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PHYTO-SYNTHESIS OF MANGANESE OXIDE NANOPARTICLES FOR THE MITIGATION OF PHYTOPATHOGENIC FUNGI *Sclerotinia sclerotiorum*

Author name and information

Nouf Albulushi[1], Hanin Alhuraibi[1], Reham Aldahasi[1], Dhuha Alsuwaid[2], Aisha Alrajhi[1] and Afrah E. Mohammed[1,*]

[1]Department of Biology, College of Sciences, Princess Nourah bint Abdulrahman University, Riyadh P.O. Box 84428, Riyadh,11671, Saudi Arabia.

[2]Health Science Research Center, Princess Nourah bint Abdulrahman University. Riyadh , Saudi Arabia

***Correspondence**: Afrah E. Mohammed; AFAMohammed@pnu.edu.sa

Keywords

Nanostructure, *Russelia equisetiformis*, Tomato, Antifungal, TEM, SEM

ABSTRACT

The green synthesis approach was used to fabricate manganese oxide nanoparticles (MnO NPs) using an aqueous extract of *Russelia equisetiformis* leaves as the study's primary aim. The biosynthesized MnO NPs were monitored using various techniques such as UV-visible spectroscopy (UV), dynamic light scattering (DLS), scanning electron microscopy (SEM), energy-dispersive X-ray spectroscopy (EDX), transmission electron microscopy (TEM), and Fourier transform infrared spectroscopy (FTIR). The latter was conducted to determine the organic ingredients in the leaves extract that could be responsible for the bioreduction and stabilization of MnO NPs that were further tested for their antifungal activity against tomato pathogenic fungus *Sclerotinia sclerotiorum*. Results indicated the successful formation of MnO NPs, as confirmed by peak absorbance of the UV–Vis spectra at 325.09 nm. The SEM and TEM analysis showed the presence of spherical nanoparticles, while the EDX analysis revealed intense signals of Mn. FTIR indicated the presence of phenol and protein that might contribute to the stability of MnO NPs as confirmed by the negative zeta potential, −0.014 mV, for particles of 211.9 nm size with a polydispersity index of 0.29 indicating good dispersion. The study also explored the potential use of biosynthesized MnO NPs against *Sclerotinia sclerotiorum* since treated fungus showed a remarkable decrease in mycelial growth, thin, deformed, and lysed hyphae when viewed under the light microscope. The promising antifungal activity results provided an important perspective for using biosynthesized MnO NPs in various applications.

INTRODUCTION

The stability of the ecosystem and agriculture is threatened by various environmental factors, such as microbial pathogens, pests, chemical pollutants, and weeds (Moore et al., 2020). As a result of the factors mentioned above, crop production has seen a severe and continuous decline (Moore et al., 2020; Prakash, 2022). Various factors, including natural disasters, pests and diseases, climate change, and human activities, can significantly impact crop yield and quality. Among these factors, pests and diseases are considered cause of crop loss. Fungal and bacterial diseases like blights and wilts can also negatively affect crops. The spread of pests and diseases can be rapid, leading to significant crop loss in a short period (Erlee, 2023; Richard et al., 2022). Phytopathogens may even further amplify losses through poor agricultural practices and overuse of pesticides, increasing the chances of toxin production in cereal crops within the food chain (Prakash, 2022). Phytopathogens, like fungi, pose a significant threat due to their saprophytic lifestyle and the limited availability of antifungal agents. Still, their harm will further increase with the progression of global warming (Nnadi and Carter, 2021). Standard management and control methods for fungi include chemical pesticides; however, this presents multiple drawbacks as they contain petrochemical derivatives, which pose extreme health hazards when accumulated in soil or groundwater.

Overuse and the lack of compliance with usage regulations also pose more significant risks due to the possible induction of resistance within fungi (Hernandez-Diaz et al., 2021). According to a survey conducted by the World Health Organization on 56 countries, 32% lack legislation covering public health pesticides, and 65% lack provisions restricting their overuse (World Health Organization and Food and Agriculture Organization of the United Nations, 2019). This could eventually lead to further repercussions through the development of pesticide-resistant and mycotoxin infection (Wang et al., 2022). Such outcomes encourage the spread of various plant phytopathogen diseases and mycotoxins, such as multiple species of *Alternaria* sp., *Sclerotinia* sp., and *Fusarium* sp. These fungi are known to cause leaf blight, withering, and decay in tomato plants, giving a high potential risk (Antwi-Boasiako et al., 2022; Chen et al., 2021; de Chaves et al., 2022). However, they could be managed by fungicide applications.

Limitations in current solutions for this problem guided us to the implementation of nanotechnology in the development of efficient compounds for treatment. Nanotechnology has gained immense attraction in recent years. It is the utilization and modification of materials on an atomic level to achieve advantageous properties that could be used in multiple desired applications (Gleiter, 2000). Nanoparticles within the 1 to 100 nm size range and of diverse shapes present distinct chemical, physical, and optical characteristics. This has broadened multiple interdisciplinary research fields in chemistry, biology, environmental science, and medicine (Scholes, 2008; Singh et al., 2018; Yilmaz and Yilmaz, 2020). Using metal nanoparticles (MNPs) is a viable option for controlling phytopathogenic fungi (Cruz-Luna et al., 2021). MNPs can be synthesized using physical, chemical, and biological methods (Chen et al., 2008); however, such approaches could have high negative environmental impacts. In contrast, green synthesis of nanoparticles utilizes plants, bacteria, fungi, and algae and has the advantages of environmental friendliness, low cost, high feasibility, and ease of production.

Although nanoparticles' variable size and aggregation tendency could be expected (Zhang et al., 2023). In the current study, the perennial shrub *Russelia equisetiformis* was used since previous studies reported its ability to form silver nanoparticles (Mohammed and Al-Megrin, 2021; Sabiha Sulthana et al., 2022). This plant, native to South America, belongs to the family Scrophulariaceae (recently Plantaginaceae). It is a ritualistic medicine used to treat diabetes, malaria, and inflammation in various parts of Nigeria (Kolawole and Kolawole, 2010). Besides, multiple anti-inflammatory, antimicrobial, and antioxidant properties were recorded (Muhammad Riaz, 2012). Despite many advantageous properties, *R. equisetiformis* has only been used as a biogenic agent in nanoparticle synthesis in limited studies (Mohammed and Al-Megrin, 2021; Sabiha Sulthana et al., 2022). Our current study focused on Manganese oxide nanoparticles (MnONPs) that have been proven to have effects against fungi. MnONPs are emerging as agents for biomedical applications such as drug delivery, antimicrobial, photothermal therapy, and anti-angiogenic, having different mechanisms of action(Haque et al., 2021). Previous studies have also proven MnONPs to be efficient antifungal agents against many types of fungi like *Candida albicans*, *Curvularia lunata*, and *Aspergillus niger* (Gillani et al., 2021). The antifungal activity of nanoparticles is influenced by various factors, including their shape, size, distribution, composition, crystallinity, agglomeration, and surface chemistry (Cruz-Luna et al., 2021). The antifungal mechanism of NPs is mainly attributed to their ability to inhibit the growth of fungi by degrading their cell walls and membranes, disrupting protein synthesis, and influencing their metabolism, signal transduction, and genetic information processing. Additionally, NPs produce reactive oxygen species (ROS), further contributing to their antifungal properties (Al-Otibi et al., 2022; Jian et al., 2022). The purpose of the current study was to utilize *R. equisetiformis* for the biosynthesis of MnONPs and assess their antifungal potential against *S. sclerotiorum*, the causal agent of Sclerotinia stem rot of tomato plants. The morphology of the fungi was observed under light microscopic after the MnONPs treatment.

MATERIALS AND METHODS

Materials

Russelia equisetiformis leaves were collected from the nursery of the Royal Commission for Riyadh City (RCRC), Riyadh, Saudi Arabia(. Potato Dextrose Agar (PDA) and, Magnesium (II) sulfate were obtained from the Laboratory of Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia. The strain of *S. sclerotiorum* (ON876490) was isolated from infected tomato (*Solanum lycopersicum*) at Health Sciences Research
Center at Princess Nourah bint Abdulrahman University

Biosynthesis of MnONPs

A 50 mL of 1 mM aqueous $Mn_sO4.H_2O$ solution was combined with 2.5 g of powdered plant extract. The resulting solution was then heated for 15 minutes at 90°C until the color changed from green to purple, indicating the reduction of metal ions. Afterward, the solution was filtered and centrifuged at 14,000 rpm for 1 hour. The precipitate was washed thrice with distilled water and centrifuged at 14,000 rpm for 30 minutes.

Characterization:

UV-Vis spectral analysis

The optical properties of MnONPs were measured using a UV-visible spectrophotometer (Thermo Fisher Scientific, USA) with an absorption spectrum between 200-500 nm.

Fourier Transform Infrared Spectroscopy (FT-IR)

FTIR spectroscopy (Perkin-Elmer, USA) analyzed the functional groups responsible for reducing and capping nanoparticles in phytoconstituents. The measurements were performed in the transmission range of 400 cm^{-1} to 4000 cm^{-1} with 64 scans.

Dynamic Light Scattering (DLS) and Zeta Potential

For size and distribution measurement of the biomolecules and determination of the stability of their colloidal properties, zeta (ζ) potential and dynamic light scattering (DLS) were used. Four measurements per sample were performed with the Zetasizer Ultra (Malvern Panalytical, UK).

Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

The morphology and topography of MnONPs were determined using a scanning electron microscope (JSM-IT500HR, JEOL, Japan) equipped with an X-ray energydispersive spectroscopy (EDX) (STD-PC80, JEOL, Japan) operated at 15 kV and were utilized. Further information about the size, shape, and crystallography of the MnONPs was obtained through a transmission electron microscope (JEM-1400 flash, JEOL, Japan).

Identification of fungal strain isolated from infected tomato fruit

The isolation process of the fungal strain was applied according to Nizamani et al. (2021). Infected tomato fruits were thoroughly washed with tap water and dried, then a piece from the infected part (2-3 mm in length) was cut at the junction of the diseased area with the help of an alcohol-sterilized sharp blade. These pieces were aseptically located at Petri plates containing sterile PDA and incubated at 25°C. The plates were monitored regularly for the development of the colonies. After that, fungal growth was purified using a single spore culture technique. Further, for fungal identification, 18S RNA gene sequencing was used. The molecular identification of fungus, DNA extraction, PCR amplification, and sequencing were done according to Mohammed et al. (2021).

Antifungal Activity of MnONPs

The agar dilution method tested biosynthesized MnONPs as antifungal agents against *S. sclerotiorum* (ON876495). A 10 mg/mL concentration was prepared from MnONPs and added to a Petri plate. Then, 9 mL of sterilized potato dextrose agar was added before solidification. An inoculum of 9 mm diameter of *S. sclerotiorum* was taken from a 7-day-old culture and placed aseptically at the center of the solidified agar. The plates were then incubated at 25°C for 3 days.

Microscopic observation of fungal growth

To investigate the impact of treatments on fungal growth. The cells from treated and control plates were collected using a sterilized loop from the Petri plates' surface containing *Sclerotinia* *sclerotiorum*. The morphology of the fungal cells was observed under a light microscope (LABOMED, SLA2000).

Statistical Analysis

Statistical analysis for antifungal investigation was carried out using GraphPad Prism and Image J 1.54d. Statistical comparisons of multigroup data were collected using Oneway (ANOVA), and only values of $p < 0.01$ were considered significant.

RESULTS AND DISCUSSION

The current study evaluated the ability to fabricate MnNOPs from *R. equisetiformis* leaf extract, and the final product was assessed for its anti-fungal properties against tomato pathogenic fungi *S. sclerotiorum.* The phyto-produced MnONPs were characterized using different approaches as follows:

UV-Vis Analysis

UV-Vis analysis was performed to investigate the optical properties and absorption maxima (λmax) of MnONPs. The concentration of NPs is a determining factor of UVVis absorption intensity. Usually, increased absorption of MNOs within the UV region implies better solubility and dispersion of the nanomaterials, meaning more efficient applicability (Souri et al., 2018). The absorption range of MnONPs is critical for their reactivity to the biological and chemical systems (Selim et al., 2020). Figure 1 shows the absorption spectrum of MnONPs suspended in the aqueous solution using UVVis. The Absorption maximum (λmax) of green synthesized MnONPs was 325.09 nm, corresponding to the characteristic band of MnONPs, confirming that the leaf extract can reduce the metal to its nanoforms. Compared to other studies, a slight shift was observed in the absorption maxima (Khan et al., 2020; Roy et al., 2018).

