HIV assays were performed at the Division of Virology, ICMR-National AIDS Research Institute, Pune. Overall, the utilization of these diverse microorganisms enabled a comprehensive assessment of the antimicrobial potential of the prepared AgNP-CR composition against a range of pathogens, including bacteria, fungi, and viruses.

2.6. Antibacterial assessment and bioassay of silver nanoparticles

The antimicrobial efficacy of silver nanoparticles synthesized from *C. roseus* was meticulously evaluated against the gram-positive bacterium *B. subtilis* and the gram-negative bacterium *E. coli*. To confirm the presence and assess the antimicrobial activity of the nanoparticles, we adopted the widely employed agar disc diffusion method. Seeded agar plates were prepared using active cultures of *E. coli* and *B. subtilis* in 50% nutrient agar medium, following established protocols (Baker et al., 2005; Sahoo et al., 2023). Sterile filter paper discs impregnated with the synthesized silver nanoparticles were carefully positioned onto the surface of the agar plates inoculated with the respective microbial cultures. After a specified incubation period, typically 24 hours, the plates were thoroughly examined for the presence of discernible zones of inhibition surrounding the discs. These zones served as visual indicators of the antimicrobial activity of the nanoparticles against the tested microorganisms. The diameter of the zones of inhibition was measured using calibrated instruments and compared to control discs containing known antimicrobial agents (gentamicin 10 µg, HiMedia, India) to gauge the relative efficacy of the silver nanoparticles. Additionally, to further evaluate the antibacterial activity, seeded agar plating was conducted and allowed to solidify. Subsequently, sterile filter paper discs were immersed in tubes containing the synthesized silver nanoparticles. These discs were carefully placed onto the seeded agar plates inoculated with *E. coli* and *B. subtilis*, following the method outlined earlier (Kim et al., 2007). Each concentration of the extract, corresponding to different time intervals up to 1 hour (10, 20, 30, 40, 50, 60, 70, and 80 minutes), was meticulously applied to the agar plates. Following application, all plates were incubated at 37 °C for 24 hours to allow for bacterial growth and the manifestation of potential antibacterial activity. After the incubation period, zones of inhibition surrounding the discs were visually observed, measured, and recorded in accordance with the protocol outlined previously (Thakkar et al., 2010).

The results from both the agar disc diffusion assay and the seeded agar plating provided invaluable insights into the antimicrobial potency of the silver nanoparticles synthesized from *C. roseus* against *E. coli* and *B. subtilis*. These findings highlight the potential applications of these nanoparticles in combating microbial infections, thus contributing significantly to the field of antimicrobial research and fostering further exploration of their therapeutic utility.

2.7. Antifungal assessment and bioassay of silver nanoparticles

The antifungal activity of silver nanoparticles synthesized from *C. roseus* was evaluated against *A. niger* and *A. fumigatus*, adhering to established protocols (Bidaud et al., 2021). Agar plates were prepared by pouring agar seeded with *A. niger* and *A. fumigatus* into petri dishes, allowing it to solidify according to the methodology described earlier (Gajbhiye et al., 2009). Filter paper discs were immersed in the respective solutions containing the synthesized silver nanoparticles and were meticulously positioned onto the seeded agar plates to ensure uniform distribution across the surface. The agar plates were then incubated at 37 °C for 24 hours to facilitate fungal growth and the potential manifestation of antifungal activity exerted by the silver nanoparticles. Following the incubation period, zones of inhibition surrounding the discs were carefully examined, measured, and recorded following established protocols (Krishnaraj et al., 2012; Monteiro et al., 2012). Ketoconazole (50 µg - HiMedia, India) discs were used for antimicrobial susceptibility testing of fungal cultures.

These evaluations provided critical insights into the antifungal efficacy of the synthesized silver nanoparticles against *A. niger* and *A. fumigatus*. The results from both the initial antifungal assessment and the bioassay underscored the potential therapeutic applications of the synthesized silver nanoparticles in combating fungal infections, thereby contributing significantly to understanding their effectiveness against fungal pathogens.

2.8. Antiviral assessment of AgNP-CR

The silver nanocomposition prepared from the leaf extract of *C. roseus*, developed by the Department of Microbiology, Dyanopasak Shikshan Mandal, Parbhani, was evaluated for its anti-HIV-1 activity at the Indian Council of Medical Research - National AIDS Research Institute, Pune (ICMR-NARI), following the standard protocol outlined by Gaikwad et al. (2023). The biological activity of AgNP-CR was assessed using high-throughput cell-based mechanistic studies, as previously described (Jadaun et al., 2022; Jadaun et al., 2023).

2.9. Cytotoxicity assay by MTT

The cytotoxic effect of the pure extract of *C. roseus* (CR-Pure) and AgNP-CR was evaluated in the TZM-bl cell line following established methodologies (Rakshit et al., 2024). Briefly, 1×10^5 adherent TZM-bl cells/well were seeded onto a 96-well plate and incubated for 24 hours with 5% CO_2 at 37 °C. The phyto-extract dilutions and their nanocomposition counterpart were applied to the cell-seeded plates in a dose-dependent manner and incubated for 48 hours. After the incubation period, the treated cells were assessed by adding 20 µl of 5 mg/ml MTT to all wells and further incubating for 3 hours to allow for MTT metabolism. The supernatant was then replaced with 150 µl of dimethyl sulfoxide (DMSO) to dissolve the formazan crystals. Following a final hour of incubation, the optical density (OD) values were recorded at 550 nm and 630 nm using a multimode plate reader. Cell viability was determined by comparing the absorbance of untreated and treated cells. The percentage of cell viability was calculated using the formula:

$$\text{Percentage of cell viability} = \frac{\text{Absorbance of treatment} - \text{Absorbance of blank}}{\text{Absorbance of control} - \text{Absorbance of blank}} \times 100\%$$

The CC_{50} was determined as the concentration at which 50% of the cells remained viable in the presence of CR-Pure or AgNP-CR, based on three independent assays.

2.10. Cell associated assay

Based on the CC_{50} values, a range of non-cytotoxic concentrations of CR-Pure and AgNP-CR were utilized, and the anti-HIV-1 activity was evaluated following established methods (Mutalik et al., 2023). Briefly, TZM-bl cells (1×10^4 cells/well) were initially infected with the HIV-1VB028 virus for 2 hours at 37 °C in a 5% CO_2 incubator, followed by treatment with various dilutions of the extracts. Additionally, 5 mg/ml DEAE-dextran was added to facilitate viral internalization. After a 48-hour incubation period, luciferase activity was measured using the BriteliteTM Plus reagent on a luminometer (Perkin Elmer, USA). The standard nucleoside reverse transcriptase inhibitor drug Azidothymidine (AZT) was used as a positive control at a known concentration of 0.49 µM/ml.

2.11. Statistical analysis of in vitro assays

The mean values from a minimum of three replicates were used for each experiment. The final results were calculated and presented as percentage inhibition graphs after subtracting the blank and comparing them with the respective controls from at least three independent experiments. Error bars indicate the standard deviation of the mean from three assays for each experiment. A p-value of < 0.05 was considered statistically significant for all assays.

3. Results and discussion

The synthesis and evaluation of silver nanoparticles (AgNPs) derived from *C. roseus* extract have significant implications for antimicrobial, antiviral, and antifungal applications (Mukunthan et al., 2011; Salleh et al., 2020). This study presents a comprehensive exploration of the synthesis process, followed by an extensive assessment of the biological activities of AgNP-CR against various pathogens.

3.1. Synthesis of silver nanoparticles (AgNP-CR)

The synthesis of silver nanoparticles (AgNPs) via the reduction of an aqueous solution of silver nitrate represents a well-established method in nanotechnology (Šileikaitė et al., 2006). In this investigation, we focused on the production of a unique composition termed AgNP-CR, derived from the extract of *C. roseus* leaves, and explored its antimicrobial potential against various pathogens. The formation of AgNP-CR was visually indicated by a distinct transition in color, initially manifesting as a pale-yellow hue, which gradually transformed into a deep reddish color within the screw-cap tubes **(Figure 1)**.

A B

Figure 1. Visual indication of AgNP-CR formation
(A) Four samples of pale-yellow aqueous leaf extract of *C. roseus* without $AgNO_3$ (control)
(B) Colour change from pale yellow to dark reddish-brown after adding 9 ml of $AgNO_3$ and heating for 5 minutes

The color transformation was meticulously observed and documented in the screw-cap tubes containing 9 ml of silver nitrate ($AgNO_3$) solution and 1 ml of plant extract before and after a brief heating period of 5 minutes **(Figure 1)**. Notably, the color change was monitored across different time intervals corresponding to the extraction process of the plant extract, ranging from 10 to 80 minutes (i.e., 10, 20, 30, 40, 50, 60, 70, and 80 minutes). This systematic comparison facilitated the observation of potential variations in the color change corresponding to different extraction durations.

The emergence of distinct color profiles at varying time intervals provided preliminary insights into the kinetics of nanoparticle formation, potentially correlating with the phytochemical composition and reductive potential of the *C. roseus* leaf extract. Furthermore, these visual cues served as initial indicators of successful nanoparti-

cle synthesis, paving the way for further characterization and evaluation of the antimicrobial properties of the AgNP-CR composition against a spectrum of microbial pathogens.

The successful synthesis of AgNPs from *C. roseus* extract, as evidenced by the color change from pale-yellow to dark reddish, represents a significant achievement. This method utilizes the reducing properties of plant extracts to convert silver ions into nanoparticles, offering an environmentally friendly approach to nanoparticle synthesis. The observed color change across different extraction times highlights the influence of synthesis duration on nanoparticle characteristics, emphasizing the importance of optimizing synthesis parameters to achieve desired nanoparticle properties.

3.2. Antimicrobial activity of AgNP-CR against gram-positive and gram-negative bacteria

In this investigation, the efficacy of AgNP-CR as antimicrobial agents was evaluated against selected bacterial strains using both agar plate and liquid medium assays, compared with the leaf extract alone (CR-Pure) and $AgNO_3$ only (Figure 2, Figure S2-supplementary file). Blank controls were utilized to ensure the validity of the results, providing a baseline for comparison and confirming that any observed inhibitory effects were attributable to the AgNP-CR and not to other variables. The findings revealed that among the bacteria tested, the gram-positive *B. subtilis* exhibited significant susceptibility to AgNP-CR compared to the gram-negative *E. coli* (Figure 2). Moreover, the inhibitory effect on bacterial growth was influenced by the duration of AgNP-CR synthesis, with *B. subtilis* being more effectively inhibited with increasing synthesis times compared to *E. coli* (Figure 3). Notably, AgNP-CR synthesized after one hour exhibited heightened efficacy in inhibiting a higher concentration of bacteria, as depicted in Figure 3.

Silver nanoparticles have demonstrated remarkable antimicrobial properties against bacteria, with minimum inhibitory concentrations (MIC) of 0.04 mg/ml for *E. coli* and 0.03 mg/ml for *B. subtilis*. This efficacy is attributed to their high surface-area-to-volume ratio and distinctive chemical and physical characteristics. Typically ranging in size from 1 to 100 nm, AgNPs exhibit an augmented surface-area-to-volume ratio as particle size decreases. Previous studies have shown that AgNPs within the size range of 10-100 nm exert potent antimicrobial effects against both gram-positive and gram-negative bacteria, similar to the strains used in this study (Morones et al., 2005). The small particle size enables AgNPs to adhere to bacterial cell walls and penetrate bacterial cells more readily, thereby enhancing their antimicrobial potency. The antimicrobial efficacy of AgNPs has been particularly noteworthy against multidrug-resistant bacteria, such as multidrug-resistant *E. coli*, as reported previously (Kar et al., 2016; Paredes et al., 2014).

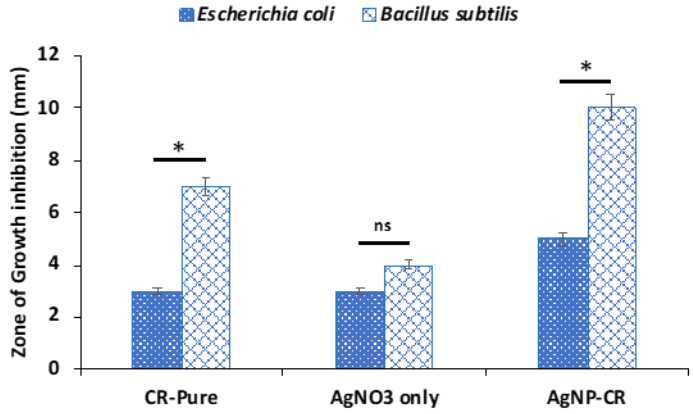


Figure 2. Antibacterial activity of nanoparticles synthesized using *C. roseus* extracts (AgNP-CR) against bacterial strains

Comparative analysis of bacterial growth inhibition on nutrient agar plates treated with CR-Pure, $AgNO_3$, and AgNP-CR demonstrates the efficacy of the synthesized nanoparticles in inhibiting the growth of both gram-negative and gram-positive bacteria. Standard deviations were calculated from three independent assays and are represented as error bars.

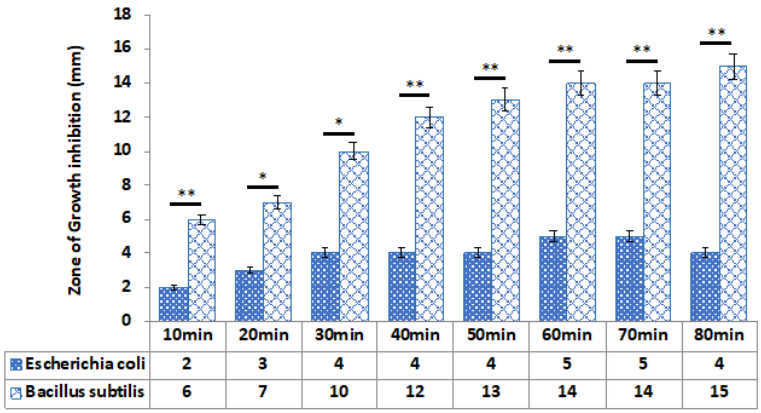


Figure 3. Effect of time interval on the synthesis of silver nanoparticles (AgNP-CR) and its antibacterial activity on *E. coli* and *B. subtilis* over 24 hours

The figure illustrates the relationship between nanoparticle synthesis duration and antibacterial efficacy, highlighting how varying synthesis times impact the antimicrobial properties of AgNP-CR against the two bacterial strains. Standard deviations were calculated from three independent assays and represented as error bars.

3.3. Antifungal activity of AgNP-CR on Aspergillus spp.

In this study, the antifungal potential of AgNP-CR against *Aspergillus* species was assessed using the agar well diffusion method. The results revealed significant zones of inhibition against both fungal strains, with no discernible difference in efficacy between the two strains at a concentration of 0.0625 mg/ml. As depicted in Figure 4, a notable increase in the inhibition zone (4 mm) was observed with green AgNP-CR compared to CR-Pure and $AgNO_3$, which exhibited smaller zones of inhibition (2 mm). These findings underscore the effectiveness of the synthesized silver nanoparticles as antifungal agents against phytopathogens. Interestingly, a distinct pattern of inhibition was observed concerning the effect of synthesis time on the antifungal activity of AgNP-CR on potato dextrose agar (PDA) medium, contrasting with the pattern observed in the bacterial study. As illustrated in Figure 5, the antifungal activity of AgNP-CR against the phytopathogens peaked at a synthesis time of 40 minutes, with an inhibition zone measuring 4 mm. However, beyond this optimal synthesis duration, the inhibitory action of AgNP-CR declined significantly, reaching its lowest point at 80 minutes. These results highlight the dynamic nature of AgNP-CR and its consequent impact on antifungal efficacy.

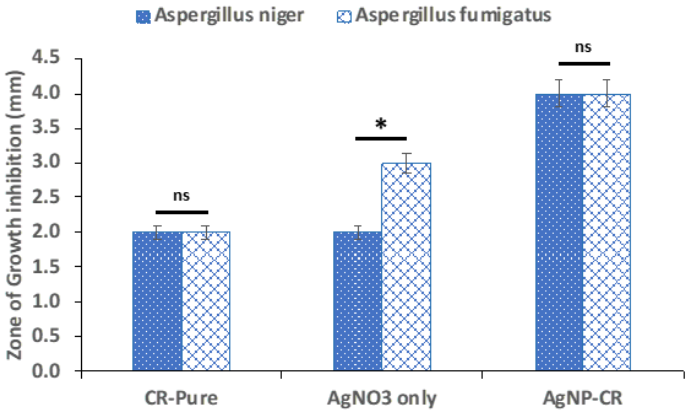
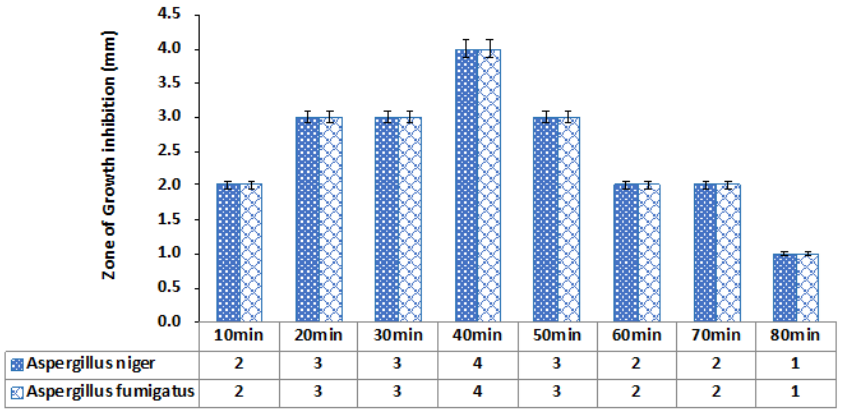


Figure 4. Antifungal activity of AgNP-CR synthesized using phytoextracts of *C. roseus* on potato dextrose agar plates against *Aspergillus* spp
The figure shows the zones of inhibition formed around the discs impregnated with AgNP-CR, indicating the nanoparticles' effectiveness in inhibiting the growth of *A. niger* and *A. fumigatus*. Standard deviations were calculated from three independent assays and are represented as error bars.



	10min	20min	30min	40min	50min	60min	70min	80min
Aspergillus niger	2	3	3	4	3	2	2	1
Aspergillus fumigatus	2	3	3	4	3	2	2	1

Figure 5. Effect of synthesis time interval on the antifungal activity of silver nanoparticles using *C. roseus* extract (AgNP-CR) against *Aspergillus* spp. on potato dextrose agar (PDA) medium incubated at room temperature for 4 days
The figure demonstrates how different synthesis durations influence the antifungal efficacy of AgNP-CR, with consistent inhibition observed for both *A. niger* and *A. fumigatus* strains. Standard deviations were calculated from three independent assays and are represented as error bars.

Furthermore, the biosynthesized AgNP-CR exhibited promising antifungal activity against the tested fungal strains, effectively inhibiting the growth of *A. niger* and *A. fumigatus*. These fungi pose significant threats to agriculture and human health by causing agricultural damage and skin-related diseases, respectively. Collectively, these findings highlight the substantial antifungal activity of AgNP-CR, demonstrating potent effects against the tested fungi in vitro. The primary mechanisms underlying the antifungal action of AgNPs include the disruption of fungal cell walls and membranes, interference with protein function, generation of reactive oxygen species (ROS), and disruption of protein structures (Jian et al., 2022). However, further research is warranted to explore the applicability of AgNP-CR as antifungal agents in field settings and to elucidate their mechanisms of action in greater detail.

3.4. In vitro cytotoxicity of AgNP-CR on TZM-bl cells

The cytotoxic effects of the *C. roseus* phyto-extract and its silver nanocomposition (AgNP-CR) on TZM-bl cells were initially evaluated using the MTT quantitative colorimetric assay. A dose-response relationship ranging from 5.0 to 0.005 mg/ml was examined to assess the impact of *C. roseus* and AgNP-CR on cellular viability, as depicted in Figures 6A and 6B. From three independent replicates, the concentrations at which 50% of cells remained viable (CC_{50} values) were determined to be 3.43 mg/ml for *C. roseus* and 0.08 mg/ml for AgNP-CR. The cytotoxicity of AgNP alone (Figure S3-supplementary file) and the FDA-approved standard drug Azidothymidine (data not shown) were also determined for experimental validation.

3.5. CR extracts and AgNP-CR mediated inhibition of HIV-1 replication

TZM-bl cells were used to screen the anti-HIV-1 activity. Based on the results of the cell viability assay, optimal concentrations of 0.5 mg/ml for CR extracts and 0.08 mg/ml for AgNP-CR were selected as the initial testing concentrations for the cell-based anti-HIV-1 assay.

In the cell-associated assay, the half maximal effective concentration (EC_{50}) values of the *C. roseus* extract and its metal nanocomposition, AgNP-CR, against HIV-1VB028 (R5, Subtype C) were determined. The *C. roseus* extract exhibited an EC_{50} value of 0.16 mg/ml, whereas AgNP-CR inhibited 50% of HIV-1 virus replication at a much lower concentration of 0.02 mg/ml. A dose-dependent inhibition of HIV-1 replication was observed for both the *C. roseus* extract and AgNP-CR in TZM-bl cells, as illustrated in Figures 7A and 7B. While the *C. roseus* extract demonstrated significant inhibition of replicating cell-associated virus across the concentration range of 0.50 to 0.005 mg/ml (Figure 7A), AgNP-CR exhibited better inhibitory effects within the concentration range of 0.080 to 0.0006 mg/ml (Figure 7B). The EC_{50} values were compared with the standard drug AZT (0.49 µM, data not shown) and AgNP alone (Figure S3B-supplementary file).

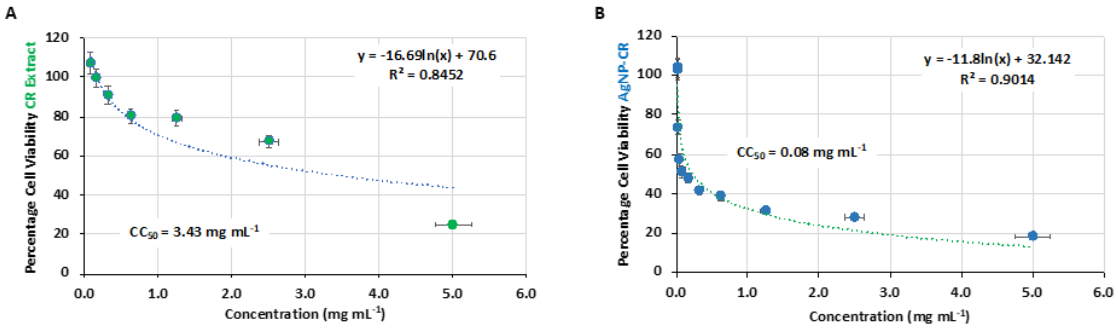


Figure 6. Determination of cell viability of *C. roseus* leaf extract and its silver nanoparticle composition

The figure depicts the impact of varying concentrations of (A) CR-extract and (B) AgNP-CR on TZM-bl cells examined through the MTT assay. The cell viability curves indicate the cytotoxic effects, with calculated CC_{50} values derived from three independent experiments, demonstrating the concentration at which 50% of the cells remain viable. Standard deviations were calculated and are represented as error bars.

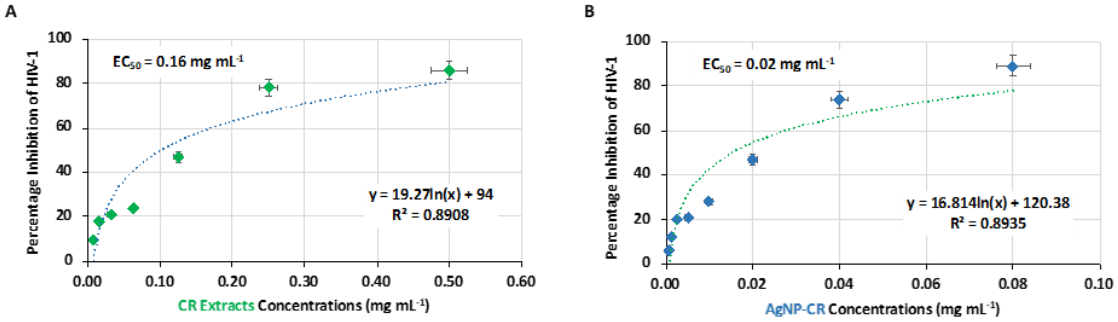


Figure 7. Anti-HIV-1 activity of silver nanoparticle composition of *C. roseus* leaf extract

The figure illustrates the percentage inhibition of HIV-1 replication by (A) CR-extract and (B) AgNP-CR in HIV-1VB028 (R5 - Subtype C) infected TZM-bl cells through Cell-Associated assays. The graphs show the average effective concentrations (EC_{50} values) required to inhibit 50% of viral infections, derived from three independent experiments, highlighting the potential of AgNP-CR as an anti-HIV agent. Standard deviations were calculated and are represented as error bars.

The antiviral assessment of AgNP-CR against HIV-1 underscores its potential in combating viral infections. The cytotoxicity assay using TZM-bl cells revealed low cytotoxicity for AgNP-CR, indicating a favorable safety profile for potential therapeutic applications. Subsequent evaluation of anti-HIV-1 activity demonstrated dose-dependent inhibition of viral replication by both the *C. roseus* extract and AgNP-CR. Notably, AgNP-CR exhibited superior efficacy in inhibiting HIV-1 replication compared to the plant extract alone, highlighting the enhanced antiviral activity conferred by the nanoparticle composition.

4. Conclusions

The synthesis of silver nanoparticles (AgNPs) from *C. roseus* (periwinkle) demonstrates significant therapeutic potential. The study revealed that AgNP-CR exhibits antimicrobial effects against both gram-positive (*B. subtilis*) and gram-negative (*E. coli*) bacteria, as well as fungal pathogens such as *A. niger* and *A. fumigatus*. Additionally, AgNP-CR showed promising anti-HIV activity, indicating potential as an antiviral agent against HIV infections. The primary mechanism of action appears to involve the disruption of microbial cell membranes, which inhibits microbial growth and viral replication. The utilization of *C. roseus* for nanoparticle synthesis not only aligns with sustainable and eco-friendly practices in nanomedicine but also leverages the plant's rich phytochemical profile. The findings underscore the multifaceted therapeutic properties of AgNP-CR, suggesting its utility in developing novel antimicrobial and antiviral agents. However, several limitations need to be addressed in future research. The precise mechanisms underlying the observed antimicrobial and antiviral activities require further elucidation. Additionally, the synthesis protocols must be optimized to enhance therapeutic efficacy and consistency. While the study highlights the superior antibacterial activity of AgNP-CR, its relatively lower antifungal activity warrants further investigation to fully understand its clinical applicability.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Statement of ethics

No animal or human specimens were used in this study, and ethical approval was waived under protocol number of NARI/EC/Approval/2022/662.

Availability of data and materials

The original contributions presented in this study are included in the article and supplementary materials. Further inquiries can be directed to the corresponding author(s).

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CRediT authorship contribution statement

Rohini Joshi: Conceptualization, Validation, Formal analysis, Investigation, Data curation, Writing manuscript, Visualization, Funding acquisition
Shiva Aithal: Validation, Investigation, Data curation, Review and editing, Supervision
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Supplementary File

The supplementary file accompanying this article is available at https://ijpbp.com/index.php/ijpbp/libraryFiles/downloadPublic/21.