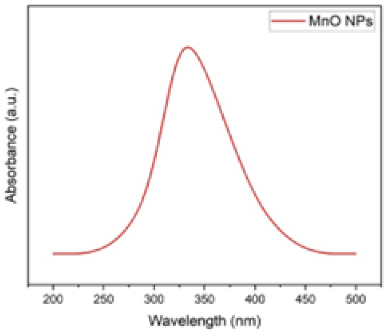

Figure 1. UV- vis spectra showing absorbance peaks of MnONPs prepared by *R.equisetiformis*

FTIR Analysis:

In Figure 2, the FTIR spectrum of *R. equisetiformis* leaves extract, and the phytofabricated MnONPs provided information about the functional groups and biomolecules involved in the fabrication process (Mohammed and Al-Megrin, 2021). The major peak at 3314 cm^{-1} was noted in the spectra of *R. equisetiformis* and MnONPs, which indicate the presence of polyphenolic-OH groups.

A peak at 1640 cm^{-1}, which belongs to C = O, was also detected, indicating amide 1 and carbonyl (C = O) stretching of proteins. Various biomolecules, such as proteins and polyphenolics, were noticed in both tested materials, demonstrating their role as capping and stabilizing agents in the NPs (Mohammed and Al-Megrin, 2021). Recent findings indicated similar peaks ranging between 3550 and 3500 cm^{-1} and 1639.2 cm^{-1} for AgNPs prepared by the same plant extract; however, different parts were used, thus confirming that the functional group determination is dependent on the plant part used (Sabiha Sulthana et al., 2022).

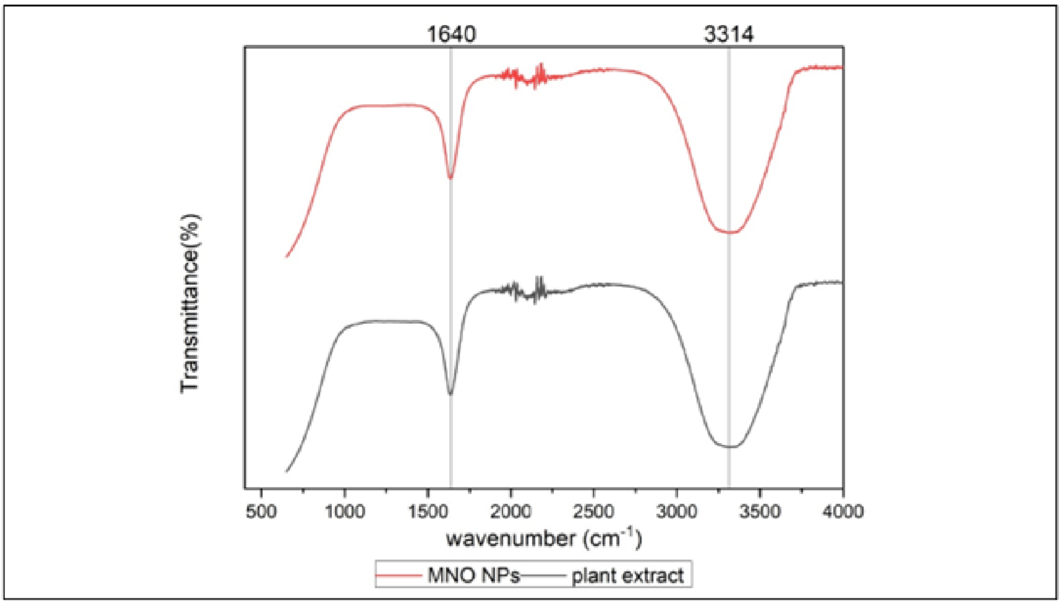

Figure 2. The FTIR spectra of *R. equisetiformis* extract, and MnONPs.

Dynamic Light Scattering (DLS) and Zeta Potential:

Dynamic light scattering was performed to obtain the measurement of particle size distributions (Babick, 2019). The size distribution of the nanomaterials is described by the polydispersity index (PDI), which specifies their uniformity. The PDI value of 0.1 to 0.25 indicates a narrow size distribution, while a PI greater than 0.5 refers to a broad distribution (Hoseini et al., 2023). MnONPs (Figure 3A) indicated stable nanoparticle synthesis through DLS with a size of 211.9 nm and a polydispersity index of 0.29. In another investigation, the solution and thickness of the stabilizing compounds surrounding metallic particles of MnO2-NPs determined its average size distribution. This investigation presented results slightly different from those of the current study; DLS Showed the average particle size was 500 nm due to possible swelling of particles in an aqueous medium and a polydispersity index of 0.34 (Khan et al., 2019). Overall, the DLS result of MnONPs confirms the formation of well-defined dimensions with efficient monodispersity due to their PDIs presenting less than 0.5. Zeta potential measurements were also performed to define the samples' colloidal stability and surface charge (Faisal et al., 2021). Particles with zeta potentials that are more positive than +30 mV or more negative than -30 mV are typically considered stable (Clogston and Patri, 2011). Currently, the zeta potential of the MnONPs in distilled water was −0.014 mV, as shown in Figure 8B, indicating particles with low negative zeta potential that may lead to coagulation over a short period. However, they are still deemed stable given their zeta values remain within the negative range but cannot remain in suspended form within solutions due to surface charge (Seidel et al., 2022). Chemically synthesized MnO2-NPs showed a high surface charge at a zeta potential of −20.4 mV (Khan et al., 2019).

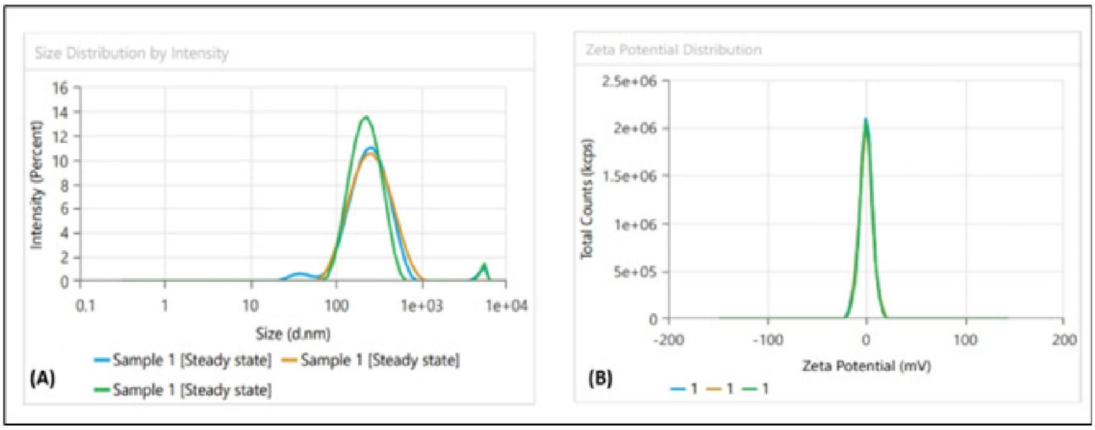

Figure 3. A) Size distribution and B) zeta potential distribution for the MnONPs fabricated by *R. equisetiformis*

SEM and TEM Imaging and EDX analysis

TEM and SEM were employed to investigate the surface morphology and porous structure of MnONPs further. Using EDX, we further studied the purity and chemical composition of the samples. TEM micrographs of MnONPs are indicated in Figure 4. Particles appeared to agglomerate as a spheroid. SEM analysis of MnONPs showed spherical, moderately dispersed, and slightly agglomerated particles (Figure 5A). Mapping (Figure 5B) revealed the presence of multiple elemental compositions like 0.22 wt% of Mn, 33.85wt% of O, and 65.92 wt% of C. Green synthesized MnONPs showed spectral signals at 0.4 keV, 5.9keV and 6.5 keV for Mn and 0.5 keV for Oxygen in Figure 5C. Similar EDX spectral signals of previous studies showed MnONPs synthesized from Indian abutilon (Ekinci et al., 2023). Overall, it can be concluded that *R. equisetiformis* leaf extract is a promising substance for effectively synthesizing nanomaterials; however, aggregation of the biomolecules produced is common in green synthesis (Zhang et al., 2023).

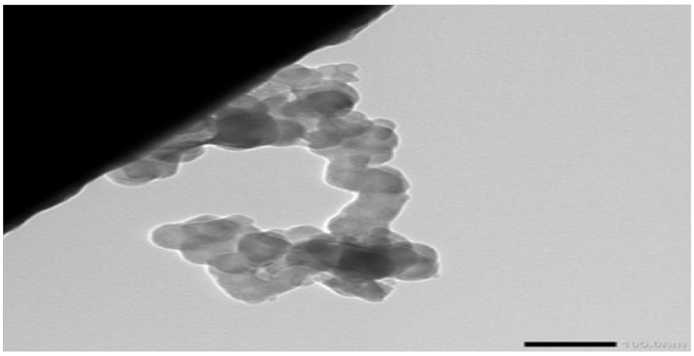

Figure 4. The TEM images of MnONPs

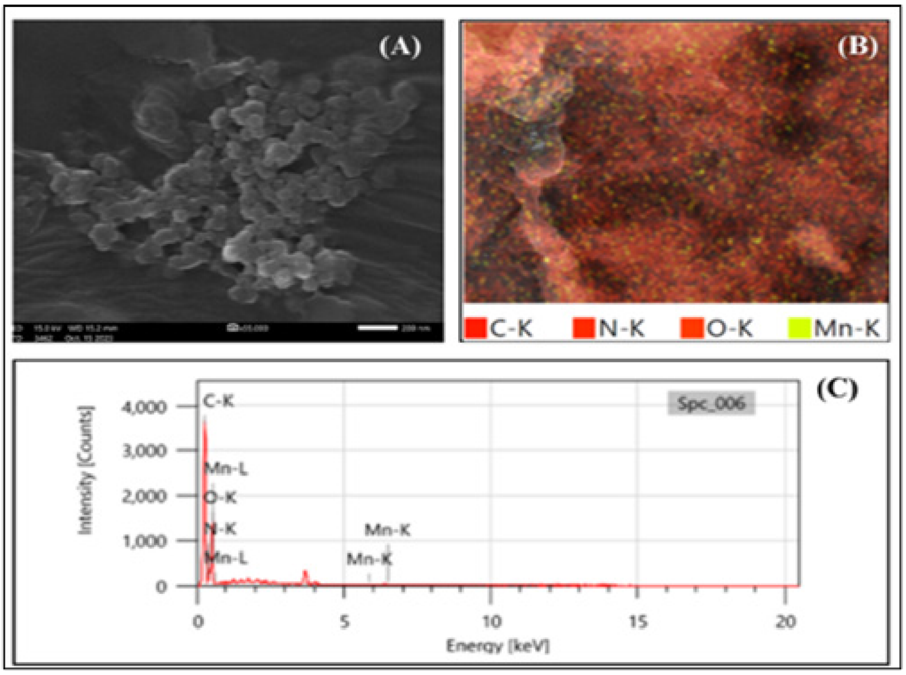

Figure 5. A) SEM image; B) element mapping and C) EDX pattern and elemental composition of MnONPs

Molecular identification of the isolated fungi and the antifungal Effect

The isolated fungal strain was detected using an 18S rRNA gene sequence compared to the sequence database at GenBank for genera or species detection by BLASTn. The presence of the fungus presented 100% similarity to *S. sclerotiorum* and the sequence has been deposited in gene Bank (ON876495). The antifungal properties of MnONPs were tested against *S. sclerotiorum* in a laboratory setting. The average growth area of the fungus from the plates treated with MnONPs was analyzed and compared to untreated control ones. The results showed that the average growth area of the fungus from treated plates was 0.02 ± 0.001 Cm, while from untreated plates, it was 0.07 ± 0.001 Cm. Findings reported the significant impact of MnONPs on the growth rate of the *S. sclerotiorum*. Similar findings were also reported for Pd-doped Mn_3O_4NPs, which showed antimicrobial activity against *S. sclerotiorum* (Vikal et al., 2023). Several theories exist concerning the mechanisms through which NPs demonstrate antifungal properties. However, despite extensive research on the antifungal activity of MNPs, the exact mechanisms remain unknown. MNPs exhibited antifungal properties by interacting with the fungal cell wall, causing structural damage and cell death (Nguyen et al., 2022). The antifungal properties of NPs can be divided into three stages: firstly, NPs attach themselves to the cell walls of fungi and enter the fungal cell using various pathways. Secondly, once inside, they spread to different locations within the cell. Lastly, they interact with various biomolecules, setting off cellular reactions that ultimately result in the death of the fungal cell (Gurunathan et al., 2022; Li et al., 2022; Rana et al., 2023).

Microscopic observation of fungal growth

Due to their suppression ability, the impact of MnONPs on the growth of the *S. sclerotiorum* was studied under the light microscope in an atrial to find any morphological changes in spore and fungal mycelia. A remarkable decrease in mycelial growth was observed (Figure 6). Thickened and septate hyphae of *S. sclerotiorum* mycelium are displayed in Figure 6A. However, thin, deformed, and lysed hyphae were observed from treated plates (Figure 6B). Similar findings were also noted for *S. sclerotiorum* treated by the biogenic AgNPs fabricated using Trichoderma Isolates, where small fragments and damage of hyphae were reported (Tomah et al., 2020).