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SHORT REPORT

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In vitro antifungal activity of extracts and alkaloid compounds from *Piper arboreum* against dermatophytes

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ABSTRACT

Piper is widely distributed in subtropical regions and species of this genus are known for their potent pharmacological activities. *Piper arboreum* Aubl. is a traditional medicinal plant popularly known as "pau-de-angola", "jaborandi", and chili pepper, demonstrating antifungal, trypanocidal, antibacterial, and antioxidant activities. The leaves of *P. arboreum* were extracted using Soxhlet and dichloromethane to obtain the extract, which was then fractionated using solvents of different polarities. Samples were analyzed using ultra-high-performance liquid chromatography coupled with mass spectrometry equipped with an electrospray ionization source. Antifungal microdilution assays were performed, and scanning and transmission electron microscopy were used to assess the invasion of the pretreated nail. The minimum inhibitory concentration (MIC) values of the extract and a dichloromethane fraction were, respectively, 62.5 µg/ml and 16.0 µg/ml against *Trichophyton rubrum*, and 125 µg/ml and 62.5 µg/ml, and 500 µg/ml and 500 µg/ml against *T. mentagrophytes*, and *Microsporum gypseum*, respectively. No growth was observed on nail fragments exposed to the extract (at concentrations > 64 µg/ml and then inoculated with spore suspension. Transmission electron microscopy revealed strong inhibition of hyphal growth and an irregular growth pattern following treatment with the extract and the dichloromethane fraction. Results demonstrated the antifungal properties of the *P. arboreum* extract and its dichloromethane fraction against dermatophytes, with the identification of three different alkaloid compounds. The cytotoxicity was specific towards the fungal cells, and morphological and ultrastructural analyses indicated damage to the cell wall and cytoplasmic membrane as the potential mechanism of action. The leaf material used to generate the extract can be taken from the plant without any detrimental effect thus enabling strategies to be implemented for the exploitation of this species.

1. Introduction

Piper is one of the most diverse genera among the basal clades of angiosperms and is widespread in tropical wet forests around the world (Dyer & Palmer, 2004), with about 2,000 species (Quijano-Abril et al., 2006). The genus *Piper* is widely distributed in tropical and subtropical regions and is known for its aromatic herbs (Biswas et al., 2022; Guerrini et al., 2009). Some species of *Piper* have already been demonstrated to have potent pharmacological activities, as well as exhibit great chemical diversity of their secondary metabolites (Tran et al., 2024; Yadav et al., 2020). Essential oils produced by *Piper* species have been found to comprise monoterpenes (germacrene A, α-pinene), sesquiterpenes (germacrene B, germacrene D, α-humulene, β-copaen-4-α-ol), phenylpropanoids (humulene epoxide II, muurola-4,10(14)-dien-1-β-ol), aldehy-

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des (cinnamaldehyde), ketones, and long chain alcohols (Cysne et al., 2005).

P. arboreum Aubl., popularly known as "pau-de-angola", "jaborandi", and chili pepper, has been shown to demonstrate antifungal, trypanocidal, antibacterial, and antioxidant activities (Bernuci et al., 2016; Regasini et al., 2009; Regasini et al., 2008). Furthermore, phytochemical studies have revealed that leaves of this species contain several amides, such as arboreumine, N-[10-(13,14-methylenedioxyphenyl)-7*(E)*-pentaenoyl]-pyrrolidine, and N-[10-(13,14-methylenedioxyphenyl)-7*(E)* (da Silva et al., 2002). Regasini et al. (2009) showed that the essential oil of *P. arboreum* is active against *Trypanosoma cruzi* protozoa and the fungal species, *Cladosporium sphaerospermum*, *C. cladosporioides*, *Candida krusei*, *C. parapsilosis*, and *Cryptococcus neoformans*.

Dermatophytosis represents the fourth cause of disease with global incidence estimated at 20 to 25% within the healthy population (Hay et al., 2014; Rouzaud et al., 2018; White et al., 2014; Yousefian et al., 2024). The high prevalence of dermatophytosis is related to the fact that the fungi are more resistant to the host's innate defense and thus can remain as a residual infection that can be lifelong if not treated correctly (Lahmer et al., 2024; Worek et al., 2014). The fungal species that cause dermatophytosis belong to three genera: *Epidermophyton*, *Trichophyton* and *Microsoprum* (Rinaldi, 2000). *T. rubrum* is the most common dermatophyte and causes dermatophytoses, such as a tine pedis and tinea corporis (Dalla Lana et al., 2016). Imidazoles, butenafine, and terbinafine are among the antifungal drugs used for the topical treatment of dermatophytosis (Baghi et al., 2016; Watanabe, 1999), while triazoles, griseofulvin, and terbinafine are used as oral systemic therapies for severe dermatophytosis (Lesher Jr, 1999; Rani et al., 2013). However, the toxicity and interactions associated with these drugs, the need for long treatment regimens, the rise of fungal resistance, and high treatment costs highlight the need for new, more efficient, and safe antifungal drugs (Bennett et al., 2000; de Pauw, 2000; Olson & Troxell, 2023).

The present study evaluated the antidermatophytic effect and cytotoxicity of *P. arboreum* leaf extract and fractions and investigated the possible mechanisms of action.

2. Materials and methods

2.1. Plant material

Leaves from *P. arboreum* were collected from 01/2016 to 06/2017 in Diamantina do Norte, Paraná, Brazil (latitude 18°14'17" S and longitude 43°36'40" W). The plant was identified by botanical professor José Tadeu Weidlich Motta and his exsiccata were deposited in the herbarium of the Botanical Museum of Curitiba. The aerial parts of *P. arboreum* were collected at the Caiuá Ecological Station, Diamond of the North-PR and identified by professor Mariza Barion Romagnolo of the Biology Department/Riparian Vegetation Laboratory/Nupelia and its exsiccata deposited at the State University of Maringá Herbarium (HUEM 15942). After collection, *Piper* leaves were weighed and dried in an air oven circulating (QUIMIS®, model Q-31), at a temperature of 36 °C. After drying, the plant samples were crushed in a knife mill (Tecnal Marconi® model TE 048), packaged, and stored in a dry place and protected from light.

2.2. Preparation and fractionation of P. arboreum extract

The leaves of *P. arboreum* were dried in a circulating air oven and ground in a knife mill, after which they were extracted using Soxhlet and dichloromethane as a solvent over 8 h to obtain the extract. A part of the extract was lyophilized and stored at –10 °C until use, while the other part was submitted to column chromatography on silica gel 60 (0.063-0.2 mm, Macherey-Nagel) and submitted to fractionation with solvents with different degrees of polarity to give six different fractions: hexane, hexane/dichloromethane 80:20, hexane/dichloromethane 50:50, dichloromethane, dichloromethane/ethyl acetate 50:50 and methanol (Achenbach et al., 1987; Chauret et al., 1996; Obici et al., 2008).

2.3. Fungal strains and growth conditions

T. rubrum ATCC 28189, *T. mentagrophytes* ATCC 11480, and *Microsporum gypseum* ATCC 14683 were cultured at 28 °C in Sabouraud dextrose agar tubes for around 25 days. *C. albicans* ATCC 10231, *C. parapsilosis* ATCC 22019, and *C. tropicalis* ATCC 28707 were grown in Sabouraud dextrose broth at 37 °C and maintained on Sabouraud dextrose agar at 4 °C. Before the assays, spores were collected in sterile saline and suspensions were adjusted to 1.0×10^5 spores/ml.

2.4. Antifungal assays

The minimal inhibitory concentration (MIC) of the extract and its fractions against fungal strains was determined according to the M38-A2 broth microdilution reference procedure of the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute-CLSI, 2008a, 2008b). Fungal inocula of 0.4×10^4 to 5×10^4 conidia/ml were prepared in RPMI 1640 medium with L-glutamine and without bicarbonate and buffered with 0.165 M 3-(morpholino) propanesulfonic acid (MOPS). Serial two-fold dilutions of the extract and fractions were performed in 96-well microdilution plates containing 100 µl of RPMI medium. After this, inoculum (5 µl) was added to each well and the plates were incubated at 28 °C for 72 h. The MIC was defined as the lowest concentration that resulted in inhibition of visual growth.

2.5. Checkerboard assay

The checkerboard test, using the microdilution method, was performed to evaluate the in vitro interaction of the antifungal fluconazole (FLU) or nystatin (NYS) with *P. arboreum* extract against *T. rubrum*. The fractional inhibitory concentration (FIC) index was determined, which is defined as the sum of the MIC of each drug in combination, divided by the MIC of the drug used alone. An FIC index of ≤ 0.5 is considered synergism, > 4 is antagonism, and > 0.5 but ≤ 4 is indifferent (Odds, 2003).

2.6. Effect on the invasion of nails

Distal fragments of normal human fingernails were collected from a healthy volunteer who was not receiving antifungal therapy (Macura et al., 2003). Nail fragments were cut into pieces approximately 2 mm × 2 mm in diameter and autoclaved at 121 °C for 15 min. The nail pieces were then saturated with the extract and fractions at different concentrations for 1 h in test tubes. After this, the surface of the nail pieces was inoculated with 50 µl of spore suspension, which were then incubated in a humidified atmosphere at 28 °C for 7–14 days.

Scanning and transmission electron microscopy (SEM and TEM) were used to assess nail invasion. For SEM analysis, the nail pieces were prefixed in a solution of 2.5% sodium glutaraldehyde and 0.1 M sodium cacodylate buffer, dehydrated in increasing ethanol concentrations, and critical point dried. The samples were then coated

with gold and analyzed on a Quanta™ 250 scanning electron microscope (FEI Company, Hillsboro, OR, USA).

For TEM analysis, the nail pieces were washed with PBS and fixed in a solution of 2.5% sodium glutaraldehyde and 0.1% sodium cacodylate buffer then postfixed in 1% osmium tetroxide (OsO_4), 0.8% potassium ferrocyanide, and 10 mM calcium chloride ($CaCl_2$) in 0.1 M cacodylate buffer. After this, the samples were dehydrated in increasing acetone concentrations and soaked in EPON resin at 60 °C for 72 h. Ultrafine sections were obtained, stained with uranyl acetate and lead citrate, and then examined on a Quanta ™ 250 transmission electron microscope.

2.7. Cytotoxicity assay

The cytotoxic effect of the *P. arboreum* extract and the dichloromethane fraction was analyzed as described in Benassi-Zanqueta et al. (2018). The tests were performed in triplicate using VERO epithelial cells (ATCC CCL81). In 96-well plates, 5×10^4 cells/well in DMEM supplemented with 10% fetal bovine serum (FBS) were added and the plates were incubated for 24 h at 37 °C and 5% CO_2. Thereafter, the well culture medium was aspirated and different concentrations of extract and fraction were added (1000 to 1.95 µg/ml). After 24 h of incubation at 37 °C and 5% CO_2, the culture medium was removed and the wells were washed twice with PBS. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Ambresco®, USA) was dissolved in 2 ml PBS and then 50 µl was added to all wells except the blank. Plates were incubated at 37 °C and 5% CO_2, protected from light for 4 h, after which the MTT was removed and 150 µl dimethylsulfoxide (DMSO; Ambresco®, USA) was added to each well. Plates were shaken and the reading was performed on an Asys Expert Plus Plate Reader (Biochrom, Cambridge, UK) at a wavelength of 550 nm. DMSO was used as a negative control. Data were calculated as the percentage of inhibition, and the concentration for 50% cellular toxicity (CC_{50}) was determined as the concentration that reduces the optical density of the treated cells by 50% relative to the untreated cells.

2.8. High-resolution mass spectrometry (HRMS) analysis

Samples were analyzed by ultra-high-performance liquid chromatography (UHPLC; Nexera X2, Shimadzu, Kyoto, Japan) coupled with HRMS (QTOF Impact II, Bruker, Billerica, MA, USA) equipped with an electrospray ionization source. The capillary voltage was operated in positive ionization mode, set at 4500 V, and with an endplate offset potential of –500 V. The dry gas parameters were set to 8 l/min at 200 °C with a nebulization gas pressure of 4 bar. Data were collected from m/z 50 to 1300 with an acquisition rate of 5 spectra/s, and the ions of interest were selected by auto MS/MS scan fragmentation. C18 column (75 × 2.0 mm i.d.; 1.6 µm Shim-pack XR-ODS III) was used for chromatographic separation. Gradient mixture of solvents A (H_2O) and B (acetonitrile with 0.1% formic acid; v:v) was as follows: 5% B for 0–1 min, 30% B for 1–3 min, 95% B for 3–12 min, maintained at 95% B for 12–15 min, and 5% B 15–17 min, at 40 °C (Chauret et al., 1996; Freixa et al., 2001; Holetz et al., 2002).

2.9. Statistical analysis

All tests were carried out in triplicate, and the data were analyzed through Analysis of Variance (ANOVA). Tukey's test was conducted, and a *p*-value of ≤ 0,05 was considered significant compared with the control group. The statistical analysis was performed with the program Graph-Pad Prism 4, USA.

3. Results and discussion

3.1. MIC Results

The MIC of the *P. arboreum* extract was first determined against various fungal species, *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *T. rubrum*, *T. mentagrophytes*, and *M. gypseum* (Table 1). The best effect was observed against *T. rubrum*, and thus, fractions of the extract were subsequently tested against this species and the other dermatophytes.

Table 1. Minimum inhibitory concentration (µg/ml) of *P. arboreum* extract and fluconazole against dermatophytes and yeasts

Yeasts	*Piper arboreum* extract	Fluconazole
C. albicans ATCC 10231	> 1000	7.8
C. tropicalis ATCC 28707	> 1000	250
C. parapsilosis ATCC 22019	500	1.9
Dermatophytes		
T. rubrum ATCC 28189	62.5	125
T. mentagrophytes ATCC 11480	125	31.25
M. gypseum ATCC 14683	500	1000

The results represent mean values for at least three separate experiments. Standard errors were less than 10%.

3.2. Fractionation and antifungal composition

The *P. arboreum* extract was submitted to fractionation with solvents with different degrees of polarity to give six different fractions: hexane (HE), hexane/dichloromethane 80:20 (HD 80:20), hexane/dichloromethane 50:50 (HD 50:50), dichloromethane (DI), dichloromethane/ethyl acetate 50:50 (DA), and methanol (ME). The extract and fractions showed activity against *T. rubrum*, in particular (Table 2). The DI fraction showed the most effective fungicidal activity (16 µg/ml) against *T. rubrum*, followed by the extract itself, both of which were more effective when compared with those of FLU (MIC of 125 µg/ml) and the other fractions, which had MICs that ranged from 125 to 500 µg/ml.

Table 2. Evaluation of the *P. arboreum* extract and its fractions against dermatophytes

Dermatophytes	Extract	HE	HD (80:20)	HD (50:50)	DI	DA	ME	FLU
T. rubrum ATCC 28189	62.5	250	125	125	16.0	125	500	125
T. mentagrophytes ATCC 11480	125	500	250	250	62.5	500	1000	31.25
M. gypseum ATCC 14683	500	> 1000	> 1000	> 1000	500	62.5	250	1000

HE: hexane, HD: hexane/dichloromethane (80:20), HD: hexane/dichloromethane (50:50), DI: dichloromethane, DA: dichloromethane/ethyl acetate (50:50), ME: methanol, FLU: fluconazole. The results represent mean values for at least three separate experiments. Standard errors were less than 10% of means.

Silva (2004) showed that *P. arboreum* presents pyrrolidine amides, which are considered markers of the species. Monoterpenes and sesquiterpenes were also identified in the essential oils of this species (Durant-Archibold et al., 2018; Navickiene et al., 2006). The pyrrolidine amides present several biological activities, particularly antifungal activity against opportunistic fungi, such as *C. sphaerospermum* (da Silva et al., 2002), *C. albicans*, *C. krusei*, *C. parapsilosis*, and *C. neoformans* (Regasini et al., 2009).

The checkerboard method was used to evaluate the association of the antifungal drugs, FLU and NYS, with the *P. arboreum* extract and the DI fraction against dermatophyte species (Table 3). The FIC indices indicated a synergic interaction between the DI fraction and both antifungal drugs against both *T. rubrum* and *T. mentagrophytes* (values ranging from 0.1 to 0.4). The extract, on the other hand, showed a synergic effect when associated with FLU or NYS against *T. rubrum*, and a indifferent effect against *T. mentagrophytes* (FIC index > 0.5). Previous reports of synergistic combinations of antifungal compounds have not been extensive or encouraging. In a review of combination therapy in systemic mycosis, Polak (1990) discussed the combination of amphotericin B with flucytosine as one of the better-established synergistic combinations of antifungal agents that have been used clinically to treat candidiasis. The combination of an azole with a polyene resulted in conflicting outcomes, depending on the species and the strain tested, and specific antagonism was observed with *C. albicans*. Since the ID fraction concentration effective in vitro is achievable in vivo, the combination of this fraction with FLU or NYS represents an attractive perspective for the development of new management strategies for dermatophytosis.

Table 3. Fractional inhibitory concentration (FIC) indices for the associate of *P. arboreum* extract or dichloromethane (DI) fractionwith fluconazole (FLU) or nystatin (NYS) against *T.rubrum* and *T. mentagrophytes*

Dermatophytes	Extract × FLU	Extract × NYS	DI × FLU	DI × NYS
T. rubrum ATCC 28189	0.270	0.344	0.155	0.209
T. mentagrophytes ATCC 11480	0.625	0.500	0.376	0.326

The results represent mean values for at least three separate experiments. Standard errors were less than 10% of means.

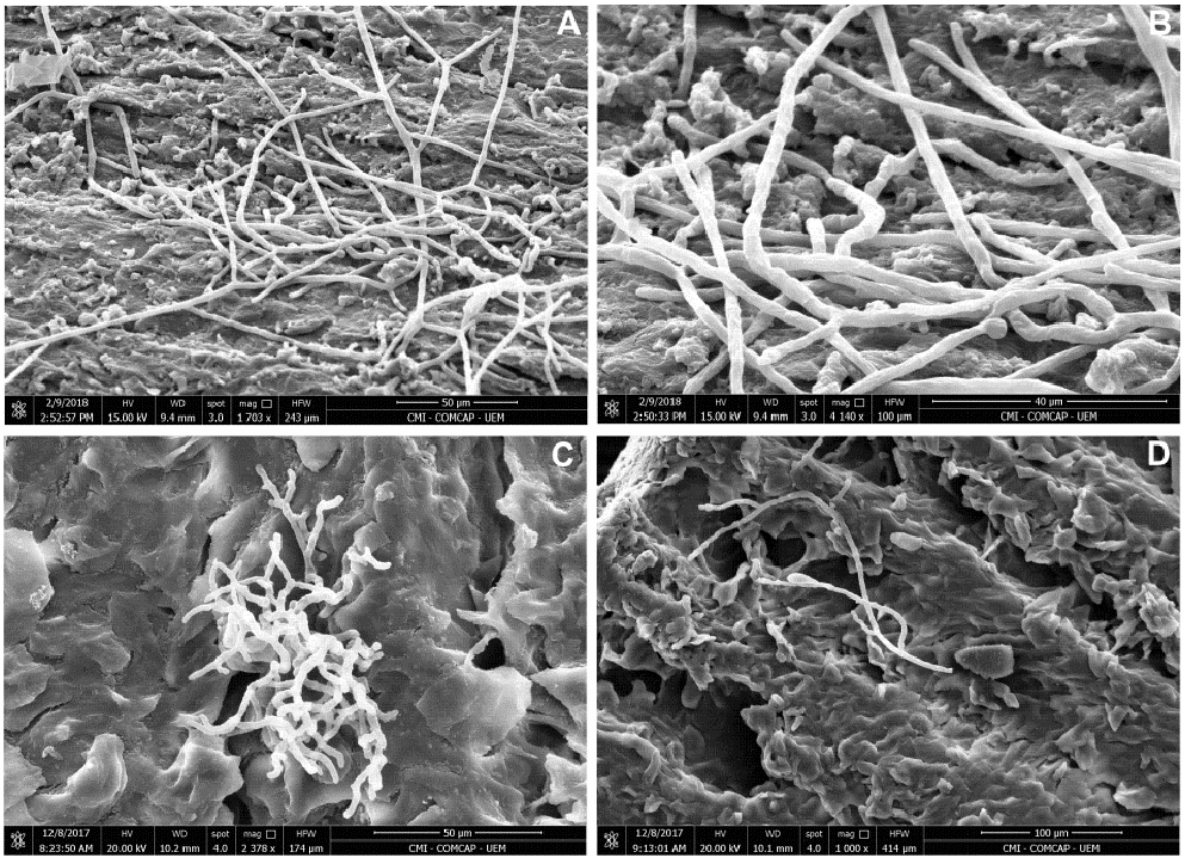

Figure 1. Scanning electron microscopy
A) and B) control cells of T. rubrum. C) *T. rubrum* treated with *P. arboreum* extract at 64 µg/ml. D) *T. rubrum* treated with the dichloromethane fraction at 32 µg/ml.

For topical treatment of dermatophytosis to be successful, it must be able to penetrate the nail plate, which is a thick barrier with a compact structure (Andrade et al., 1996; Butler et al., 2024; Walters et al., 1981). The nail pieces were saturated with *P. arboreum* extract and dichloromethane fraction and then inoculated with a *T. rubrum* spore suspension. Strong inhibition of hyphal growth was observed for cells pretreated with both the extract and the DI fraction (Figures 1C and 1D, respectively).

T. rubrum treated with 64 and 32 µg/ml of the extract and 16 µg/ml of the DI fraction presented short hyphae and irregular growth. In TEM, control cells had intact membranes and cell walls, with dense cytoplasm (Figure 2A). After treatment with 64 and 32 µg/ml of extract and 16 µg/ml of DI fraction (Figure 2B, 2C and 2D), structural changes were visible including alterations in the space between the cell wall and membrane, damage to the cell wall and cytoplasmic membrane, changes to the cytoplasm, and the destruction of organelles. The changes could lead to fungal growth inhibition. Similar structural changes were observed on nails that had not been exposed to extract or fractions that had intact membranes and cell walls, with dense cytoplasm (Figure 2A). Structural changes were visible for cells on pretreated nails (64 and 32 µg/ml of the extract and 16 µg/ml of the DI fraction (Figures 2B, 2C, and 2D, respectively), which included alterations in the space between the cell wall

and membrane, damages to the cell wall and cytoplasmic membrane, changes to the cytoplasm, and the destruction of organelles. These changes could lead to fungal growth inhibition. Similar structural changes were observed by Ridzuan et al. (2018) for *T. rubrum* treated with hydroxychavicol, a phenolic compound of betel leaf (*Piper betle*), and miconazole. Growth inhibition and short hyphae were also observed in *T. rubrum* after treatment with natural compounds, such as copaiba oil and copalic acid (Nakamura et al., 2017).

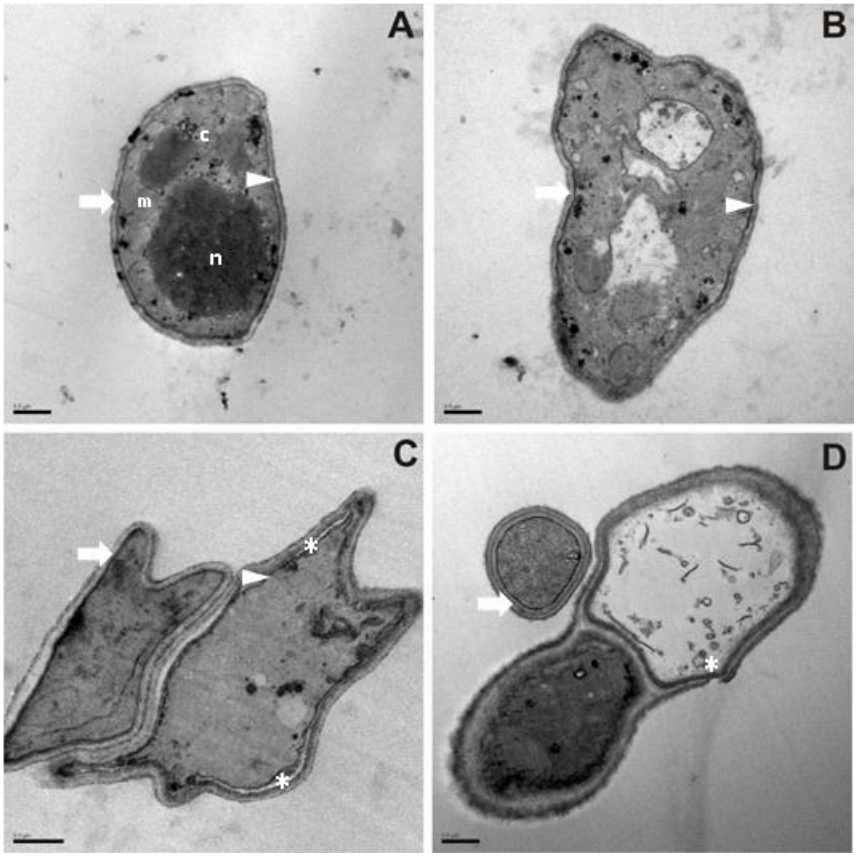

Figure 2. Transmission electron microscopy

A) Control cells of *T. rubrum*. B) *T. rubrum* treated with *P. arboreum* extract at 64 µg/mL. C) *T. rubrum* treated with *P. arboreum* extract at 32 µg/mL. D) *T. rubrum* treated with the dichloromethane fraction at 16µg/mL. In the micrographs, the following structures are indicated: nucleus (n); mitochondria (m); cytoplasm (c); white arrow: cell wall; white arrowhead: cytoplasmic membrane; membrane and cell wall damage (*).

Dermatophytosis is a superficial fungal infection with a worldwide morbidity rate of approximately 20%, which is caused by more than 30 species belonging to three main genera (*Epidermophyton*, *Microsporum*, and *Trichophyton*) (Gupta et al., 2024; Jones et al., 1973; Seebacher et al., 2008). According to a study carried out at the Gregorio Marañón hospital in Madrid, Spain, the most common species that caused dermatophytosis were *T. rubrum* (60%), *T. mentagrophytes* (21%), and *M. canis* (10%) (Rupérez et al., 2013), which emphasizes the importance of new treatments against these fungi (Mayorga et al., 2016).

Plants have a significant role in the development of new drugs to combat microbial infections. The hydroethanolic extract of the leaves of *P. regnellii* was previously shown to have antimicrobial activity against the bacteria *Staphylococcus aureus* and *Bacillus subtilis* and the yeasts *C. krusei* and *C. tropicalis* (Holetz et al., 2002). Recently, Nascimento et al. (2015) isolated amide piperetine from the roots of *P. arboreum* and evaluated the activity of extracts, fractions, and essential oils against *S. aureus*, *M. gypseum*, and *Epidermophyton floccosum*, showing potent antibacterial and antifungal activity.

The potential cytotoxic effects of the *P. arboreum* extract and the DI fraction were tested on VERO cells; both of which showed CC_{50} values higher than the values of the MIC values, indicating that the extract and DI fraction were more toxic to the fungal cells than to the VERO cells (Table 4).

Table 4. Cytotoxicity test of the *P. arboreum* extract and the dichloromethane (DI) fraction in VERO cells, as assessed by the MTT method

Dermatophytes	CC_{50} (µg/ml)
P. arboreum extract	148.33 ± 20.21
DI fraction	91.67 ± 2.89

The results represent mean values for at least three separate experiments. Standard errors were less than 10% of means.