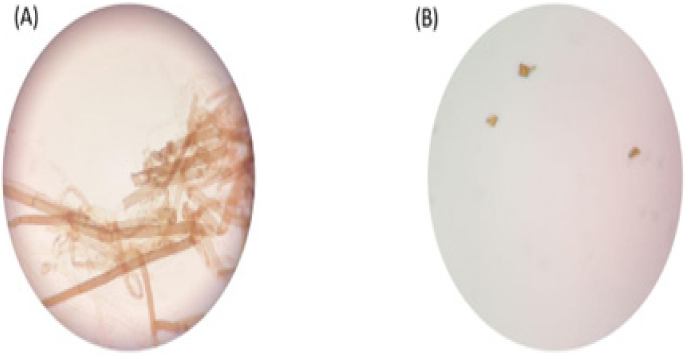

Figure 6. Morphology of *S. sclerotiorum* mycelia. A) is the control and B) is the treated fungi

CONCLUSIONS

The current study has proposed a cost-effective, eco-friendly way of producing stable MnONPs. The method involved using the leaves of *R. equisetiformis*, which are abundant in South America. The polyphenolic compounds present in this leaf extract helped form and stabilize the MnONPs. The biosynthesized MnONPs were found to be spherical. The study also found that the biosynthesized MnONPs had excellent antifungal activity against *S. sclerotiorum*, indicating their potential as an effective treatment for pathogenic fungi. More fungal strains should be tested at different concentrations of MnONPs to better understand their effectiveness as anti-fungal nanomaterials.

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Declaration of interests

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

Availability of data and materials

All data supporting our findings are contained within the manuscript. Further details can be provided upon written request to the corresponding author

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College of Science

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ADULT INTESTINAL TOXAEMIA BOTULISM IN ULCERATIVE COLITIS PATIENT

Author name and information

ElSagad Eltayeb [1,2], Afrah E. Mohammed [3], Aisha Alrajhi[3], Enass M. Abdalla[4], Nassir Alhaboob Arabi[1], Sanaa A. Abdelaziz[5], and Kamal H. Eltom[4]*

[1] Ibn Sina Specialised Hospital, Alamarat, Street 17–21, 12217 Khartoum, Sudan; sagadgady@yahoo.com (E.E.), nassir_alhaboob@yahoo.com (N.A.A).
[2] Faculty of Medicine, Al Neelain University, Street 53, 11111 Khartoum, Sudan
[3] Department of Biology, College of Science, Princess Nourah bint Abdulrahman University, Riyadh 84428, Saudi Arabia; afamohammed@pnu.edu.sa
[4] Department of Animal Health and Safety of Animal Products, Institute for Studies and Promotion of Animal Exports, University of Khartoum, Shambat 13314, Khartoum North, Sudan; kamal@uofk.edu (K.H.E.); enassmukh17@gmail.com (E.M.A.)
[5] Department of Microbiology, Faculty of Veterinary Medicine, University of Khartoum, Shambat 13314, Khartoum North, Sudan; sabdelaziz262@gmail.com

***Correspondence:** Kamal Eltom; keltom@gwdg.de

Keywords

botulinum neurotoxins, *Clostridium*, inflammatory bowel disease, gastrointestinal disease

ABSTRACT

Botulism is caused by neurotoxins produced by diverse members of the genus *Clostridium* and is rare in humans. Among the major forms is the adult intestinal toxaemia botulism caused by the in situ production of the neurotoxin from the toxigenic *Clostridium* spp. colonizing the intestines. Crohn's disease an inflammatory bowel disease, (IBD) is considered a predisposing factor for colonization of the intestines by *C. botulinum*. Here, we report the first case of botulism in a patient with ulcerative colitis (UC) who visited the gastrointestinal clinic complaining of intermittent diarrhea, abdominal distension, and acute pain. In addition, she experienced symptoms of cranial nerve palsies)diplopia and blurred vision(that lasted for a short time. *C. botulinum* was isolated from the stool of the patient, which led to the confirmed diagnosis of adult intestinal toxaemia botulism. Long antibiotic therapy and UC (another form of IBD) had likely promoted colonization by *C. botulinum*, leading to the symptoms of botulism that were overlooked by those of UC.

INTRODUCTION

Botulism is a rare but severe disease caused by a group of neurotoxins produced mainly by *Clostridium botulinum* in addition to many other members of the genus *Clostridium* (e.g., *C. butyricum*, *C. baratii*, etc.). The botulinum neurotoxins (BoNT) are serologically classified into seven distinct serotypes (A – G) (Zhang et al., 2017), and they are classified depending on their physiological properties and 16S rRNA gene sequences into four different groups (I – IV) (Austin et al., 2003). Groups I and II are associated with human disease, which have various forms: foodborne, infant, adult intestinal colonization, and wound botulism; group III is associated with animal disease, while group IV is not related to any disease to date. Botulism symptoms can vary from typical GIT symptoms (nausea, abdominal pain, vomiting, diarrhea) to neuro-muscular toxicity symptoms: symmetric descending acute flaccid paralysis progressing to respiratory compromise (Carrillo-Marquez et al., 2016). Due to the rarity of the disease, only a few cases of adult botulism due to colonization of the intestines are reported to occur. Antimicrobial therapy for extended periods promotes colonization by disrupting the intestinal microflora/ microbiome. Other risk factors for intestinal colonization are conditions related to the intestines, such as surgery, anomalies, inflammatory bowel diseases (IBD), and foodborne botulism (Harris et al., 2020). In the literature, only a few cases of botulism in Crohn's disease (CD) patients are reported (Griffin et al., 1997; Sheppard et al., 2012). Botulism is diagnosed by detecting the neurotoxin in the serum of a suspected patient or the food sample, isolating the neurotoxigenic *Clostridium*, or detecting its DNA.

Here we report a case of adult intestinal toxaemia botulism in an ulcerative colitis patient by isolating *C. botulinum* from the stool of the patient.

Case Report Patient information

A 43-year-old- female visited the GIT clinic at Ibn Sina Specialized Hospital complaining of standing stabbing abdominal pain, abdominal distension for 10 days, loss of weight for the last month, intermittent diarrhea (5 - 6 times a day), stool tinged with blood, and dry mouth with cracks in the lips. In addition, she suffered from diplopia and blurred vision, but she did not mention them on the first visit to the GIT clinic. The patient was non-alcoholic and non-smoker. She was in antibiotic treatment for 10 days with ciprofloxacin and metronidazole.

Clinical findings

Upon physical examination, pallor and abdominal distension were evident.

Timeline

In 2005, the patient suffered from very severe abdominal pain in the epigastrium and right and left lumbar regions associated with abdominal distension, vomiting, and diarrhea. Later, the symptoms were rectal bleeding and mucus discharges. Subsequently, the condition was diagnosed as ulcerative colitis. The patient was also diagnosed with diabetes in the last 2 years.

Diagnostic assessment

The patient underwent an endoscopy and colonoscopy after the physical examination. Endoscopy and colonoscopy reports described extensive oesophageal candidiasis and LA grade B oesophagitis; no gastritis or duodenitis; normal mucosa and vascular pattern; neither piles nor anal mass were noticed. Duodenal and colonic biopsies were sent for histopathology to exclude dysplastic changes. The histopathology report of duodenal mucosa biopsies described typical villous architecture; the lamina propria showed mild chronic inflammatory cell infiltrate; no evidence of villous atrophy, increased intraepithelial lymphocytes, or crypt hyperplasia. Colonic mucosa biopsies revealed unremarkable crypt architecture, the lamina propria showed mild chronic inflammatory cell infiltrate; no cryptitis, crypt abscesses, dysplasia, or malignancy. Anaerobic stool culture yielded the growth of gram-positive rods. PCR with 16S rDNA primers followed by sequencing identified the organism as *Clostridium botulinum*.

Therapeutic intervention

The patient was under a regimen of IBD treatment with mesalazine) Pentasa tabs (and insulin for diabetes. The patient was advised to continue with this medication.

Follow-up and outcomes

The symptoms of cranial nerve palsies (diplopia and blurred vision) improved with time and subsided completely, but the abdominal pain persisted.

DISCUSSION

Botulism is diagnosed by detecting *C. botulinum* or any other neurotoxigenic clostridia or neurotoxin in the samples (Harris et al., 2020; Angulo et al., 1998) . Here, we report a case of adult intestinal toxaemia botulism by isolating *C. botulinum* from the stool of a patient with UC. As a known UC patient, she visited the GIT clinic complaining only of symptoms related to UC and did not mention any other symptoms. However, when the stool culture results were obtained, the patient was contacted to enquire about any symptoms of neurotoxicity. The patient disclosed that she had experienced diplopia and blurred vision, which lasted for a short period. Unlike the aggressive type A botulism, chronic cases of visceral botulism are associated with a short-lived BoNT E (Rodloff and Kruge 2011). In a thorough literature review conducted by Harris, Annibali and Austin (Harris et al., 2020) on adult intestinal toxaemia botulism, only three cases were found that relate to IBD, specifically CD. They suggested that CD is predisposed to intestinal colonization by the neurotoxigenic Clostridium; IBD has been associated with dysbiosis, leading to a change in the intestinal microbiome (Barko et al., 2018), which may favour the conditions for colonization by these bacteria. Prolonged antibiotic therapy affects the equilibrium of the microbial communities in the intestines, which may favor colonization by non-usually hosted bacteria, such as *C. botulinum*. It is a common practice in Sudan that people take antibiotics without proper diagnosis and medical prescription. In addition, the patient in the present report came from a rural area where health-care facilities are limited and, therefore, people take medicines on advice from their relatives and older adults. The patient had visited Ibn Sina Hospital as a referral center for GIT after being under antibiotic therapy for 10 days. It was unclear whether this therapy predisposed the patient to colonization by *C. botulinum*. However, the contrary could be possible. Metronidazole is active against anaerobic bacteria and is a known regimen for reducing the risk of *Clostridioides difficile* infection. It may have shortened the colonization by *C. botulinum* and reduced its burden, so the neurotoxicity symptoms resolved sooner. The source of the infection was also unclear. The area from which she came (East Nile region) is known for animal botulism, according to reports by a field veterinary doctor (Omima Osman, Department of Animal Health and Epidemics Control, Khartoum State, personal communication). The patient had no contact with animals, but contamination of food or water directly by this soil-borne organism or by fecal material from animals harboring human toxigenic serotypes is possible.

Adult intestinal toxaemia botulism is rare, and few reports are available in the literature (Rodloff and Kruge 2011; Harris et al., 2020). It is more likely that many cases are mistaken or overlooked due to the mild symptoms, as in the present case. Therefore, we screened stool samples from patients over 6 months to look for other possible cases of intestinal colonization by *C. botulinum*.

It was astonishing that a sample from another diarrhoeic patient yielded growth of *C. botulinum*, as identified by initial culture and 16S rDNA PCR followed by sequencing. Unfortunately, the patient's record was incomplete, and his contact details were incorrect. Therefore, it was not possible to obtain any further information about him.

CONCLUSIONS

The symptoms of the overlaying disease (UC) in this case were more or less similar to those of early intestinal toxemia botulinum when excluding those of neurotoxicity (cranial nerve palsies). The case described here represents the first case of this disease in a patient with UC. It points out that many cases of adult botulism resulting from intestinal colonization by BoNT-producing *Clostridium* species are overlooked.

Abbreviations

UC	Ulcerative Colitis
IBD	Inflammatory Bowel Disease
CD	Crohn's Disease
BoNT	Botulinum Neurotoxin
GIT	Gastrointestinal Tract
PCR	Polymerase Chain Reaction

Declarations

Ethics approval and consent to participate

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee, of Ibn Sina Specialized Hospital. The patient gave written and oral consent for the use of her anonymous data and samples. Informed consent was obtained from all subjects whose samples were involved in the study.

Consent to publish.

Written informed consent was obtained from the patient for publication of this case report and any accompanying images. A copy of the written consent is available for review by the Editor-in-Chief of this journal.

Availability of data and materials

All data supporting our findings are contained within the manuscript. Further details can be provided upon written request to the corresponding author

Competing interests

All authors have no reported conflicts of interest.

Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Ibn Sina Specialized Hospital.

Informed Consent Statement

The patient gave written and oral consent for the use of her anonymous data and samples. Informed consent was obtained from all subjects involved in the study.