3.3. Chemical composition

iHRMS ionization processes are more robust and allow the construction of hypotheses based on literature data, having a remarkable level of confidence since the purification of chemical compounds are high-cost process often not feasible to Three compounds were identified in the crude *P. arboreum* extract and the DI fraction analyzed by UHPLC-HRMS/MS (positive ion mode). The identification of these compounds was proposed from a review of the genus *Piper*, in addition to the mass error value. Only molecular formulas with ≤ 5 ppm of error were considered in this study (Brenton & Godfrey,

2010).These compounds were putatively identified as the alkaloids, *n*-[10-(13,14-methylenedioxyphenyl)-7*(E)*-pentanoyl]-pyrrolidine ($C_{16}H_{17}NO_3$[M+H]), 4-butoxy-N-(furan-2-ylmethyl)benzamide ($C_{16}H_{19}NO_3$ [M+H]), and arboreumine ($C_{25}H_{40}N_2O_5$ [M+K]). The identification was established based on the fragmentation pattern of the main ion by MS/MS analysis, and compared to those already described in the literature (Table 5). In a previous paper, Navickiene et al. (2000) described various amides bearing isobutyl, pyrrolidine, dihydropyridone, and piperidine moieties that have been isolated from Piperaceae species. The amides isolated from stems of *P. hispidum* and from seeds of *P. tuberculatum* were active against the fungus *Cladosporium sphaerospermun* as evaluated by direct bioautography.

Table 5. Constituents of the *P. arboreum* extract and the dichloromethane fraction (Data of the compounds identified by UHPLC-HRMS/MS)

Molecular formula	Exact mass (*m/z*)	Precursor ion (*m/z*)	Mass error (ppm)	t_R/min
$C_{16}H_{17}NO_3$ (M+H)	272.1281	272.1277	1.47	4.76
$C_{16}H_{19}NO_3$ (M+H)	274.1437	274.1431	2.19	4.78
$C_{25}H_{40}N_2O_5$ (M+K)	487.2568	487.2575	1.44	5.50

t_R: Retention time. The results represent mean values for at least three separate experiments. Standard errors were less than 10% of means.

4. Conclusions

In the present study, the *P. arboreum* extract and its dichloromethane fraction demonstrated antifungal activity against dermatophytes. Furthermore, three different alkaloid compounds were identified. The cytotoxicity was specific towards the fungal cells, and morphological and ultrastructural analyses indicated damage to the cell wall and cytoplasmic membrane as the potential mechanism of action. For topical treatment of dermatophytosis to be successful, it must be able to penetrate the nail plate. The nail pieces saturated with the *P. arboreum* extract and dichloromethane fraction and then inoculated with a *T. rubrum* spore suspension show a strong inhibition of hyphal growth was strongly inhibited on nails pretreated with both the extract and DI fraction. In terms of conservation, the results showed that leaf material could be useful for antifungal uses, and it could be used without any detrimental effect on the plant. However, extracts may be derived from whole plants or specific parts of plants such as leaves, stems, barks, roots, flowers, and/or fruits, and may be either total extracts or selective extracts. Each extract may contain hundreds of different compounds with complexity providing its challenges in terms of both batch variability and defining mechanisms of action. We suggest more conclusive studies to evaluate these biological components.

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Conflict of interest

The authors confirm that there are no known conflicts of interest.

Statement of ethics

In this study, no method requiring the permission of the "Ethics Committee" was used.

Availability of data and materials

All data generated or analyzed during this study are included in this published article. On request, the associated author can provide more information.

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Supplementary File

None.

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RESEARCH ARTICLE

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Ameliorative effect of zingerone on cadmium-induced nephrotoxicity in adult wistar rats

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ABSTRACT

Exposure to heavy metals like cadmium has been reported to cause severe kidney damage through oxidative stress and inflammation. Zingerone is a bioactive compound present in ginger, it contains significant anti-oxidative and anti-inflammatory properties. This study aims to investigate the anti-oxidative and therapeutic role of zingerone on cadmium-induced nephrotoxicity. Thirty (30) adult male rats were divided into 6 groups (A-F) of 5 rats each (n = 5) randomly [A: normal control (normal saline), B: cadmium-exposed (5 mg/kg of cadmium only), C: zingerone-alone, D-F: 5 mg/kg of cadmium + 50 mg/kg, 100mg/kg, 200 mg/kg of zingerone, respectively]. Nephrotoxicity was induced by oral administration of cadmium chloride ($CdCl_2$), followed by zingerone treatment orally. Renal function markers (serum creatinine and urea level), oxidative stress markers (superoxide dismutases, catalase, malondialdehyde), and histopathological investigations of the kidney were assessed to evaluate the effects. Cadmium administration resulted in significant renal dysfunction, characterized by elevated serum creatinine, urea, and kidney malondialdehyde levels, along with reduced antioxidant enzyme activities (superoxide dismutase and catalase). Histopathological evaluation showed extensive kidney damage characterized by renal tubular damage, necrosis, and inflammation. Zingerone treatment significantly ameliorated these alterations, restoring renal function markers, reducing oxidative stress, and improving the histological architecture of the kidney. These findings suggest that zingerone exerts an anti-oxidative and therapeutic effect against cadmium-induced nephrotoxicity. According to these findings, zingerone shows potential as a therapeutic approach for kidney impairment caused by exposure to heavy metals.

1. Introduction

The kidney is usually a major target of harmful destruction from exposure to chemicals, drugs, and other toxicants, it is a vital organ required to perform several essential functions, such as detoxification, homeostasis, excretion of dangerous compounds, and regulation of extracellular fluids (Augustine et al., 2023; Finn & Porter, 2003; Stevens et al., 2006). Nephrotoxicity is the term used to describe the quick decline in kidney function due to the harmful effects of drugs and chemical exposure. Exposure to heavy metal and accumulation of heavy metal in the body can trigger toxicity to the kidney and result in the decline of several functions of the kidney (Al-Naimi et al., 2019).

Cadmium (Cd) is a toxic heavy metal, reported to be highly dangerous to human health and is, therefore, a serious public health problem (Fatima et al., 2019). In the surroundings, Cd is omnipresent. The primary contamination causes are ambient air, cigarette smoke, welding, and

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polluted food and beverages (Chargui et al., 2011; Munisamy et al., 2013). Its use as a corrosive reagent in industry, and as a stabilizer in color pigments, PVC products and Ni-Cd batteries are continuous sources of Cd (Genchi et al., 2020). House dust is a possible source of Cd exposure in locations with polluted soils (Hogervorst et al., 2007). The metallothioneins (MTs) family of ubiquitous small cysteine-rich proteins, whose specific role is to regulate zinc metabolism, protect humans from long-term exposure to low amounts of Cd (Peana et al., 2022). MTs are crucial for preventing DNA damage, oxidative stress, and ion toxicity from various heavy metals (Matović et al., 2015; Peana et al., 2022). If the body absorbs more Cd than MTs, the metal can accumulate, making the situation even more difficult. This buildup will mostly occur in the kidneys (30%) and liver (30%), with the remaining Cd being distributed across the other organs and has an exceptionally long half-life of 10–30 years (Bernhoft, 2013).

Cadmium is absorbed in the body and accumulates in the kidney and it triggers oxidative stress by generating free radicals. The accumulation of these free radicals will lead to cellular damage to the kidney. The oxidative stress and cellular damage pathways lead to inflammation in the renal tubules, tubular damage, and distortion of the glomeruli, which will result in diminished renal functions (Das & Al-Naemi, 2019; Salama et al., 2019). Cadmium-induced oxidative stress is linked to DNA damage, protein alteration, and lipid peroxidation. Among other illnesses, exposure can result in neurotoxicity, liver damage, and kidney problems (Emeka et al., 2023; Kim et al., 2018; Salama et al., 2019). Research indicates that the widespread low environmental exposure to Cd that now exists in industrialized nations may hurt people's kidneys and bones (Bernard, 2008). Various investigations have revealed that oxidative pressure, inflammation, and apoptosis may be the mechanisms of Cd-induced damage to the kidney and other organs (Alibakhshi et al., 2018; Wachira et al., 2019). Oxidative stress and the generation of reactive oxygen species (ROS) are caused by Cd and are often counteracted by enzymatic and non-enzymatic anti-oxidative barriers (Alibakhshi et al., 2018; Jamakala & Rani, 2014).

Scientific research is increasingly embracing the use of medicinal plants and herbs like ginger, *Curcuma longa* (Akinyemi et al., 2018), *Ficus exasperata* (Oviosun et al., 2023), and garlic (Anusuya et al., 2013) in curbing renal damage caused by toxins (Ekor, 2014). Ginger (*Zingiber officinale* Roscoe, family: Zingiberaceae) is the underground rhizome of the ginger plant (Ahmad et al., 2015; Emeka et al., 2023). Zingerone, also known as vanilla acetone, is found in ginger at about 9.25% (Ahmad et al., 2015). Zingerone is primarily present in dry ginger, but cooking or drying also converts gingerol (another component in ginger) into zingerone by retro aldol reaction (Zhang, 2012). According to reports, zingerone has antioxidant, anti-inflammatory, anti-obesity, oxidative stress antagonist, anti-emetic, anti-diuretic, and anti-nausea properties during chemotherapy (Ahmad et al., 2015; Kumar et al., 2014; Mashhadi et al., 2013).

Zingerone, an antioxidant found in ginger, can prevent kidney damage by decreasing oxidative stress triggered by an imbalance of ROS. ROS, such as superoxide, anions, hydrogen peroxide, and hydroxyl radicals, can harm renal cells, lipids, proteins, and DNA. They can also cause inflammation by activating signaling pathways (Alibakhshi et al., 2018; Türk et al., 2022). Antioxidants-rich bioactive compounds like zingerone can mitigate oxidative stress, inflammation enhanced mitochondrial function, and protect the kidney (Alibakhshi et al., 2018; Kandemir et al., 2019; Türk et al., 2022).

This study explores the ameliorative potential of zingerone, a bioactive compound derived from ginger, in mitigating Cd-induced renal toxicity in rats. Despite extensive research into Cd's nephrotoxic effects and various antioxidants as therapeutic agents, the role of zingerone is greatly underexplored. By investigating its antioxidant mechanisms, this study provides novel insights into various dose-dependent zingerone's efficacy and pathways, contributing to the development of safer and natural therapeutic strategies for heavy metal-induced nephrotoxicity. This study evaluated the ameliorative effect of various doses of zingerone on Cd-induced kidney toxicity.

2. Materials and methods

2.1. Experimental animals

Thirty (30) adult rats (10-12 weeks old) weighing between 180-200 g, were purchased from the animal farm of the University of Nigeria, Nsukka. The rats were kept in a well-ventilated standard animal cage in the Department of Anatomy, University of Nigeria in March 2022. The experimental rats were acclimatized for 2 weeks in standard laboratory conditions, with 37 ˚C temperature and, a 12-hour light/dark cycle, and they were allowed unrestricted access to water and food.

2.2. Chemical procurement

We purchased standard analytic products of cadmium ($CdCl_2$) (CAS No: 7440-439) and zingerone (CAS No: 122-48-5, with purity ≥ 98%) from Sigma-Aldrich USA.

2.3. Preparation of zingerone and cadmium solution

Zingerone and $CdCl_2$ were dissolved in normal saline solution and administered daily. Zingerone was administered at varying doses of 50 mg/kg, 100 mg/kg, and 200 mg/kg (Alam, 2018; Alibakhshi et al., 2018; Amin et al., 2021; Safhi, 2015). $CdCl_2$ was administered at a dose of 5 mg/kg (Vijaya et al., 2020). Both $CdCl_2$ and zingerone were administered orally using an oral cannula.

2.4. Exprimental design

Six groups of five (5) rats each were formed by dividing the rats according to their weight. The sample size was calculated following the ARRIVE guidelines and using the resource equation method. As shown in Table 1, group A received normal saline daily for 21 days as the normal control group. Group B served as the negative control group which received 5mg/kg of Cd for 7 days, group C received 100 mg/kg of zingerone only, groups D, E, and F were treated with 5 mg/kg of Cd for 7 days and treated with 50 mg/kg, 100 mg/kg, 200 mg/kg of zingerone, respectively for (14) fourteen days (Alam, 2018; Alibakhshi et al., 2018; Amin et al., 2021; Safhi, 2015; Vijaya et al., 2020).

2.5 Animal sacrifice, blood collection, and tissue collection

Under mild anesthesia using chloroform, the animals were sacrificed by cervical dislocation twenty-four hours after their last treatment. A midline surgical incision was made in the abdominal cavity to expose the abdominal viscera. The right and left kidneys were isolated and excised. The excised kidneys were rinsed in distilled water and preserved in 10% formal saline solution before histological examination. Blood samples were obtained through retro-orbital puncture using a capillary tube. Using capillary tubes we collected blood retro-orbitally, this was done before using chloroform for mild anesthesia to enable us to collect samples that would be free from any possible contamination. The blood sample was sent to the

laboratory for a standard biochemical analysis of a kidney function test (Augustine et al., 2023; Oviosun et al., 2023).

2.6. Biochemical analysis (oxidative stress markers- SOD, MDA, CAT)

For biochemical analysis of SOD, MDA, and CAT activity, harvested kidney samples were frozen immediately by placing them on ice (−70 ˚C) and thereafter homogenized. The level of SOD in the kidney homogenate was calculated using the method of Bannister and Calabrese (1987) as reported by Ceretta et al. (2012). CAT levels in the kidney were determined by the methods of Aebi (1984) as previously described by Ceretta et al. (2012). The lipid peroxidation was measured in the homogenate from kidney by quantifying the level of MDA formed by 2-thiobarbituric acid (TBA) reaction as thiobarbituric acid reactive substances (TBARS) using the method of Conti et al. (1991) previously reported by (Chiş et al., 2016; Sevastre-Berghian et al., 2017).

Table 1. Experimental grouping and design

Experimental group	Number of rats	Administration	Treatment
A	5	Normal saline	Normal saline (5ml) for 21 days
B	5	5 mg/kg $CdCl_2$ only	5 mg/kg of $CdCl_2$ for 7 days
C	5	100 mg/kg of zingerone only	100 mg/kg of zingerone only for 21 days
D	5	5 mg/kg $CdCl_2$ +50 mg/kg of zingerone	5 mg/kg $CdCl_2$ for 7 days + 50 mg/kg of zingerone for 14 days
E	5	5 mg/kg $CdCl_2$ +100 mg/kg of zingerone	5 mg/kg $CdCl_2$ for 7 days + 100 mg/kg of zingerone for 14 days
F	5	5 mg/kg $CdCl_2$ +200 mg/kg of zingerone	5 mg/kg $CdCl_2$ for 7 days + 200 mg/kg of zingerone for 14 days

2.7 Kidney function test (serum urea and serum creatinine level)

The blood samples were allowed to clot for about 2-3 hours and centrifuged to obtain serum. Serum creatinine and urea levels were measured using standard laboratory procedures and measurements were recorded as mg/dl. The method outlined by Kandemir et al. (2019) was used to measure the levels of urea and creatinine in the serum.

2.8 Histological evaluation

The kidney was fixed with 10% phosphate-buffered formaldehyde for 24 hours and subjected to routine histological investigation using standard procedures according to Spencer et al. (2012). The tissue was then subjected to the routine method of fixation, dehydration, clearing, filtration, and embedding. Embedded tissue was sectioned at 5 microns using a rotatory microtome to obtain tissue ribbon sections, stained with routine hematoxylin and eosin, and examined with the aid of an Olympus binocular light microscope (Olympus USA).

2.9. Data analysis

GraphPad Prism version 8.01 for Windows (GraphPad Software, USA) was used to analyze data obtained from this study. One-way analysis of variance (ANOVA) was used to compare the differences between groups followed by Tukey's Post Hoc test. Values were presented as mean ± standard Error (SEM) and $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. The effect of zingerone on serum creatinine and urea level

Figure 1 and Figure 2 show the effect of zingerone on serum creatinine and urea levels respectively, with values expressed as mean ± SEM.

The result shows that there was a statistically significant elevation in the values of serum creatinine and urea levels in group B (administered with 5 mg/kg of Cd only) ($p \leq 0.05$) compared to rats in group A (normal saline). Elevated levels of Cd can accumulate in the body leading to dysfunction of the mitochondria and ROS production. Cd toxicity may lead to damage to various organs through mechanisms like oxidative stress and inflammation (Das & Al-Naemi, 2019). The accumulation of Cd within the body system triggers toxic effects and damage to the kidney function and structure (Kim et al., 2018; Lee et al., 2014; Vukićević, 2012). The result of this study showed that there was a remarkable elevation in the values of serum creatinine and urea level in rats exposed to only Cd, this findings are in concurrence with previous reports which reported that Cd toxicity can relatively affect the kidney and lead to elevation of serum creatinine and urea (Kim et al., 2018; Lee et al., 2014; Shati, 2011). The level of serum creatinine and serum urea remarkably reduced in groups C-F and post-treatment groups which received 5 mg/kg of Cd + 50 mg/kg, 100 mg/kg and 200 mg/kg of zingerone compared to the rats in group B ($p \leq 0.05$). This suggests that administration of zingerone to rats in other treatment groups improved renal function, with serum creatinine and urea levels significantly reduced. Various studies reported zingerone's role in regulating kidney function parameters like serum creatinine and urea level (Dawood et al., 2022; Hosseinzadeh et al., 2020).

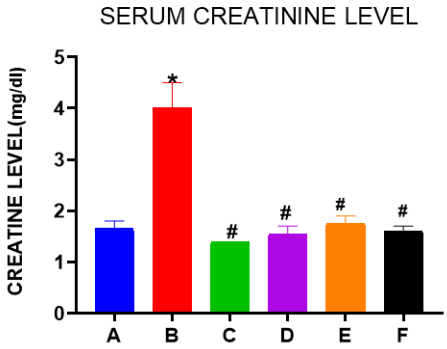

Figure 1. Effect of zingerone on serum creatinine level
Values expressed as mean ± SEM.* indicates significant difference compared with group A at $p \leq 0.05$. # indicates significant difference compared with group B at $p \leq 0.05$

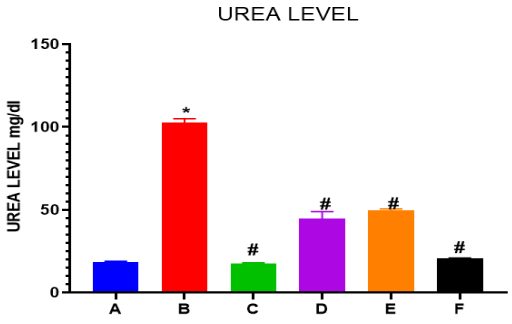

Figure 2. Effect of zingerone on urea level
Values expressed as mean ± SEM.* indicates significant difference compared with group A at $p \leq 0.05$. # indicates significant difference compared with group B at $p \leq 0.05$

3.2. The effect of zingrone on anti-oxidative parameters (SOD, CAT and MDA)

Figures 3-5 show the level of SOD, CAT and MDA. In this study, it was observed that there was a reduction in the level of SOD and CAT in Group B (Cd of 5 mg/kg only) compared to group A. The MDA level in group B was significantly elevated, when statistically compared to group A. Groups C-F treated with various doses of zingerone showed a significant reduction in the level of MDA.

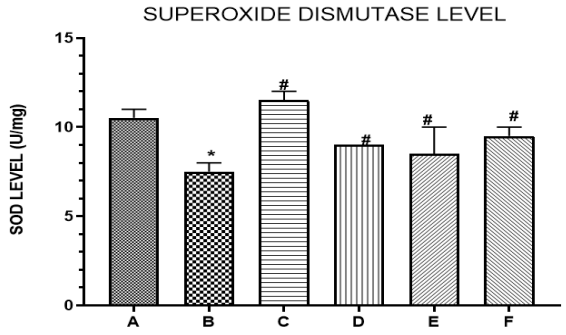

Figure 3. Effect of zingerone on SOD activity
Values expressed as mean ± SEM. * differs significantly compared with group A at $p \leq 0.05$. # differs significantly compared with group B at $p \leq 0.05$

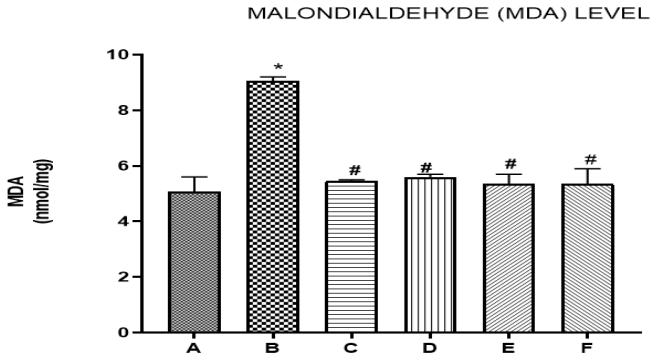

Figure 4. Effect of zingerone on MDA activity
Values expressed as mean ± SEM. * differs significantly compared with group A at $p \leq 0.05$. # differs significantly compared with group B at $p \leq 0.05$

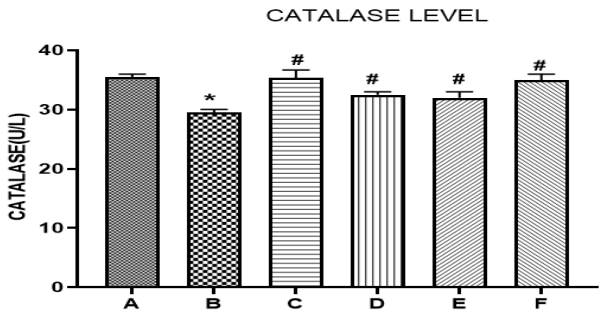

Figure 5. Effect of zingerone on CAT activity
Values expressed as mean ± SEM. * indicate significant difference compared with group A at $p \leq 0.05$. # indicate significant compared with group B at $p \leq 0.05$

Nephrotoxicity arising from Cd exposure is a complex process that has been reported to trigger oxidative stress, inflammation, and cellular damage to the kidney (Onwuka et al., 2011; Yan & Allen, 2021). The significant decrease in SOD, CAT levels and increase in MDA levels observed in rats administered only Cd are consistent with the findings of previous authors who reported that Cd causes oxidative damage as a result of mitochondrial dysfunction, DNA damage, inflammation, decreased activity of antioxidant enzymes and increased lipid peroxidation (Das & Al-Naemi, 2019; Salama et al., 2019). An increase in redox imbalance, DNA damage, and oxidative degradation of proteins and lipids are the primary underlying mechanisms of Cd-related renal impairment (Yan & Allen, 2021). Administration of zingerone in rats treated with zingerone only, post-treated with 50 mg/kg, 100 mg/kg, and 200 mg/kg of zingerone shows a significant increase in antioxidant enzyme activities (SOD, CAT) and decrease lipid peroxidation (MDA). This highlights the zingerone's reportedly strong antioxidant qualities for scavenging free radicals and enhancing tissue structure (Ahmad et al., 2015; Alibakhshi et al., 2018; Kandemir et al., 2019; Mehrzadi et al., 2021).

3.3. The role of zingerone on histological examination of the kidney tissue

Group A (normal control group) showed normal kidney histology with the glomeruli, renal tubules, and cortex clearly shown to be normal. Histological examination of the kidney showed that there were structural changes in the kidney of rats administered with 5mg/kg of Cd only, with histology of the kidney showing tubular necrosis, inflammation degeneration of renal tubules, basal membrane disruption, and congestion of red blood vessels (Figures 6-7). These histological findings of rats treated with Cd only are also linked with the significant increase in the level of serum creatinine, urea, and deregulation of antioxidant enzymes observed. The histological alterations observed in rat's exposure to Cd may also be linked to the accumulation of free radicals in the renal tissue of the rats treated with cadmium (Kandemir et al., 2019; Olubunmi et al., 2017). This observation conforms with previous studies which reported that Cd distorts kidney histology, reduces renal function, and induces oxidative stress (Olubunmi et al., 2017; Onwuka et al., 2011). Group C (treated with zingerone only) showed normal histology of the kidney. Experimental rats in Group C were given only zingerone and rats in the treatment group were treated with 5 mg/kg Cd and 200 mg/kg zingerone. The microanatomical features of the kidneys were found to be very normal with no signs of necrosis or deterioration in the glomeruli and renal tubules. However, in the groups post-treated with low doses (50 mg/kg, 100 mg/kg of zingerone) after Cd administration, there were mild alterations in the histology of the kidney, with signs of cellular improvement in the degeneration compared to the group exposed to Cd only. These observations show that a high dose of zingerone exhibits great potency for restoring the histology of the kidneys of Cd-induced nephrotoxic rats.

According to Kandemir et al. (2019) and Mani et al. (2016), zingerone mitigated drug and chemically induced kidney toxicity, by enhancing the histology of the kidney tissue, reducing serum creatinine, and urea levels, and decreasing oxidative stress. This aligns with the findings of this current study, as our data showed that administration of zingerone greatly improved Cd-induced toxicity by regulating serum levels of creatinine, urea level, and oxidative stress and greatly improving the microanatomy of the kidney. The modulatory effect of zingerone on Cd toxicity was also observed to be dose-dependent, as our result showed that 200 mg/kg of zingerone proved to be more effective.

4. Conclusions

The result from this study revealed that Cd-induced nephrotoxicity was characterized by increased levels of serum creatinine, urea, oxidative stress, and a decrease in renal function. However, zingerone administration showed great potential in modulating Cd-induced renal damage, evidenced by a reduction in serum creatinine, and urea levels, a decrease in oxidation of lipids, and increased activity of antioxidant enzymes in kidney tissue of rats administered

with Cd. Zingerone also restored the histology of the kidneys of Cd-induced nephrotoxic rats. The outcome of our research indicates that zingerone possesses a dose-dependent therapeutic effect against Cd-induced renal toxicity. This highlights the role of zingerone as a promising therapeutic agent in mitigating heavy metal-induced renal damage.

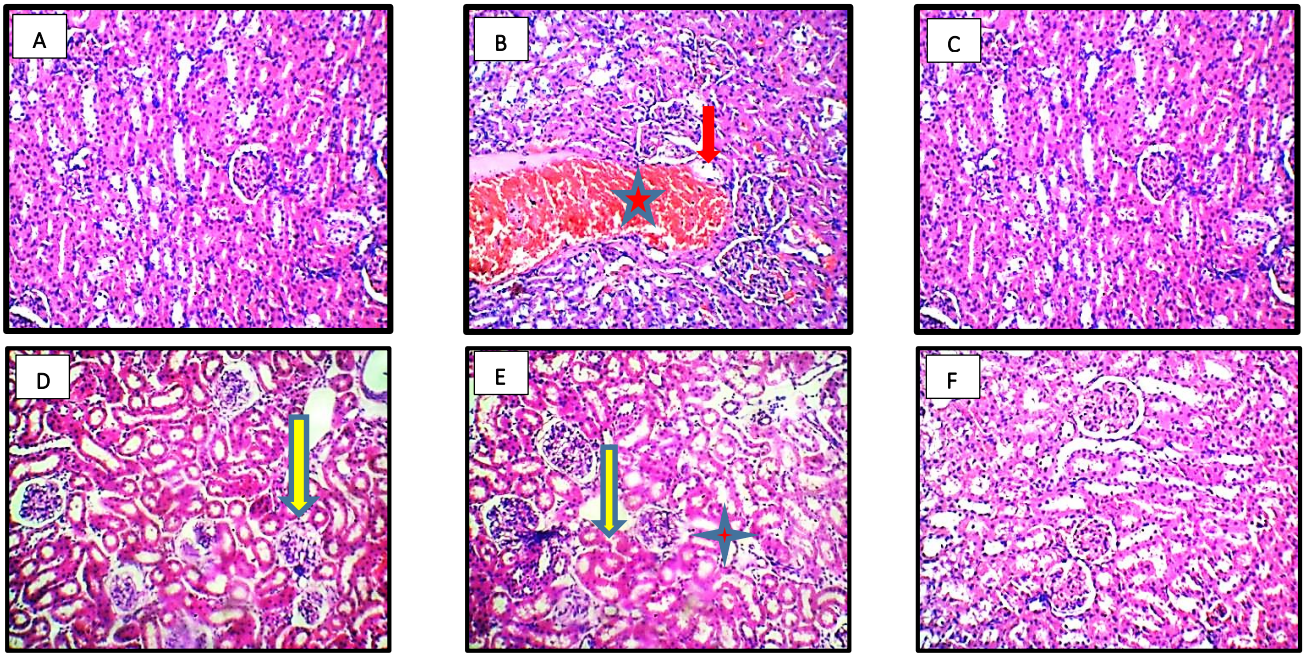

Figure 6. Kidney histology demonstrated by H&E (x 100, scale bar: 100 µm)

The renal tubules, renal cortex, glomeruli and mesangial cells as well as renal parenchyma are clearly seen across the treatment groups. **Group A** showed normal kidney, **group B** animal treated with Cd only showed tubular necrosis, inflamation of the tubules, glomeruli distortion and alterations in renal tubules (indicated by red arrow and star), **group C** showed a normal histology of the kidney, **groups D & E** showed mild strutural alteration in the kidney (indicated by yellow arrow and star symbol), **group F** showed a normal histoloy of the kidney with no signs of necrosis or distortion of the glomeruli and renal tubules.