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College of Science

REMOVAL OF TETRACYCLINE FROM WATER WITH FLUORESCENT N-DOPED CARBON DOTS PRODUCED BY *Acacia tortilis* SEEDS

Author name and information

Jumanah Ghannam and Afrah E. Mohammed*

Department of Biology, College of Sciences, Princess Nourah bint Abdulrahman University, Riyadh 11671, Saudi Arabia

***Correspondence:** Afrah E. Mohammed; AFAMohammed@pnu.edu.sa

Keywords

Carbon dots; Nanofabrication; Green one-pot synthesis; *Acacia tortilis*; Doxycycline; Oxytetracycline

ABSTRACT

Tetracyclines are ranked as the second most used antibiotics worldwide and pose significant risks to human health and the environment when present in aquatic locations. The current study investigated the application of nitrogen-doped carbon dots (N-CDs) to remove tetracyclines (Doxycycline and Oxytetracycline) from aqueous solution. The N-CDs were derived from *Acacia tortilis* seed extract and applied at different concentrations (10, 20, 30 mg/ mL) to water samples containing 10 mg/mL of antibiotic. The effectiveness of this treatment was evaluated over periods of 16 and 24 hours using a UV-visible spectrophotometer. Characterization of the N-CDs was conducted through UV, Fourier-transform infrared spectroscopy (FTIR), dynamic light scattering (DLS), transmission electron microscopy (TEM), and energy-dispersive X-ray spectroscopy (EDX). The results revealed that N-CDs produced from *A. tortilis* seed extract were of high quality, where the UV-Vis spectral profile showed peaks ranging from 251–293 nm and an average ζ-potential of -31.6 mV. FTIR indicated functional groups at peaks of 3300 cm^{-1} and 1631 cm^{-1} that corresponded to O-H and C=C. Such N-CDs revealed removal efficiencies for doxycycline and oxytetracycline, which reached 8.98% and 19.5%, respectively, with the highest efficiency observed at 30 mg/ mL concentration after 24 hours. N-CDs' ability to remove antibiotics from water is time- and concentration-dependent. This research demonstrates a promising, eco-friendly approach for mitigating antibiotic pollution in an aqueous solution, contributing to developing effective water treatment technologies.

INTRODUCTION

The contamination of aquatic systems with antibiotics leads to potential hazards that affect both human health and the ecosystem (Wang et al., 2023). Antibiotics are mainly used in human medical therapy and agriculture livestock as growth promoters and disease controllers (Kovalakova et al., 2020). Antibiotic residuals have frequently been detected in the surface groundwater and drinking water (Liu et al., 2017). Antibiotic residuals reach aquatic systems by discharging wastewater treatment plant effluent, which can flow into surface water bodies or infiltrate groundwater containing significant concentrations of non-metabolized residuals (Carvalho and Santos 2016; Martínez 2008). Antibiotics have the potential to endure in aquatic environments for extended durations (Guo et al., 2020). For instance, tetracyclines (TCs) are not easily metabolized by humans and animals, leading to more than 70% of the original substance being excreted and consequently entering the environment (Xu et al., 2021). TCs cannot be easily degraded naturally because of their chemical stability. However, the byproducts of their degradation can sometimes be more toxic than the original substance (Xiao et al., 2023).

Furthermore, the presence of antibiotics in the surface water leads to the development of bacterial resistance and mutation (Wang et al., 2017). Antibiotic resistance is designated a significant global health threat by the World Health Organization, endangering humans and ecosystems (Leonard et al., 2015). Additionally, antibiotic residuals may trigger endocrine disruption, and there's a risk of harmful effects arising from unidentified byproducts (Zeng et al., 2018). Doxycycline (DO) and Oxytetracycline (OTC) are members of the tetracycline group that are used in treating various bacterial infections, including veterinary uses (Zaidi et al., 2019). Li et al. (2008) stated that many OTC and related substances remain in the water even after wastewater plant treatment. However, groundwater showed tetracyclines residual at low concentrations (MacKie et al., 2006). Since removing antibiotic residues by conventional wastewater treatment methods is inefficient (Langbehn et al., 2021), the emergence of antibiotic residues interfering with the development of aquatic species is also an expected disaster, and these antibiotic residues may build up in the food chain, significantly impacting human health leading to conditions such as nephropathy, joint disease, central nervous system defect, mutagenicity, and changes in photosensitivity (Kovalakova et al., 2020).

Various methods have been employed to remediate water, such as ozonation (Sánchez-Polo et al., 2008) and chemical reduction (Chen et al., 2012). These methods have many disadvantages; chemical methods are expensive and could produce toxic residuals (Mehrjouei, Müller, and Möller 2014). Recently, the adsorption method offered several benefits above other methods due to its lower power consumption, easy handling, and environmental friendliness (Ahmad et al., 2014). Nanoparticles (NPs) have attractive properties due to their tiny sizes, huge surface area, and high sensitivity to their bulk material counterpart (Bhilkar et al., 2023).

Carbon dots (CDs) are a new class of spherical, crystalline carbon nanomaterials, ranging in size from 1 nm to 10 nm (Jing et al., 2023). CDs attract research attention due to their unique characteristics, such as biocompatibility, dispersibility, high chemical stability, and low environmental hazard, rendering them promising light-emitting materials (Hong et al., 2022; Liu et al., 2020). Green resources are preferred for CD production due to their affordability, ready availability, stability, straightforward process, safety, eco-friendliness, and abundant carbon sources (Jing et al., 2023). Limited studies have tested the ability of CDs to mitigate tetracyclines from aqueous solution; however, combinations of CDs with other materials have been employed for this purpose. Guo et al. (2017) prepared CDs/g-C3N4/ZnO nanocomposite through a thermal impregnation process, which showed efficient TC degradation (100%) in 30 minutes. Green synthetic protocols have been established for synthesizing N-CDs from various natural sources as green precursors by utilizing different plant parts. For instance, Atchudan et al. (2016) have prepared N-CDs from *Prunus persica* fruit extract. Furthermore, Korah et al. (2022) successfully synthesized N-CDs using Curcuma amada extract.

For the current study, the seeds of *A. tortilis* were selected as a carbon source for synthesizing nitrogen-doped carbon dots (N-CDs). *A. tortilis* is a tree known as Umbrella thorn, is a halophyte, drought resistant, and grown in Ethiopia, Yemen, Sudan, Somalia, part of Kenya, Tanzania, and Saudi Arabia (Meresa et al., 2016; Noumi and Chaieb 2012). *A. tortilis* seeds extract consists of 5.30% moisture, 3.99% ash, 9.19% fat, 14.31% fiber, 27.21% protein and 45.30% carbohydrates (Embaby and Rayan 2016). Hence, this study seeks to synthesize N-CDs using *A. tortilis* seed extract as a carbon source for the first time and assess their characteristics. While applied at varied concentrations, the N-CDs were investigated to remove DO and OTC from aqueous solutions. The study also examined how the treatment duration influences the effectiveness of mitigation.

MATERIALS AND METHODS

2.1 Chemical reagents

Doxycycline hyclats and oxytetracycline hydrochloride were provided by Saudi Pharmaceutical Industries (SPI). Acetone, methanol, and urea were obtained from the laboratory at Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia. *Acacia tortilis ssp. Spirocarpa* seeds are provided by RCRC Seed Bank in Riyadh, Saudi Arabia.

Biosynthesis of green, fluorescent N-CDs

To prepare N-CDs, 5 grams of plant seed powder were mixed with 50 mL of distilled water and stirred for 60 min. The mixture was then filtered using Whatman filter paper to eliminate undissolved particles, resulting in the collection of 20.0 mL of filtrate. Subsequently, 2.0 g of urea was introduced to the filtrate, and the resulting solution was heated in a crucible at 180°C for 2 hours. Once the crucible had cooled, the solid product was scraped and transferred into a centrifuge tube with 15 mL of acetone to separate N-CDs from any remaining unreacted materials. The successful formation of N-CDs was confirmed using a UV lamp, which typically causes N-CDs to fluoresce under its light, as demonstrated in Figure 1. The mixture was then centrifuged at 5500 rpm for 15 minutes, after which the supernatant was carefully removed, ensuring the pellet remained intact at the bottom. This pellet underwent multiple washing cycles with acetone and methanol (90:10) followed by centrifugation at 5500 rpm for 15 minutes in each cycle. Finally, the obtained N-CD pellet was stored in a well-sealed container for future use.

Figure 1. Fluorescence properties of the greenly prepared N-CDs mediated by seed extract of *A.tortilis* under UV light at 365 nm

Characterization Methods

In this study, the microstructure and morphology of the N-CDs were investigated using advanced techniques. Transmission electron microscopy (TEM) analysis was conducted using the JEM-1400-Flash instrument to provide detailed insights into the microstructural characteristics of the N-CDs. The products' shape, size, and morphology were analyzed using scanning electron microscopy (SEM, JSM-IT500HR). The EDX analysis with 15 kV accelerated voltage was applied (STD-PC80). Furthermore, ultraviolet-visible (UV-Vis) spectroscopy, carried out with a Thermo (Scientific Evolution 201 instrument, Serial Number: 5A4T346003, China), allowed the monitoring of the absorbance spectra and detection of the maximum surface plasmon resonance (SPR) of the N-CDs, which fell within the 250-450 nm range. To gain an understanding of the size and distribution of the N-CDs, Dynamic Light Scattering (DLS) measurements were performed using the NANO ZSP instrument (Malvern Instruments Ltd, Serial Number: MAL1118778, ver 7.11, UK) and three measurements per sample were performed. Moreover, Fourier-Transform Infrared Spectroscopy (FT-IR) was carried out using the SPECTRUM100 instrument (Perkin-Elmer, USA). This technique effectively analyzed the functional groups of biomolecules in the sample by measuring their infrared absorption and emission spectra. 64 scans per sample were done in the range between 400 and 4000 wavenumber cm^{-1}

Adsorption Experiments Effect of N-CDs Dose on Antibiotic Adsorption

A 10 mL solution containing individual OTC and DC at a 1.25 mg/mL concentration was tested using different N-CD concentrations (10, 20, and 30 mg). After that, the mixture was sealed using plastic wrap and agitated on a constant-temperature shaker at 180 rpm at room temperature. Finally, the sample was collected for measurement. An antibiotic solution was used as a control.

Effect of N-CDs treatment duration on antibiotic adsorption

The measurement process was conducted at two distinct time intervals: first, after 16 hours and then again after 24 hours. This approach was employed to thoroughly investigate the impact of contacting time on the adsorption process over specified durations.

Detection of antibiotic

The residual antibiotics concentration in the solution was determined by UV spectrophotometry. 1 mL of each concentration, including control, was centrifuged for 10 min, and the residual concentration of OTC and DC in the liquid was detected by UV spectrophotometer, where OTC was detected at 303 nm and DC at 358 nm. These wavelengths were selected based on the previously identified OTC and DC adsorption wavelengths. For the measurements, a standard curve has been created from the stock solution prepared by dissolving 0.025 g of individual DC and OTC in 25 mL of distilled water. For DC, precise volumes of 20, 40, 50, 60, and 80 µL were carefully transfered from stock solution into 1mL Eppendorf tube, and the volumes were adjusted to 1.0 mL using distilled water. The linear standard curve range was constructed at 0.5-2 mg/ mL (R2 = 0.999). For OTC, 10, 20, 30, 60, and 70 µL were carefully trannbsfered from stock solution into 1mL Eppendorf tube, and the volumes were adjusted to 1.0 mL using distilled water. The linear standard curve range was constructed as 0.25- 1.75 mg/mL (R2 = 0.999). The removal efficacy (RE%) was determined using the following formula:

$$Removal\ Efficacy\ (\%) = \left(\frac{Initial\ Concentration - Final\ Concentration}{Initial\ Concentration}\right) \times 100 \quad (1)$$

Statistical analysis

The statistical analysis, including the mean, standard deviation, and relative standard deviation, were computed using Microsoft Excel 2019. Spectra for FTIR and UV-Vis were generated using OriginPro® 2023b.

RESULTS AND DISCUSSION

Characterization of N-CDs

Results from the current study indicated that *A. tortilis* seed extract was a viable material to fabricate N-CDs that were further tested for their catalytic activity to eliminate DC and OTC from an aqueous solution. The absorption behavior of N-CDs was observed by UV-vis spectroscopy. Figure 2 showed the absorption as a broad peak around the range of 251–293 nm, which is attributed to n→ π^* transition of the carbonyl group in the CDs (D'Souza et al., 2016) or it could be due to typical absorption of an aromatic π system (Lu et al., 2019). A similar absorption range of CDs prepared from gelatin was observed between 250–290 nm (Liang et al., 2013). In addition, to determine the electrical charges on the surface of N-CDs and their colloidal stability, a zeta potential test has been applied. Figure 3 illustrates -31.64 mV as the average of ζ-potential. The negative zeta potential guaranteed excellent colloidal stability for N-CDs (Zulfajri et al., 2019). Negative zeta potential values might indicate the presence of negatively charged carboxyl and hydroxyl functional groups (Abd Rani et al., 2021) on the N-CDs surface.