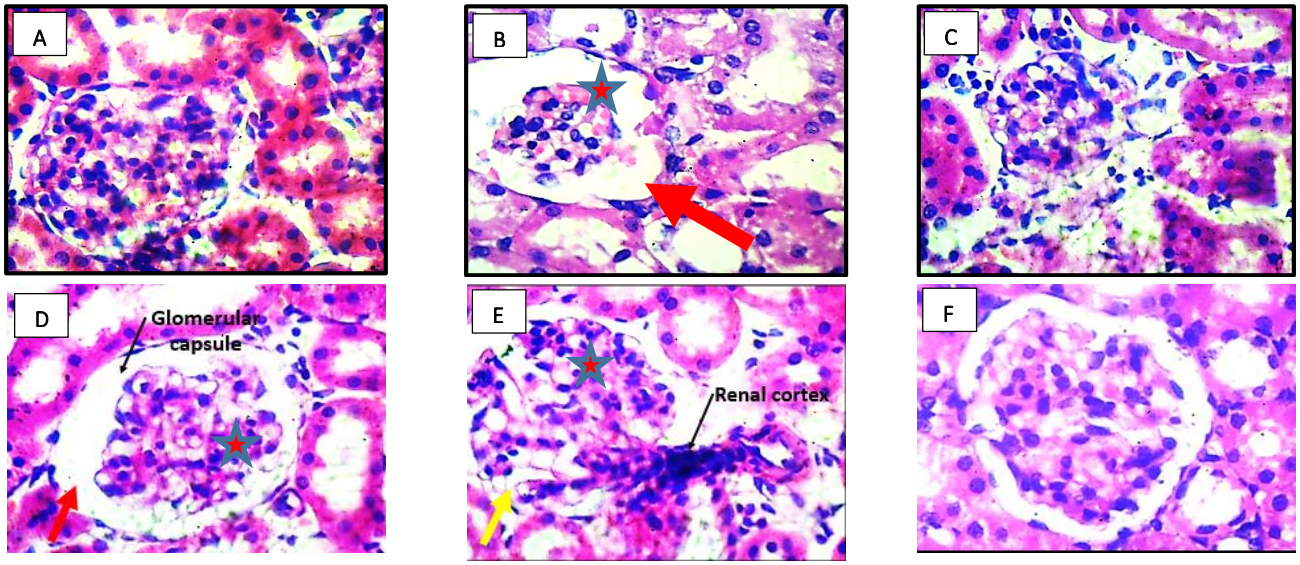

Figure 7. Histology section of kidney (H&E staining at x 400, scale bar: 100µm) showing the cortex,renal tubules, glomeruli, renal parenchymal cells, and mesangial cells across the treatment groups

Group A: The kidney shows normal micro-anatomical features. **Group B:** Animal treated with Cd only showed tubular necrosis,basal membrance disrution, glomeruli damage,inflamation and alterations in renal tubules (indicated by red arrow and star). **Group C:** The kidney appears normal, with normal microanatomical features. **Groups D & E:** Showed mild strutural alteration in the kidney microanatomy (indicated by yellow arrow and star). **Group F:** Showed a normal histology of the kidney with no signs of necrosis or distortion of the glomeruli and renal tubules.

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Conflict of interest

The authors confirm that there are no known conflicts of interest.

Statement of ethics

The current investigation adheres to the procedures established by the Institutional Animal Ethics Committee (IAEC) and the ARRIVE Guideline. Ethical approval for this study was obtained from the Research Ethics Committee of the College of Medicine, University Of Nigeria (2020-NNHREC/05/01/2008-FWA00002458-1RB00002323).

Availability of data and materials

All data generated or analyzed during this study are included in this published article. On request, the associated author can provide more information.

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Godson Emeka Anyanwu: Research design, Supervision, Formal Analysis, Investigation, Methodology
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Supplementary File

None.

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SHORT REPORT

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Extraction of phenolic compounds and antioxidant activity analysis of *Ficus carica* L. seed oil using supercritical fluid technology

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ABSTRACT

The rationale behind this study was to investigate the potential of fig (*Ficus carica* L.) kernel oil as a source of bioactive compounds, particularly focusing on its phenolic compounds, due to the increasing interest in plant-based oils with antioxidant properties for use in functional foods and nutraceuticals. The primary objective was to identify and quantify the active phenolic components present in fig kernel oil. Utilizing an additional co-solvent in the supercritical fluid extraction (SFE) process, specific phenolic compounds, such as 4-hydroxybenzoic acid, 3-hydroxybenzoic acid, and syringic acid, were exclusively identified in the CO_2 + ethanol (IC-2-1) sample. Furthermore, other notable compounds, including vanillin, verbascoside, ferulic acid, luteolin 7-glucoside, hesperidin, rosmarinic acid, quercetin, and kaempferol, were detected in both the IC-2-1 and CO_2 (IC-1-1) samples. These findings suggest that fig kernel oil with its rich phytochemical profile, is a promising alternative oil source and has significant potential as a functional food ingredient. Further research on the SFE of fig seeds and oil is recommended to expand its applications and potential health benefits.

1. Introduction

Fig (*Ficus carica* L.) is a member of the Moraceae family and grows from Turkey to Afghanistan, with around 800 species. It has been cultivated for health and food purposes in temperate climates since ancient times (Abbasi et al., 2013; Barolo et al., 2014). Leading producers include Turkey, Morocco, Egypt, Spain, Greece, California, Italy, and Brazil (Abbasi et al., 2013). Figs are extensively studied for their nutritional value, health benefits, and therapeutic applications, including antipyretic, anti-inflammatory, hepatoprotective, hypoglycemic, anticancer, and antioxidant properties (Badgujar et al., 2014; Joerin et al., 2014; Mawa et al., 2013; Stepek et al., 2004). Furthermore, figs have been found to assist with anxiety, sleeplessness, blurred vision, and appetite loss (Argon et al., 2020).

Traditional medicine uses *F. carica* to treat gastrointestinal and respiratory ailments, as well as for its anti-inflammatory and antispasmodic properties. This has inspired research into its chemical composition, including phytosterols, phenolic compounds, fatty acids, and other secondary metabolites (Baygeldi et al., 2021).

Polyphenols, flavonoids, and anthocyanins possess significant antioxidant capacity. The anthocyanin content found in figs is thought to aid in maintaining healthy blood lipid levels. It plays a crucial role in preventing conditions such as obesity, diabetes, cardiovascular disease, and spe-

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cific types of cancers (Wojdyło et al., 2016).

Additionally, analyses have investigated sterols, phenolics, volatiles, antioxidant capacity, antimicrobial properties, and fatty acids in various parts of the plant, such as latex, fruits, leaves, and roots (Jeong & Lachance, 2001; Mahmoudi et al., 2016).

A study was conducted on fig kernel oil utilizing the cold press extraction method. The analysis revealed that fig kernel oil is abundant in linolenic acid (omega-3, 40.25%), linoleic acid (omega-6, 31.28%), and oleic acid (omega-9, 17.0%). It also contained a small amount of palmitic acid and trace levels of other fats, with no aflatoxin detected. Furthermore, fig kernel oil had a significantly high concentration of gamma-tocopherol (4090.70 ± 383.30 mg/kg) compared to other sources of edible oils (Tarlacı, 2021).

Due to its abundant omega-3 fatty acid content, figs and fig kernel oil play a crucial role in moisturizing and nourishing the skin from within, preserving its elasticity. These sources are also rich in minerals such as potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), and copper (Cu), as well as vitamins A, E, and K, all of which exhibit potent antioxidant properties. Furthermore, the calcium present contributes to the maintenance of bone health (Cihat Icyer et al., 2017).

Studies show its protective effects against colon (Campbell et al., 2003), breast, and prostate cancers, with high serum levels reducing prostate cancer risk by five times (Helzlsouer et al., 2000). Gamma-tocopherol's strong antioxidant activity, particularly against nitrogen radicals, also inhibits colon cancer cell proliferation (Campbell et al., 2003). In contrast, alpha-tocopherol is linked to a reduced risk of bladder cancer (Jiang et al., 2000).

Gamma-tocopherol, unlike alpha-tocopherol, inhibits cyclooxygenase-2 (COX-2), providing anti-inflammatory benefits that may prevent Alzheimer's and atherosclerosis. It also acts as a diuretic, helping to lower blood pressure and reduce pancreatic cell loss, potentially decreasing Type 1 diabetes risk (Bharti et al., 2013; Jiang et al., 2001). Clinical trials show that gamma-tocopherol reduces cholesterol more effectively than alpha-tocopherol (13% vs. 5% in four weeks). Low gamma-tocopherol levels are linked to increased coronary artery disease risk, unlike alpha-tocopherol. Additionally, Swedish individuals with double the gamma-tocopherol levels had a 25% lower cardiovascular mortality rate compared to Lithuanians (Kristenson et al., 1997).

Radical scavenging activity refers to the ability of a substance to neutralize free radicals, which are unstable molecules that can cause oxidative damage to cells and tissues. This activity protects cells and tissues against oxidative damage, reducing chronic disease risks. Natural antioxidants, such as vitamins C and E, flavonoids, and polyphenols, are widely used in food preservation, skincare, and pharmaceuticals as safer alternatives to synthetic options (Devasagayam et al., 2004). The mechanisms of radical scavenging include electron donation, hydrogen atom transfer, the formation of non-radical species, and metal chelation. Various methods are employed to assess radical scavenging activity, including 1,1-diphenyl-2-picrylhydrazyl (DPPH·), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS·+), ferric reducing antioxidant power (FRAP), and oxygen radical absorbance capacity (ORAC) assays. The DPPH· assay is commonly used to evaluate antioxidant activity, where the color of DPPH· changes upon reduction by antioxidants. The ABTS assay involves the reduction of the ABTS·+ radical cation, with the decrease in absorbance measured at 734 nm (Al Mousa et al., 2023; Hassane et al., 2022b; Re et al., 1999).

SFE is an environmentally friendly alternative for triglyceride extraction, successfully applied to seeds such as apricot (Özkal et al., 2005), palm (Zaidul et al., 2007), canola (Dunford & Temelli, 1997; Temelli, 1992), sesame (Namiki et al., 2002; Xu et al., 2005), flax (Bozan & Temelli, 2002), and grape (Beveridge et al., 2005), as well as nuts like walnut (Oliveira et al., 2002; Salgın & Salgın, 2006) and almond (Marrone et al., 1998). Carbon dioxide (CO_2) is the preferred supercritical fluid due to its nontoxicity, low cost, and high purity, widely accepted in the food and pharmaceutical sectors (Taribak et al., 2013).

Since fig kernels are too small to be chewed or eaten, they cannot be used as a useful food source. Due to their minuscule size, fig seeds cannot be efficiently masticated or utilized as a substantial food source. They resist digestion within the gastrointestinal tract and are typically excreted intact in the feces. Germencik, where the fig kernel is taken from, is a district of Aydın Province in the Aegean Region of Turkey. Its geographical coordinates are approximately 37.8739° north latitude and 27.6064° east longitude. The district, which is approximately 25 km from Aydın provincial center, is located on the fertile lands of Western Anatolia. *F. carica*, is the most produced fig variety in Turkey, especially in Aydın region. While cold press and soxhlet extraction are common methods for extracting compounds from kernel figs, SFE is a promising green technology with several advantages; however, no studies have reported on the bioactive compounds in this fig variety of seeds extracted using supercritical fluids. Therefore, the objectives of this study were to analyze the phenolic compounds extracted from *F. carica* seed oil using SFE and to evaluate their antioxidant activity, initiating research on this species as a potential functional food.

2. Materials and methods

2.1. Raw material and sample preparation

Fig seeds were obtained from dried figs supplied from Germencik Organic Oleogustus Gıda Ltd. Şti. in August. These figs were cultivated in the Aydın region. The dried figs were pre-treated by soaking in warm water for 30 minutes, followed by sieving and shade-drying for 72 hours. Two kilograms of fig kernels were subjected to a drying process in an oven (ILD-EKH-120, 1500W, Türkiye) and subsequently, ground using a grain mill (Emir Industrial Kitchen Products, EMR-Ö-01, 1.5 kg, Türkiye). The ground material was then sieved to achieve a particle size of less than 0.30 mm. The resulting ground fig kernel was stored in a dark environment at room temperature until further analysis.