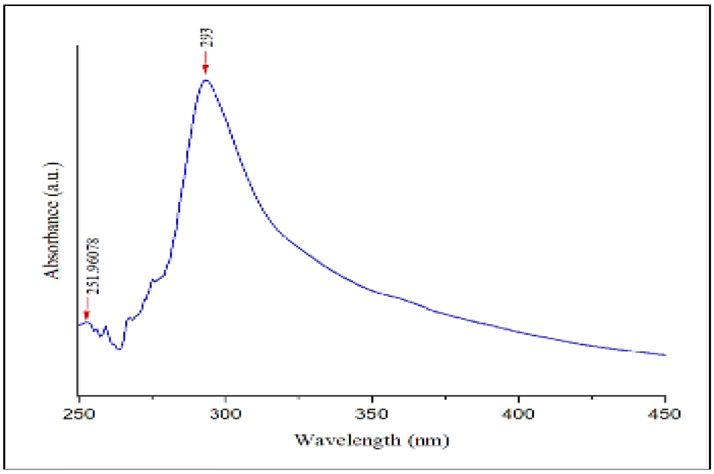

Figure 2. The analysis of UV-Vis absorption spectra for the N-CDs prepared using *A. tortilis* seed extract.

Moreover, the FTIR spectrum was acquired to determine the surface functional groups of the obtained N-CDs. Figure 4 represents the FT-IR spectrum of N-CDs, highlighting absorption bands in the range of 3300 and 1631 cm^{-1}. Bands at 3300 cm^{-1}. correspond to the stretching vibrations of OH (hydroxyl) and NH (amine) groups, which might be responsible for the hydrophilic characteristics of the N-CDs. Liu et al. (2021) suggested that peak at 1631 cm^{-1}. could be attributed to the amide bonding from C=C stretching. Similar peaks were observed in Kalanidhi and Nagaraaj's (2021) study when N-CDs were prepared with betel leaves. Thus, the author suggested the presence of functional groups rich in oxygen and carbon that indicate the formation of N-CDs rich in alcohol and amines. Such functional groups might have a role in N-CDs' ability to adsorb antibiotics (Ahuja et al., 2022; Sadegh et al., 2017). Further characterization of N-CD distribution was evaluated by transmission electron microscopy (TEM). Figure 5 a show well-dispersed N-CDs at 500 nanoscales. Figure 5 B represents the aggregation of semi-circular shapes at a 50 nm scale. The tiny circular structures within these aggregations at a 20 nm scale are the N-CDs with an average diameter of 7nm, as shown in Figure 5 c. Similar observations have also been found when N-CDs were prepared with Jackfruit seed extract (Raji et al., 2019). Figure 6 a-c represents an SEM image for N-CDs fabricated by *A.tortilis* seed extract. Figure 6 a identifies a smooth surface with a folded structure. Sabet & Mahdavi (2019) reported N-CDs quantum dots using grass extract, revealing tiny particles clustered together, forming larger, chunkier structures. This aggregation happens because the tiny carbon particles may have high surface energy.

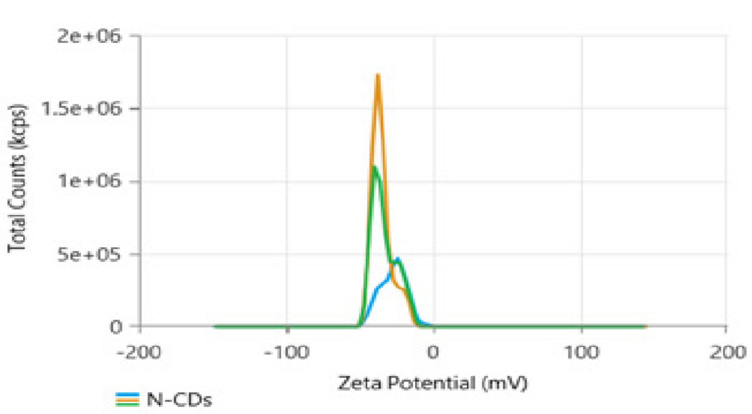

Figure 3. Average zeta potential of -31.64 mV for the N-CDs prepared using seed extract of *A. tortilis*. The figure presents the mean zeta potential of three readings.

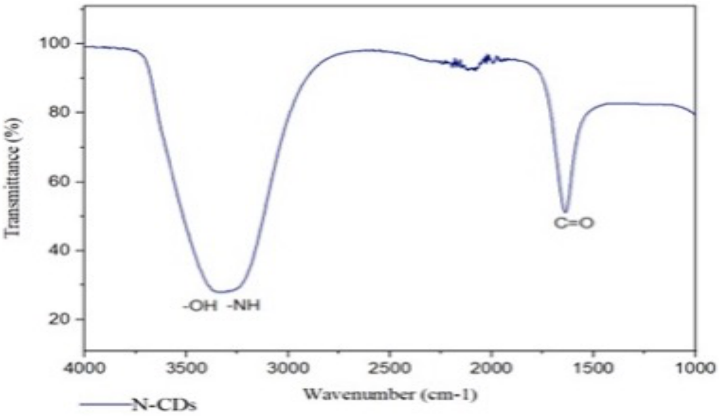

Figure 4. FTIR spectrum of N-CDs prepared by *A.tortilis* seed extract where two major peaks appeared (between 3300 to 3500 cm^{-1} and at 1631cm^{-1}).

Moreover, elemental mapping in Figure 6 b represented the even distribution of C and O elements on the N-CDs surface. Furthermore, EDX spectra in Figure 6 c showed the presence of C and O elements. Perumal et al. (2022) obtained closely related SEM analysis results when preparing N-CDs from Red Malus floribunda fruits.

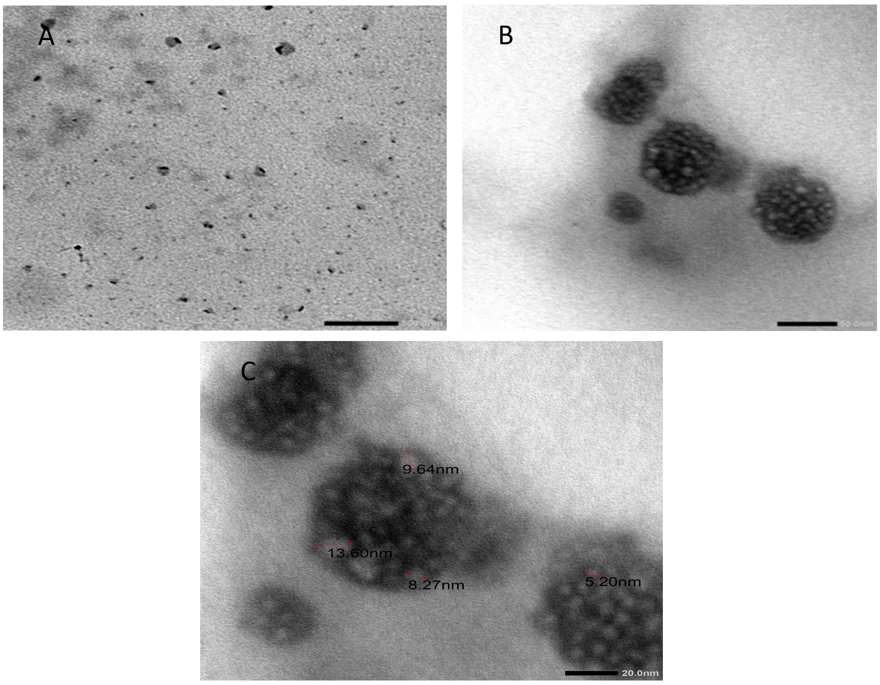

Figure 5. TEM images of N-CDs prepared by *A. tortilis* seed extract showed welldispersed N-CDs (a), three main clusters of N-CDs (b), and the same clusters at higher magnification (c).

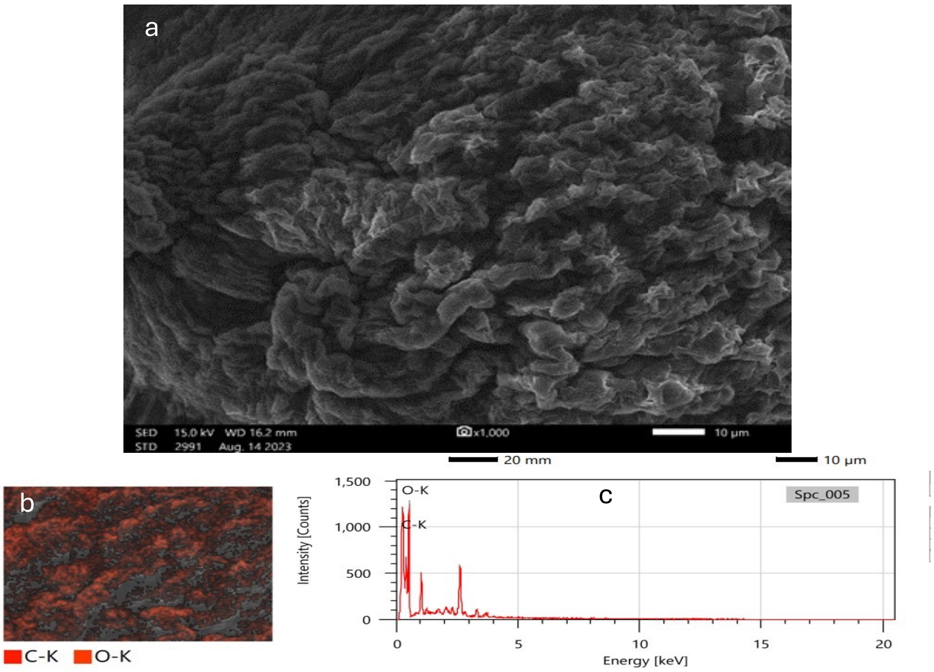

Figure 6. SEM image for N-CDs prepared by *A. tortilis* seed extract (a), EDX elemental mapping (b), and EDX spectrum (c).

Effect of Dose and time on the efficacy of N-CDs on antibiotics adsorption

The current study aimed to assess the efficacy of prepared N-CDs in mitigating antibiotic contamination in water. The concentrations of DC in water before and after treatment with three different concentrations of N-CDs (10 mg, 20 mg, and 30 mg) at two separate time intervals, 16 and 24 hours, were presented in Table 1. The results obtained after 16 hours indicated a noteworthy trend as the dosage of N-CDs increases, the removal efficacy also increases where the removal efficacy percentages were 5.19%, 6.33%, and 7.40% for N-CD concentrations of 10 mg, 20 mg, and 30 mg, respectively. Such findings highlighted the dose-dependent effectiveness of N-CDs in mitigating DC from contaminated water. The results obtained after 24 hours showed removal efficacy percentages of 6.09%, 6.35%and 8.98% for N-CD concentrations of 10 mg, 20 mg, and 30 mg, respectively. A similar pattern of dose-dependent effectiveness was observed at both exposure durations. Notably, the removal efficacy percentages increased after 24 hours compared to 16 hours for all tested concentrations, where the highest removal efficiency was observed in N-CD concentrations at a concentration of 30 mg.

The concentrations of OTC in water before and after treatment with varied concentrations of N-CDs at two distinct time intervals, 16 and 24 hours, are displayed in Table 2. After 16 hours of treatment, it was observed that the removal efficacy percentages for OTC increased as the dosage of N-CDs escalated. Removal percentages were 1.5%, 2.5%, and 4.1% for N-CD concentrations of 10 mg, 20 mg, and 30 mg, respectively. This initial data indicated that higher doses of N-CDs led to more efficient removal of OTC from the water. The results obtained after 24 hours also demonstrated a dosedependent pattern. The removal efficacy percentages after 24 hours were 6.1%, 5.1%, and a notable 19.5% for N-CD concentrations of 10 mg, 20 mg, and 30 mg, respectively. Reduction percentages were increased compared to the 16-hour treatment for all N-CD concentrations, with the highest removal efficacy observed in N-CD concentrations at 30 mg.

A study conducted by Karaca et al. (2023) indicated the usage of N-CQDs/TiO2 for the removal of their study despite using a relatively high dose (0.2 g/L) of Tetracycline; the adsorption of TC molecules on the N-CQDs/TiO2 surface was only 1.52% over a 120-minute duration. Variations compared to the current findings could be related to the different N-CDs used. However, the initial concentration of TC in the solution can also impact adsorption efficiency. Ekande & Kumar, (2023) explored the effectiveness of N-CDs prepared at high carbonization temperatures ranging from 700°C to 1000°C. He indicated their capacity to adsorb approximately 95% to 98% for Ciprofloxacin (CIP) and TC within 5 minutes. Such a high adsorption rate compared to our materials' efficiency could be attributed to the differences in the carbonization temperature and source since they utilized Polyaniline polymer as the starting material. Variations in carbonization may lead to differences in the composition of active sites and functional groups on the surface of the N-CDs, which ultimately influences the adsorption rates.

The adsorption system employed by N-CDs as an adsorbent for removing both DC and OTC antibiotics might be achieved through π–π electron acceptor–donor interactions, as described by Ekande & Kumar, (2023). The interaction of the OH− group with the benzene rings in the antibiotic (DC and OTC) structure may lead to more electrons, resulting in π–π electron acceptor–donor interaction between the antibiotics and N-CDs (Sun et al., 2020). This agrees with FTIR results that indicated the presence of hydroxyl and amide groups on the surface of N-CDs that actively contribute to removing DC and OTC from contaminated water. Different mechanisms have been widely employed to mitigate antibiotics from aqueous solution. For example, Qu et al. (2020) used ZnO/N and S-CQDs under simulated sunlight for 20 min and natural sunlight for 50 min, respectively, where 92.9% and 85.8% of ciprofloxacin (CIP) were removed. Herein, this variation in the removal efficacy and the reduction in the adsorption as time increases could be attributed to the mechanism itself. During the photocatalytic process, the surface of the N-CDs captures photons originating from the light source, and these absorbed photons then provide the energy needed to create electron-hole pairs (Liu et al., 2019). A hole emerges when an electron undergoes excitation from the valence band to the conduction band. Subsequently, this hole engages in reactions with either water or hydroxyl ions, ultimately resulting in the creation of hydroxyl radicals (Alkian et al., 2020). These hydroxyl radicals readily react with organic molecules, converting them into more friendly substances such as CO_2 and H_2O (Syafei et al., 2017).

Table 1. The concentration of doxycycline (mg/mL) and the removal efficacy of N-CDs prepared by *A.tortilis* seeds extract at different concentrations (10, 20, and 30 mg) after 16 and 24 hours. Data presented are mean ± SD.

	Control	N-CDs (10 mg/mL)		N-CDs (20 mg/mL)		N-CDs (30 mg/mL)	
		16h	24 h	16h	24 h	16h	24 h
Mean	354.1±2.8	335.7±0.4	332.5±0.2	331.7±2.4	331.6±0.5	327.9±0.4	322.3±0.6
RSD	0.8	0.1	0.06	0.7	0.1	0.1	0.1
RE %		5.19%	6.09%	6.33%	6.35%	7.40%	8.98%

Table 2. The concentration of oxytetracycline (mg/mL) and the removal efficacy of N-CDs prepared by *A.tortilis* seeds extract at different concentrations (10, 20, and 30 mg) after 16 and 24 hours. The data presented mean ± SD.

	Control	N-CDs (10 mg/mL)		N-CDs (20 mg/mL)		N-CDs (30 mg/mL)	
		16h	24 h	16h	24 h	16h	24 h
Mean	319.5±3.4	314.5±1.7	300.0±2.8	311.4±3.7	303.2±3.03	306.4±2.4	257.1± 0.6
RSD	1.0	0.5	0.9	1.2	0.9	0.7	0.2
RE %		1.5%	6.1%	2.5%	5.1%	4.1%	19.5%

CONCLUSIONS

This study demonstrated the feasibility of producing cost-effective, environmentally friendly fluorescent N-CDs by the principles of sustainable green development using *A.tortilis* seed extract. Through TEM analysis, the synthesized sample showed a small size where the ζ-potential average was -31.6 mV. The findings also unveiled the presence of essential phytochemical compounds in the seed extracts of *A.tortilis*; as evidenced by FTIR analysis, such compounds may play a crucial role in the mitigation process. The prepared N-CDs exhibited a removal efficiency of 8.98% for DC and

19.5% for OTC. Higher efficiency could be obtained when a higher concentration is applied. This research underscores the promise of employing green methods for synthesizing N-CDs, offering an environmentally friendly approach to address the removal efficiency of antibiotics from water resources.

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Declaration of interests

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

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Princess Nourah Bint
Abdulrahman University
College of Science

Princess Nourah Bint
Abdulrahman University
College of Science

PREVALENCE OF DIFFERENT GASTROINTESTINAL PARASITES IN HORSES AROUND THE WORLD

Author name and information

Wadha Obaid Alsahli, Afaf Aqeel Alhazzaa, Rawan Saif Alotaibi, Dalal Abdullah Alotaibi, Rania Mohammad Aldosari, Wafa Almegrin*

Department of Biology, College of Science, Princess Nourah bint Abdulrahman University, P.O. Box 84428, Riyadh 11671, Saudi Arabia

*Correspondence: Wafa Almegrin; waalmegrin@pnu.edu.sa

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ABSTRACT

Gastrointestinal parasites (GIPs) are a common concern in horses, and if left untreated, they can cause significant health problems. Virtually all horses are affected at some point in their lives. Strongyles, roundworms, and tapeworms are examples of common parasites. Weight loss, diarrhea, colic, and lethargy are all indications of an infected horse. To prevent the spread of parasites, horse owners must follow regular deworming programs and conduct proper pasture management. Regular deworming, pasture management, and good hygiene practices are essential in preventing and controlling GIPs in horses. Early detection and treatment are crucial to avoid serious health problems.

INTRODUCTION

Humans have long tamed horses, and since then, they have become loyal friends and reliable partners in daily life, whether pets or companion animals. It is known that horses are essential to people in many ways, including wars or police duty, horseback riding, polo games, ceremonies, crowd control, recreation, farming, and research purposes (Ola et al., 2019), which explains why the horse's industry is an important part of the American, Mexican, Brazilian, Chinese and Middle Eastern economy. The total economic inflow in Brazil is expected to be 16.15 billion, with approximately 3,000,000 direct and indirect jobs created. At this stage, the economic importance of horses becomes clear (Bianchi et al., 2019). In Saudi Arabia, the horse population is estimated to be more than 33,000, and more than 500 horses are imported annually from different countries such as the United Arab Emirates, the United States, and Europe to be used for various purposes, including husbandry activities, transportation, racing, showing, and breeding (Alanazi et al., 2018).

Horses suffer from various infectious diseases, including viruses, bacteria, and parasitic diseases; parasitic infections are among the infectious diseases responsible for causing significant mortality and morbidity in these animals (Adeppa et al., 2016). Colic in horses significantly contributes to economic loss within the equine industry. A vast number of potential risk factors have been implicated as a cause of gastrointestinal disease in horses, such as Displacement of the intestines, obstructions, impactions, perforations, and ruptures, which are the leading cause of death in horses, including intestinal parasites (Bianchi et al., 2019). Intestinal parasites cause severe illness and death in horses of all species. Several intestinal worms are common, and in any group of horses, specific individuals might host vast numbers of parasites, which can cause sickness (Proudman et al., 2000). The gastrointestinal system is believed to be the site of around two-thirds of animal parasitic infections. Coccidiosis is a severe illness that affects horses and has had a significant negative impact on the global economy. Intestinal parasitism is a substantial source of financial loss in the horse breeding industry (Ola et al., 2019). Endoparasite infections are estimated to affect more than 90% of horses in Ethiopia and Mexico. *Strongyle*, *Cyathostomess*, *Triodontophorus* species, *Strongyloides westei*, *Parascaris equorum*, *Dictyocaulus arnfieldi*, *Oxyuris equi*, *Gastrodiscus*, and *Fasciola* species are the most often recognized gastrointestinal helminths of equines in various sections of the nation (Mathewos *et al*., 2021). Equine bloodworms, or *Strongylus vulgaris*, are known as "the horse killer" and the most dangerous gastrointestinal parasite (Nielsen et al., 2021).

Peritonitis is the most common symptom of the clinical sickness produced by this parasite, and studies have demonstrated that this condition is linked to *S. vulgaris* positivity, confirming that this parasite can kill animals (Pihl et al., 2018). *Cyathostomins* infect nearly all horses, and infections with 10 or more species per horse are common. Despite possible species bias in disease development and anthelmintic resistance, species-specific knowledge is scarce (Bellaw & Martin, 2020). *Cyathostomins* form in the lumen and wall of the large intestine, and horses seldom acquire substantial protective immunity. As a result, substantial *Cyathostomin* loads are possible in animals of all ages. *Cyathostomins* cause various clinical symptoms, including impaired performance, slower development rates, weight loss, coarse hair coat, asthenia, diarrhea, and various forms of colic (Pergrine et al., 2014). Therefore, in this study, we comprehensively reviewed the knowledge of gastrointestinal parasites (GIPs) in horses worldwide. There are numerous published studies worldwide to detect and investigate GIPs in horses.

Africa

A considerable amount of literature has been published in Africa; Nigeria is well known for maintaining annual examinations on GIPs in horses, starting from Kaduna, Nigeria. A study was carried out by Umar et al. (2013) to estimate the prevalence of GIPs in horses used for cadet training. A total of 48 horses were examined regularly. Fecal samples were collected and processed by flotation and sedimentation techniques. GIP encountered were ciliates (81.3%), *Strongylus* spp. (68.8%), *Oxyuris equi* (27.1%), *Strongyloides* spp. (25%), *Dictyocaulus* spp. (10.4%) and *Parascaris equorum* (6.3%). Male horses were significantly more infected than female horses.

In Central Nigeria, the prevalence and impact of helminths in domestic animals has been established, 1,508 fecal samples from various breeds of domestic animals: cattle, sheep, dogs, pigs, horses, rabbits, and goats were carefully examined using the concentration technique. Out of the 13 horses, samples indicated *Strongylus* spp. (92.3%) while *Triodontophorus tenuicollis* (7.7%). All samples were positive for different parasites. The study revealed that GIPs and their eggs are still endemic in the study area, indicating a neglect of simple management practices that negatively impact livestock production in Nigeria (Dogo et al., 2017).

Coccidiosis has a significant impact on life stocks worldwide. A study in some of Nigeria's states was designed to determine the species diversity and prevalence of GIPs in horses and the risk factors associated with Eimeria infections in horses. One hundred five fecal samples were collected from horses in Kwara and Niger States. Simple floatation and concentration techniques were used as detection techniques. An overall prevalence of (62.86%) was recorded. 11 GIPs consisting of 7 nematodes, 2 trematodes, 1 protozoan, and 1 cestode were detected. *Strongylus* spp. (33.33%) and *Eimeria* spp. (28.57%) were the most prevalent. 43.81% of the sampled population had multiple GIP infections (Ola et al., 2019).

Research at Jos Plateau's determined the impact of intestinal parasites on the social economy. Fecal samples were collected from 108 horses: 25 male horses and 83 females. The samples were examined using standard flotation, sedimentation, and McMaster fecal egg counting techniques. The overall prevalence of GIPs was (82.41%). The GIPs in this study were mostly *Strongylus* spp., *Strongyloides westeri*, *Trichonema* spp., and *Eimeria leuckarti* (Ogbein et al., 2022).

A study conducted by Alaba et al. (2022) identified the prevalence and association between factors (sex, age, and breed) and GIPs in Ibadan polo horses using the sodium chloride flotation method. Feces samples from 56 polo horses were examined for GIP. *Strongylus* spp., *Strongyloides* spp., *Parascaris equorum*, and *Eimeria* spp. were the four GIPs that were seen, and there was no correlation between prevalence/EPG and age, sex, or breed of horses. However, mature females and exotic breeds were more likely to develop severe illness.

In Ethiopia, Oromia. A study was conducted by Fikru et al. (2005) on 388 samples to estimate the number of equine GIPs. The study indicated the overall prevalence of *Strongyles* was (92.8%), *Parascaris quorum (17.1%)*, *Dictyocaulus arnfieldi (2.6%)*, and *Oxyuris equi* (2.1%). Also, in Oromia, a study of GIPs of equines was conducted and aimed to estimate the prevalence of helminth parasites in equines. A total of 146 fecal samples were collected from randomly selected horses and examined with direct smear, flotation, and sedimentation techniques. All age groups were infested with identical parasite species. Out of 146 fecal samples examined, five species of helminths were reported. The overall prevalence of GIT parasites was (96.6%). The most common parasites encountered were *Strongyles* spp. (68.71%), *Parascaris equorum* (29.2%), *Oxyuris equi* (10%), *Anoplocephala* spp. (5.15%) and *strongyloides westeri* (0.76%). There was no significant difference in the prevalence of Helminthiasis between sexes and ages (Roba & Hiko, 2022).

A study in Mekelle town and its surroundings, Ethiopia, aimed to determine the prevalence of significant GIPs of horses in and around Mekelle (Quiha & Wukro). Four hundred fecal samples were collected and examined by sedimentation, flotation, and direct methods. The overall prevalence was found to be 59.3%, including mixed infections. Among the parasites determined, the prevalence of *strongyles* spp. (27%), *Oxyuris equin* (8.8%) *Anoplocephala* spp. (2%), *Parascaris* *equirem* (1.8%), *Gstrophilus* sp. (1.3%), *Strongyloid westeri* (0.8%), *Fasciola* spp. (0.5%), *Eimeria* species (0.5%) and mixed infections (16.8%). A significant association was observed between the horses' body condition and the months of the study period. The prevalence of gastrointestinal parasitic eggs with the age group was not significantly associated (Belete & Derso, 2015).