2.2. Supercritical fluid extraction (SFE)

SFE is a technique that uses fluids above their critical temperature and pressure, combining gas and liquid properties to enhance solubility. CO_2 is commonly used as the supercritical fluid. The process involves pressurizing the fluid, directing it into an extraction cell with the sample, and dissolving the target compounds. After extraction, reducing pressure or increasing temperature returns the fluid to a gaseous state, leaving the dissolved compounds behind for collection. SFE is highly efficient, eco-friendly, and widely applied in the food, pharmaceutical, and cosmetic industries to extract oils, essential oils, and bioactive compounds (da Silva et al., 2016; McHugh & Krukonis, 2013).

The SFE system (Polat Extraction Technology, Türkiye) consisted of a CO_2 cylinder, a recirculating chiller, CO_2 and co-solvent pumps, an

extraction vessel, a heat exchanger, a separating vessel, an automated back pressure regulator, and a controlling PLC (Figure 1). In both extractions, 720 g and 719 g of fig kernels were placed in the extractor. Four main parameters—temperature (T, ℃), pressure (P, bar), methanol concentration (MeOH, % cosolvent-solvent ratio), and CO_2 flow rate (qCO_2, g/min) — were varied for the two extractions (Figure 1).

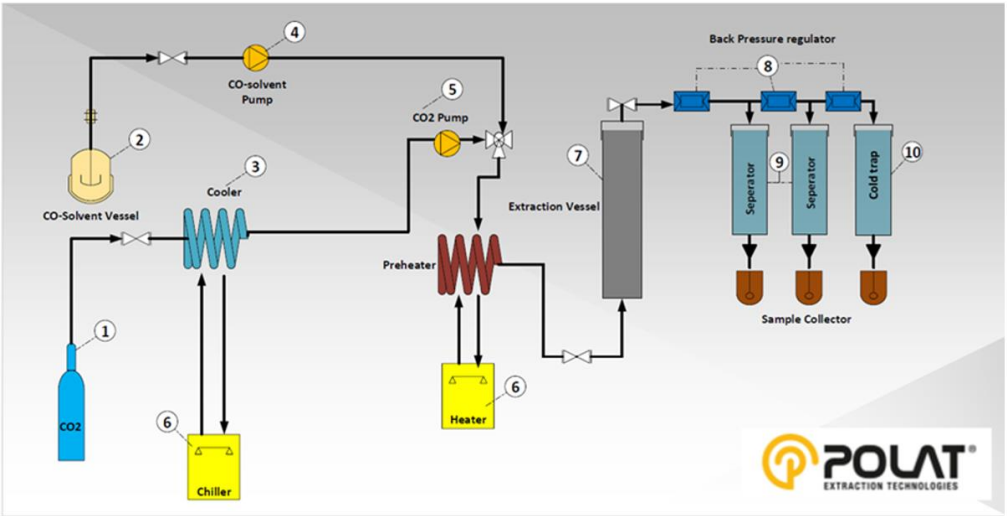

Figure 1. Schematic representation of the Polat supercritical fluid extraction technology
(reproduced with permission from Polat Makina A.S.)

2.3. Supercritical CO_2 (SC-CO_2) extraction of fig seed oil

SC-CO_2 extraction is an advanced and eco-friendly method used to extract fig seed oil, utilizing CO_2 in its supercritical state. In this process, CO_2 is pressurized and heated above its critical point (31 °C and 73 atm), where it behaves as both a liquid and a gas, allowing it to penetrate fig seeds and dissolve oils efficiently.

The purchased fig kernels were stored at 25 °C until extraction. Fig seed oil was obtained by the SC-CO_2 extraction method. The obtained oil was stored at 4 °C until analysis.

2.4. Design of experiments (DoE) and process optimization

DoE is a structured approach for planning and analyzing experiments to understand the impact of various factors on a process. Commonly applied in process optimization, DoE identifies optimal conditions to enhance efficiency, yield, or quality. In SC-CO_2, it aids in fine-tuning parameters like pressure and temperature to maximize oil yield and preserve essential compounds (Antony, 2023).

For this experiment, a laboratory-scale SFE system manufactured by Polat Extraction Technologies was used. Extraction was performed in two processes using CO_2 IC-1-1 and IC-2-1 as solvents. A schematic representation of the extraction process is given in Figure 1. For the extraction process IC-1-1, 729 g of fig kernels were placed in the extractor. It was carried out at 45 °C temperature, 300 bar pressure, CO_2 flow rate of 50 ml/min, and extraction time of 180 minutes. For the extraction process IC-2-1, 728 g fig kernels were placed in the extractor. It was carried out at 45 °C temperature, 300 bar pressure, CO_2 flow rate of 50 ml/min, extraction time of 180 minutes, and co-solvent flow rate of 5 ml/min (methanol). The extract obtained for IC-1-1 was stored at +4 °C for analysis. The solvent of the extract-methanol mixture obtained for IC-2-1 was removed under vacuum, and the remaining fig kernel oil was stored at +4 °C for analysis. Phenolic compounds and antioxidant activity were determined in two different studies (Table 1).

2.5. Analysis of phenolic compounds

Phenolic compounds, known for their antioxidant properties, are analyzed to determine their concentration in plants. Common methods include HPLC and spectrophotometry. This analysis is important for assessing the antioxidant capacity and health benefits of plant extracts, such as fig seed oil (Prior et al., 2005).

Table 1. Supercritical fluid extraction parameters

Sample	Conditions			
	P (bar)	T (°C)	MeOH (%)	qCO_2 (g/min)
IC-1-1	300	45	-	50
IC-2-1	300	45	4	50

Phenolic compounds of fig kernel oil were determined by liquid chromatography-mass spectrometry [LC (Agilent 1260 Infinity)-MS/MS (Agilent 6420 Triple Quadrupole LC-MS/MS)].

LC-MS working principle: In the initial quadrupole filter, molecules are separated based on their mass-to-charge (m/z) ratio and then subjected to fragmentation using a specific high-purity gas known as collision gas. The second quadrupole filter is responsible for the identification and quantification of the ions generated from this fragmentation process. Conditions used for LC-MS analysis include mobile phase (water and organic solvents), column selection (usually reversed phase C18), flow rate (0.2-1.0 ml/min), temperature (25-40 °C), ionization source (ESI or APCI), and mass detector settings (positive/negative mode, MS/MS).

2.6. Radical scavenging activity

Radical scavenging activities of the extracts were determined using DPPH· and ABTS·+ cation radicals (Kocak et al., 2016).

For the evaluation of radical scavenging activity against DPPH·, a sample solution (1 ml) was mixed with 4 ml of a 0.004% methanol solution of DPPH·. The absorbance of the sample was measured at 517 nm after a 30-minute incubation period at room temperature in the dark.

For assessing the scavenging activity against the ABTS·+ cation radical, the ABTS·+ radical cation was generated by reacting a 7 mM ABTS solution with 2.45 mM potassium persulfate and allowing the mixture to incubate in the dark at room temperature for 12-16 hours. Before starting the assay, the ABTS solution was diluted with methanol to achieve an absorbance of 0.700 ± 0.02 at 734 nm. A sample solution (1 ml) was then added to 2 ml of the ABTS solution and mixed thoroughly. The absorbance of the sample was recorded at 734 nm after a 7-minute incubation at room temperature.

2.7. Statistical analysis

The assays were conducted in triplicate using different portions of the samples to ensure accuracy and reproducibility. The descriptive statistical analysis was adopted to calculate the mean and standard deviation, and the results were presented as mean ± SD. Followed by one-way ANOVA (analysis of variance), Tukey's significant difference post hoc test, and Student's *t*-test with α= 0.05 were employed to determine the statistical significance between the species [IBM SPSS Statistics for Windows, version 22.0 (IBM Corp., Armonk, N.Y., USA)].

3. Results and discussion

The oil content and composition of seeds can vary due to factors like seed variety, climate, geographic origin, ripeness, size, pollination, and extraction methods. Given the significant oil yield observed in samples of fig seeds, these seeds could be deemed a promising oil source and potentially marketed as a value-added product (Argon et al., 2020; Hssaini et al., 2020). Since cold pressing is a pressing method rather than an extraction method, not all components can be fully extracted.

One study defined the fixed oil content of dried seeds as 30%, while another study reported that the oil content of four different fig seed varieties from Morocco ranged from 21.54 to 29.65% (Hssaini et al., 2020). Different fig seeds from Türkiye resulted in lower or similar oil yields of 14.08, 18, and 23.67% for different cultivars (Ergun & Bozkurt, 2020).

It is incorrect to compare the fig kernel oil obtained by SFE with the oils obtained by cold pressing, which is the other method. Nevertheless, we can compare our fig kernel results with other studies. The amounts of oil obtained by cold pressing method from different seeds are as follows: blackcurrant (16.5-30.4%), gooseberry (15.6-35.2%), lime (22.1-31.9%), passion fruit (18.5-30.4%), pear (16.3-31.7%), blackcurrant (22.8-30.1%), pumpkin (27.83-45.4%), honeydew (25.0-32.3%), and watermelon (22.1-36.65%) (Alves et al., 2021).

For the first time, fig kernel oil was obtained from two different studies using SFE technology. Fig kernel oil yields were 21% for IC-1-1 and 32% for IC-2-1. SFE technology has many advantages over cold pressing. These are solvent, temperature, selectivity, efficiency, purity, and shelf life. The biggest advantage of SC-CO_2 extraction is that it takes place in a closed system and extracts are not exposed to oxidation.

Supercritical extraction demonstrates superior yield performance compared to cold pressing due to its enhanced solubility and selectivity under controlled conditions. By optimizing parameters such as pressure, temperature, and solvent type, it achieves higher extraction efficiency, particularly for valuable bioactive compounds. Unlike cold pressing, which may leave a significant portion of extractable material behind, supercritical extraction minimizes losses, ensuring maximum recovery (Picot-Allain et al., 2021).

As a food, *F. carica* fruits are consumed fresh or dried, or used as jam. *F. carica* is generally recognized as an excellent source of minerals, vitamins, carbohydrates, and dietary fiber (Veberic et al., 2008). However, as far as is known, the kernel oil of *F. carica* has not been standardized to date. Fig kernels ingested with fruit consumption cannot be broken down in the body and excreted in the feces, and their content cannot be utilized.

The supercritical extract of fig seed oil was subjected to DPPH· and ABTS·+ radical scavenging activity assays, both of which yielded relatively low results. These findings suggest that the antioxidant capacity of the extract is limited (Hassane et al., 2022a). The low radical scavenging activity could be attributed to several factors. One possible explanation is that the supercritical extraction conditions (e.g., temperature, pressure) might not have been optimal for the efficient extraction of phenolic compounds or other bioactive molecules responsible for antioxidant activity **(Table 2)**.

Table 2. Antioxidant activities of the supercritical fluid extract of fig kernels

Assays	IC-1-1	IC-2-1	Trolox
DPPH· scavenging (IC_{50}: mg/ml)	$> 20^b$	$> 20^b$	0.07 ± 0.001^a
ABTS·+ scavenging (IC_{50}: mg/ml)	$> 20^b$	$> 20^b$	0.13 ± 0.002^a

Different superscripts in the same row indicate significant differences ($p < 0.05$).

In the study for the determination of phenolic compounds of fig kernel oil by SFE, additional co-solvent was used, and therefore 4-hydroxybenzoic acid, 3-hydroxybenzoic acid, and syringic acid were determined only for IC-2-1. Vanillin, verbascoside, ferulic acid, luteolin 7-glucoside, hesperidin, rosmarinic acid, quercetin, kaempferol were determined for both IC-2-1 and IC-1-1 studies **(Table 3)**.

Table 3. Analysis of phenolic compounds in the supercritical fluid extract of fig kernels

Compound	RT (min)	Concentration (µg/g extract)	
		IC-1-1	IC-2-1
4-Hydroxybenzoic acid	12.2969	nd	1.19 ± 0.02
3-Hydroxybenzoic acid	12.3972	nd	1.09 ± 0.01
Syringic acid	12.8542	nd	0.27 ± 0.01
Vanillin	13.3116	30.03 ± 0.01^a	14.88 ± 0.05^b
Verbascoside	13.6066	0.20 ± 0.04^a	0.08 ± 0.001^b
Ferulic acid	14.0203	2.26 ± 0.08^b	2.72 ± 0.07^a
Luteolin 7-glucoside	14.3277	0.49 ± 0.01^a	0.04 ± 0.001^b
Hesperidin	14.5005	0.11 ± 0.01^a	0.05 ± 001^b
Rosmarinic acid	14.6973	0.60 ± 0.21^a	0.11 ± 0.01^b
Quercetin	15.6494	1.77 ± 0.16^a	0.25 ± 0.01^b
Kaempferol	16.1847	0.48 ± 0.02^a	0.21 ± 0.01^b

Different superscripts in the same row indicate significant differences ($p < 0.05$). nd: not detected

4. Conclusions

This study demonstrates the potential of fig seeds as a valuable source of oil, particularly when extracted using SFE using Polat Extraction Technology, Türkiye. SFE was shown to be more efficient than cold pressing in terms of oil yield and product purity. While the specific oil content of fig seeds can vary, the results indicate the commercial viability of fig seed oil production.

The oil was found to contain essential fatty acids and a unique phenolic profile, including compounds with antioxidant and anti-inflammatory properties. However, the radical scavenging activity was relatively low. Future research should focus on optimizing extraction parameters to enhance the recovery of bioactive compounds, particularly phenolic compounds, and further investigate the potential health benefits of fig seed oil.

This study represents a pioneering effort in utilizing SFE to extract bioactive compounds from fig seeds. The identification of various phenolic compounds, such as 4-hydroxybenzoic acid, syringic acid, and quercetin, highlights the potential of fig seed oil as a functional food ingredient. By valorizing a traditionally underutilized byproduct, fig seeds can contribute to a more sustainable and health-conscious food system.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Statement of ethics

In this study, no method requiring the permission of the "Ethics Committee" was used.

Availability of data and materials

All data generated or analyzed during this study are included in this published article. On request, the associated author can provide more information.

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Supplementary File

None.

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