Another study in Mekelle was intended to estimate the prevalence of common equine strongyles. Approximately 25 grams of study horse rectal stool samples were drawn, labeled, and taken to the laboratory for joint examination. A flotation technique was used to separate the parasitic eggs from the feces, followed by microscopy to identify the eggs of robusts based on morphology. Of the 384 samples collected, 204 were positive for robusts, with an overall prevalence of 53.13%. The prevalence of consequential types in horses was estimated at 53%. Accordingly, among the six risk factors considered, only three factors (age, type of administration, and body condition scores) were found to strongly influence the incidence of infection (Negash et al., 2021). Belay et al. (2016) studied horses around Kombolcha town to estimate the prevalence of gastrointestinal tract (GIT) helminth infections and to identify the common ones. Direct fecal smear, sedimentation, and floatation techniques were used to determine the eggs of parasites in feces. A total of 384 horses, donkeys, and mules were examined for GIPs. The overall prevalence of GIPs was (73.2%); 281/ 384 with (57.0%): 73 /128 in horses. Prevalence of *Strongyle* spp (44.5%); *Parascaris equorum* (3.1%); *Anoplocephala* spp. (3.1%) and *Oxyuris equi* (2.3%) in horses.

Another study conducted by Mathewos et al. (2021) determined the prevalence of gastrointestinal tract (GIT) in horses in the Hawassa district. A total of 214 fecal samples were collected from randomly selected equines (112 donkeys and 102 horses) and examined using standard techniques: flotation, sedimentation, and modified Baermann. GI helminth eggs were confirmed using 10x and 40x magnification power. The prevalence of GI helminths in horses was 63% (65/102). Out of all positive samples, different species of parasites were identified, like *Strongyle* spp. (56.1%), *Strongyloides westeri* (35.5%), *Parascaris equorum* (25.2%), *Anoplocephala perfoliata* (15.8%), *Oxyuris equi* (9.3%), *Fasciola hepatica* (8.8%), *Gastrodiscus* spp. (5.6%), and *Dictyocaulus arnfieldi* (1.8%).

In South-eastern Ethiopia, a study was conducted in and around Bekoji, utilizing direct fecal smear, flotation techniques, and larval cultures to identify species and assess the incidence of GIPs in donkeys and horses. In this study, horses made up 90%. The coprological research revealed mixed parasite infections, *Nonmigratory strongylids*, *migratory strongylids*, *Parascaris equorum*, and *Oxyuris equi*, respectively; the prevalence of gastrointestinal nematode parasites was 87%, 8.3%, 2.5%, 1.4%, and 0.8%. *Nonmigratory Strongylids* + *Migratory Strongylids* were more common (51.5%) in mixed infections. Poorly maintained horses were 2.94 times more likely to be infected than donkeys. There is a significant relationship between the species and physical characteristics of animals and the prevalence of gastrointestinal nematodes. The prevalence of gastrointestinal nematode parasites did not differ statistically significantly according to the sex or age of the horses (Mathewos et al.,2022).

Also, in the Alage region, 384 horses from three separate peasants were sampled to identify the incidence of Strongyle in horses in and around the Alage district and its risk factors. Laboratory analysis employed the flotation method. A diagnosis of *strongylosis* was made in 67.19 percent of the patients. The infection magnitudes in Naka, Dilbato, and Koricho peasant associations were

64.1%, 68%, and 69.5%, respectively. Male horses (68.1 percent), young (84.4 percent), and in poor physical condition (90 percent) also had higher levels of animal-related prevalence. Statistics showed that the sickness was significantly associated with age and body condition ratings (Alemayehu et al., 2022).In Libya, Elmajdoub et al. (2022) Investigated the Prevalence of GIPs of Equestrian Clubs Horses; fecal samples were obtained from 50 randomly chosen horses of different sexes and ages and analyzed using sedimentation techniques. The overall prevalence of eggs, larvae, or cysts was (98.0%). However, the most abundant parasites that were detected were *Anoplocephala* spp. (17%), *Parascaris equorum* (17%), *Moniezia* spp (20%) and *Cryptosporidium parvum* (33%). Among other constraints facing horses in Lesotho are helminth infections, leading to significant health issues that limit their work potential and efficiency. A study was conducted to determine the prevalence and abundance of the parasite population of horses in the Maseru district, Lesotho. A total of 720 fecal samples were collected and analyzed using the sodium chloride flotation technique to detect the presence of GIPs. The overall prevalence of Nematode was (88.8%), and the overall prevalence of Cestodes was (9.2%) while for coccidia, the overall prevalence was (4.2%). These results demonstrated that nematodes are major health-threatening parasites for horses in the Maseru district of Lesotho (Kompi et al., 2020).

South America

Heading to South America, Morales et al. (2012) conducted a study in Venezuela to estimate the prevalence of GIPs in horses. Eight hundred and ninety-four fecal samples were collected from a breeding center (494 females and 400 males) and examined using McMaster flotation methods. The most abundant species were *Strongyles* (62%), *P. equorum* (8%), *Oxyurus equi* (1%) and 30% were negative for the presence of parasite eggs. In Brazil, a study aimed to describe the pathological findings of fatal parasite-induced enteritis and typhlocolitis caused by *cyathostominae*, *Eimeria leuckarti*, *Balantidium coli*, and *Strongyloides* westeri in horses. Results showed that ten horses died during this research, with *cyathostominae*, *Eimeria leuckarti*, *Balantidium coli*, and *Strongyloides westeri* being the main reasons for mortality in horses (Bianchi et al., 2019).

Recently, a study done in Chile, Araucania, focused on the general prevalence of *Cryptosporidium* spp. Fecal samples from 100 randomly selected horses were taken from rural Mapuche communities from four municipalities in the Araucana Region. The widespread prevalence of *Cryptosporidium* spp. was 67.0%, with 51.0% in males and 49.0% in females. The prevalence by the municipality was 60.0%, 80.0%, 64.0%, and 64.0% in Curarrehue, Lonquimay, Padre las Casas and Teodoro Schmidt, respectively. The prevalence by age was 95.4% of horses tested positive for *Cryptosporidium* between birth and 6 years, with 27.3% in the age group between 7 and 10 years. There was no presence of *Cryptosporidium* spp. in the age group older than 11 years, showing a significant relationship between the age of the animal and the presence of *Cryptosporidium* (Tuemmers et al., 2023).

North America

A study conducted in central Mexico by Romero et al. (2020) evaluated the prevalence of gastrointestinal parasite infection and risk factors in horses. Stool samples from 218 horses from different regions were analyzed using the flotation technique. Among the 218 examined samples, 103 (47.24%) were positive with several GIPs, with *Strongylus* spp. being the most prevalent (23.85%), followed by *Trichostrongylus* spp. (21.56%) and *Parascaris* spp. (11.93%).

Europe

A study in Greece aimed to investigate and compare parasites found in feces from stabled and grazing horses. A total of 223 fecal samples were collected from horses from diverse parts of Central and Northern Greece, 150 from stables, and 73 from grazing wild horses. Parasitologic investigation was performed by applying three fecal examination techniques (zinc flotation and direct smears) to detect the presence of parasites. Seventy-seven out of the 223 were positive (34.5%). The most common parasites seen in the stabled horses were eggs of *strongyles*, *Strongyloides* spp. *Anoplocephala* spp. *Habronema* spp. and *Parascaris equorum* and oocysts of *Eimeria* spp. and *Cryptosporidium* spp., in the grazing horses, *Anoplocephala* spp. *Strongyles* spp. were significantly more frequent in stable horses than in wild grazing horses (Papazahariadou et al., 2009).

In the same year, a study in Spain by Francisco et al. (2009) determined the effect of some essential factors (stress, age, and gender) on helminth infections; stool samples were analyzed with fecal examination techniques. The main strongylid genera identified were *Trichonema* spp. and *Cyalocephalus* spp. (small strongyles), *Strongylus* spp., and *Triodontophorus* (large strongyles). The prevalence of gastrointestinal Nematoda was 89% and 1% Cestoda. The percentage of horses with strongyloid parasites was (89%), *Parascaris* spp. (11%), and (3%) for *Oxyuris* spp. The highest prevalence for ascariosis was observed in the youngest horses (less than three years); most females were significantly more infected than males.

In Poland, a study was conducted to determine the prevalence of infection with GIPs in local horses and those imported from the Netherlands. The prevalence and rate of infection were selected based on coproscopic examination using Willis-Schlaf and McMaster methods. Fecal samples were tested for the presence of *Cryptosporidium* spp. using a modified staining method. The prevalence of infection with GIPs in Polish Koniks imported from the Netherlands and those from Poland was 100%. Results identified *Cyathostominae* (94.74%), *Strongylidae* (89.47%), and *Parascaris equorum* (5.26%) roundworms. Domestic Polish Koniks were found to harbor *Strongylidae* (100%) and *Cyathostominae* (100%) Nematodes, as well as *Cryptosporidium* spp. protozoa (2.27%). Local horses were more infected with *Cyathostominae* and *Strongylidae* than horses imported from the Netherlands (Pilarczyk et al., 2010).

A study was conducted in Warmia and Mazury Regions in Poland. Sokół et al. (2013) aimed to determine the prevalence of parasitic infections in horses kept in individual and agrotouristic farms during the pasture season. Five hundred and twelve Fecal samples were examined. Individual farms (64.3%) were infected. Horses from agrotouristic farms (51.7%) were infected. *Cyathostominae* eggs were found in most samples. Eggs of *Strongylus* spp., *Parascaris equorum*, *Strongyloides westeri*, and tapeworm of *Anoplocephala* were found in some individuals. The number of infected horses from individual farms was higher than from agrotouristic farms because of the excellent care paid for cleaning pastures and the regular deworming.

A study in Romania aimed to provide insight into the intestinal parasite burdens in Romanian horses using coprological examination. A total of 158 horses were examined. Fresh fecal samples were collected and analyzed for the presence of GIPs using the sodium chloride flotation technique. The overall prevalence of intestinal parasites in the positive horses were strongyles (87.97%), *Parascaris equorum* (13.9%), *Strongyloides westeri* (5.06%), and *Eimeria leuckarti* (1.90%). The highest prevalence and intensity rate belonged to strongyles (Ioniță et al., 2013). After three years, Buzatu et al. (2016) conducted a study to describe the community and epidemiology of intestinal parasitic infections in working horses in Romania. Four hundred fifty-nine fecal samples were allocated and examined quantitatively using the sodium chloride flotation method. 75.40% were positive for *strongyle* infection, *Parascaris equorum* (7.40%), *Eimeria leuckarti* (7.20%), *Strongyloides westeri* (3.70%), and *Anoplocephala* spp. (2.61%). Young horses (1-5 yrs) were the most infected for all the species, except *Anoplocephala* spp., with which horses over 10 years old were more frequently infected.

In Germany, a study by Rehbein et al. (2013) conducted the prevalence, intensity, and seasonality of GIPs in abattoir horses. Four hundred fecal samples were completed. 310/400 horses (77.5 %) were demonstrated harboring GIPs. The following parasites were found: *Gasterophilus intestinalis* (2.25%), *Gasterophilus nasalis* (0.25%), *Trichostrongylus axei* (11.0%), *Habronema majus* (8.0%), *Habronema muscae* (26.5%), *Habronema* spp. (5.5%), *Parascaris equorum* (11.3%), *Anoplocephala perfoliata* (28.5%) and *Paranoplocephala mamillana* (1.0%).

In the capital of Germany, Berlin. Gehlen et al. (2020) did a study on the occurrence of intestinal helminths in 620 adult horses with a history of colic to investigate the association between colic and helminth infection. The highest infection rates were seen for strongyles (41.8%), followed by *Anoplocephala perfoliata* and *Parascaris* spp. (both 0.8%) and (1.1%) *S. vulgaris*. This study used the FLOTAC method, employing a saturated salt solution.

One study investigated the abundance and risk factors of strongyle egg shedding in horses in Italy. Overall, the results showed that approximately (40%) of all horses in Italy shed *strongyle* eggs and that almost (90%) of stables have at least one infected animal. In addition, most parasite eggs are found in just a small fraction of the horse population, confirming the need for improved parasite control strategies (Scala et al., 2020).

In Estonia, Tartu County, a study by Oinonen et al. (2022) aimed to assess the prevalence of GIPs, emphasizing strongyle infection in horses. Fecal samples were collected from 102 randomly selected horses from six different stables. All the samples were analyzed by a modified McMaster method for the presence of gastrointestinal parasite eggs. The prevalence of GIPs in horses was (67,65%). Only strongyle-like eggs were detected in fecal samples. The majority were identified as small strongyles.

Ilić et al. (2022) conducted a study to determine the prevalence of helminths and the factors affecting it by examining 548 samples from horses of different breeds, ages, and sexes from four regions in Serbia. The total prevalence of helminths was (77.19%): *P. equorum* (8.57%), *O. equi* (3.65%), *strongylid* eggs (71.17%), and *Anoplocephala* spp. (0.91%).

A study in Ireland by Elghryani et al. (2023) aimed to determine the prevalence of helminth infections in the Irish horse population using fecal egg counts. For the 2700 horses included in the study, the prevalence of gastrointestinal helminth infections was *Strongyles* spp. (52.40%), *Parascaris* spp. (4.22%), *Anoplocephala* spp. (2.59%), and *Strongyloides westeri* (0.89%).

Australia has over 400,000 wild horses, the largest wild equids population in the world, in early 2019, Harvey et al. (2019) collected a total of 289 fecal samples from horses to do a systematic review of gastrointestinal nematodes of horses from Australia to test for any parasites present. Total strongyle egg counts were (89%) 257 out of 289; the high prevalence of *S. vulgaris* suggests observing is required and essential when adopting wild horses or when domestic horses graze in environments inhabited by wild horses. The main GIPs reported in Australian horses across different regions of Queensland, New South Wales, Victoria, and Western Australia were cyathostomins (at least 28 species), *Draschia megastoma*, *Habronema muscae*, *H. majus*, *Oxyuris equi*, *Parascaris equorum*, *Strongyloides westeri* and *Trichostrongylus axei*. Some *Cyathostomins* species (e.g., *Cyathostomum catinatum, Cylicocyclus nassatus*, and *Cylicostephanus calicatus*) have been found more frequently (more than 70%). Samples were diagnosed using the traditional McMaster egg counting technique (Saeed et al., 2019).

Asia

An investigation in Western China by Liu et al. (2022) determined the abundance of the gastrointestinal parasite in Yili horses. A total of 124 fecal samples from randomly selected horses were analyzed. 118 out of 124 came positive with the following proportions of overall parasite abundance: *Strongylus equinus* (82.26%), *Strongylus edentatus* (23.39%), *Cyathostomum coronatum* (34.68%), *Cyathostomum subcoronatum* (18.55%), *Cylicostephanus longibursatus* (56.45%), *Cylicostephanus calicatus* (75.81%), *Cylicocyclus radiatum* (3.23%), *Cylicocyclus nassatus* (72.58%), *Cylicocyclus ultrajectinus* (3.23%), *Cylicocyclus elongatum* (6.45%), *Cylicodontophorus bicoronatum* (8.87%), *Cylicodontophorus euproctus* (13.71%), *Cylicodontophorus pateratum* (23.39%).

A study was performed in Siri Lanka to determine the GIPs in domesticated and feral horses. Seventy-three fecal samples were collected and examined by iodine mounts direct saline, Sheather's modified sucrose flotation, simple test tube flotation, and sedimentation by McMaster counting technique for the positive samples. All the feral horses (14) were infected. In the domesticated horses, free grazers had a higher prevalence of GIP infections (46.7%) than the stabled ones (18.2%). There was no significant difference between protozoan and helminth infections. Six species of parasites were recorded: *Anoplocephala* sp., *Parascaris equorum*, *Strongylus* sp. *Isospora* sp. *Entamoeba* sp. and *Giardia* sp. were recorded (Dissanayake et al., 2017).

India is one of the biggest countries that value horses, according to a previous study in Kashmir Valley aimed to identify and assess the parasitic infestations in horses under unorganized practices in Kashmir. Nine hundred thirty-five fecal samples were examined by sedimentation and floatation techniques. The overall infestation was 93.26%. Most of them had more than one type of parasitic infection. *Trichonema* sp. (96.78%), *Strongylus* sp. (81.19%), *Triodontophorus* sp. (41.39%), *Dictyocaulus* sp. (14.10%), *Oxyuris* sp. (9.40%), *Paranoplocephala* sp. (8.14%), *Strongyloides* sp. (6.19%), *Parascaris* sp. (4.01%), *Amphistome* sp. (0.91%) and *Eimeria* sp. (0.34%) (Pandit et al., 2008). Another study in Jabalpur showed an overall prevalence of gastrointestinal nematodes in horses (59.25%) from 135 fecal samples. More prevalent was found in unorganized stables (65.45%) than in organized stables (32%). Species of nematodes identified in the study included single infections like *Strongyles* (25%) and *Parascaris equorum* (18.75%), followed by mixed infections like *Strongyles* and *Parascaris equorum* (47.50%). The sugar floatation technique detected Nematode and Cestode eggs (Yadav et al., 2014). Two studies were conducted to ascertain the incidence of GIPs in horses in Karnataka and Maharashtra. For Karnataka, 100 horse fecal samples were collected and examined by direct smear and sedimentation methods. Among all the 100 samples analyzed, (84.0 %) were found positive for various gastrointestinal helminths. Out of 84 positive cases, (52.38 %) were found positive for *Strongylus* spp., (10.71 %) *Parascaris equorum*, (7.14 %) had *Gastrodiscus* spp., (4.76 %) harbored *Oxyuris equi, and the remaining* (25.0 %) had a poly-infection of *Strongylus* spp., *Strongyloides* spp., and *Gastrodiscus* spp. (Adeppa et al., 2016).

In Maharashtra, 1,304 fecal samples from several species of horses collected at five different locations in the Mumbai and Pune districts were analyzed. For four nematode species, the prevalence of helminth infection was determined to be 20.63% overall. *Strongyles* (10.81%), *Strongyloides westeri* (13.19%), *Parascaris equorum* (0.23%), *Dictyocaulus arnfieldi* (0.23%); two Trematodes, *Amphistomes* (1.38%), and *Schistosoma indicum* (0.31%); and only one species of nematode, *Anoplocephala* spp. (0.07%) (Matto et al., 2016).

Valibasha et al. (2019) conducted a study to estimate the prevalence of GIPs in horses in three districts of Karnataka, India. Of the 692 collected fecal samples, 220 (31.80%) animals were positive for GIPs. The parasites with high prevalence rate were *Parascaris equorum* (14.59%); followed by *Strongyle* (11.99%); The prevalence in unorganized farms (80.30%) was higher as compared to organized farms (26.68%).

In another region of India, a recent study showed the prevalence of gastrointestinal helminths in the horse population of Rajasthan. A total of 175 horses were examined, and (30.85%) of horses were found positive for gastrointestinal helminth infection. Among various GI helminth infections reported in this study, *Strongyles* spp. (48.14%); were the most prevalent gastrointestinal helminths, followed by *Parascaris equorum* (16.66%); the sex prevalence found the highest prevalence (37.77%) in females and lowest (23.52%) in males. Samples were subjected to flotation and sedimentation techniques (Nagar et al., 2022).

In Punjab, a study by Sumanpreet et al. (2018) determined the presence of parasitic ova. The overall prevalence of GIPs was 19.06%, according to a microscopic examination of *Strongyle* sp., *Parascaris equorum*, mixed infection (*Strongyloides westeri* and *Strongyle* sp.), *amphisomes*, *Eimeria* sp., and *Trichuris* sp. are some of the GIPs that have been identified.

In another study held in and around Akula, 21 adult horses, regardless of sex, were screened for intestinal nematode infection by examining the stool sample by sedimentation and flotation methods. Out of which, 12 fecal samples were found positive for mixed and single infection of *Strongylus* spp. and *Parascaris* spp. (Katre et al., 2020).

In the Bangladesh National Zoo, Dhaka, Bangladesh, a study was performed to identify the prevalence of GIPs in horses using 48 fecal samples. The overall prevalence was 97.92 %. The highest prevalence was in *Parascaris equorum* (77.1 %), *Hymenolepis nana* (70.8 %), *Isospora* spp., (62.5 %), *Ancylostoma duodenal* (50 %), *Toxocara* spp. (39.6 %), *Entamoeba* spp. (35.4 %), *Trichuris* spp. (25 %), *Fasciola hepatica* (20.8 %) in *Capillaria* spp. (16.67 %), *Taenia* spp. (8.3 %) and *Opisthorchis sinensis*, 4.2 % in *Moniezia benedeni* and *Thysaniezia* sp. There was a significant difference in prevalence between female and male horses. The highest prevalence was recorded in female horses. (Khanum et al., 2021).

Oli et al. (2018) aimed to determine the prevalence of GIPs in horses in seven villages of the Rukum district of Nepal. A total of 105 fecal samples from horses were examined using concentration techniques. The overall prevalence of GIPs was (84.76%). Among them, *Strongylus* spp. showed the highest prevalence (51.42%), followed by *Eimeria* spp. (20%); *Trichostrongylus* spp. (14.28%);

Trichonema spp. (13.33%); *Parascaris equorum* (10.47%); *Balantidium* spp. (9.52%); *Dictyocaulus* spp. (8.57%); *Triodontophorus* spp. (7.61%); *Gastrodiscus* spp. (6.66%); *Oxyuris equi* (4.76%); *Entamoeba* spp. (3.80%); *Schistosoma* spp. (1.90%); and unidentified larvae (7.61%). Fast forward three years, Fecal samples collected again from 105 randomly chosen horses were examined using concentration techniques. The overall prevalence of GIPs was found to be (81.90%). Among all the identified parasites, *Strongyles* showed the highest prevalence (68.57%), followed by *Strongyloides* spp. (23.80%) and *Parascaris equorum* (14.28%) (Devkota et al., 2021).

In Eastern Indonesia, Sumba, a study was conducted on horses to identify the various endoparasites and the frequency and severity of parasite attacks. The findings revealed that 6 species of endoparasites, including *Strongylus* sp., *Trichonema* sp., *Dictyocaulus arnfieldi*, *Strongyloides* sp., *Triodontophorus* sp., and *Ascaris* sp., were discovered in the 18 Sumba horses that were tested. *Strongylus* sp. was 86% prevalent. *Strongyloides* spp. had a prevalence of 22%, *Trichonema* spp. had a prevalence of 38%, *Dictyocaulus arnfieldi* had a prevalence of 53%, and *Ascaris* spp. had a prevalence of 14% (Sinaga et al., 2022).

Middle East

As for the Middle East, few recent studies are available to the public. For example, In the Konya region, Turkey, Uslu & Guclu (2007) conducted a study to show the prevalence of the endoparasites of horses of different ages from the Konya region. One hundred eleven fecal samples were collected and examined using Baermann-Wetzel, floatation, and sedimentation techniques. The samples were 100% encountered by *Strongylidae* (100%), *Parascaris quorum* (10.81%), *Strongyloides westeri* (7.2%), *Fasciola* sp. (3.6%), *Anoplocephalidae* (2.7%), *Oxyuris equi* (1.8%), *Trichuris* sp. (0.9%), *Dicrocoelium dendriticum* (0.9%), *Eimeria leucarti* (4.5%), and *Eimeria* sp. (12.61%), respectively. Years later, in the same region, Ceylan et al. (2020) carried out another study to identify the gastrointestinal helminths of wild horses. Flotation, sedimentation, Baermann-Wetzel, and stool culture techniques were used to assess the fresh feces of 66 horses. The 66 stool samples tested were all positive for helminth, and the following parasites were found: *Trichonema* spp. (100%), *Poteriostomum* spp. (45.5%), *Strongylus vulgaris* (34.9%), and *Strongylus equinus* (27.3%) were also found in the stool samples. *Oxyuris equi* (3.0%), *Parascaris equorum* (1.5%), *Strongyloides westeri* (1.5%), *Strongylus edentatus* (7.6%), *Triodontophorus* spp. (4.6%), *Gyalocephalus* spp. (4.6%), *Oesophagodontus* spp. (9.1%), and *Strongylus edentatus* (4.6%) were also found at lower levels. There were 23 wild horses (34.9%) with multiple helminth infections, 18 (27.3%) with three species, 15 (22.7%) with one species, 7 (10.6%) with four species, and 3 (4.5%) with five species.

A study in Iraq included hundreds of fecal samples from horses and donkeys (44 horses and 56 donkeys). The results showed a 100% positive infection of horses and donkeys. As for horses only, the prevalence of *Strongylidae*, *Parascaris equorum*, *Strongyloides westeri*, *Trichostrongylus axei*, *Oxyuris equi*, *Cryptosporidium* spp., *Balantidium coli* and *Eimeria* spp. were (50%), (40.90%), (22.72%), (25%), (11.36%), (20.45%), (15.90%) and (6.81%) respectively. The samples were examined using the direct smear, sugar-saturated flotation, and sedimentation methods (Wannas et al., 2012). One year later, in Erbil province, North Iraq, Zangana et al. (2013) conducted a study to determine the prevalence of intestinal parasites in horses in different private breeding stables in Erbil province, north Iraq. Ninety-two fecal samples were collected and examined by flotation concentration, cellophane tape, and acid ether methods. The rate of prevalence was 70.6%. *Strongylus vulgaris*

(29.35%), *Parascaris equorum* (19.56%), *Oxyuris equi* (8.7%), *Strongyloides westeri* (5.43%), *Anoplocepha* spp. (4.35%) and *Eimeria leukarti* (3.26%). There was no significant difference in the infection rate between the sexes. The prevalence rate is higher in ages less than 5 years (84%) than in ages more than 5 years (61.1%). The clinical signs of infected horses with *P. equorum* and *S. vulgaris* rough coat revealed emaciation, anemia, dehydration, colic pain, and perianal scratching.

A study aimed to describe the prevalence and species distribution of horse intestinal parasites in Israel. Four hundred eighty-five fecal samples were collected and examined by floatation and egg counts. *Strongyle* eggs were found in (24%). *Ascarids* were found in (5%). Singular flatworm eggs (family *Anoplocephala*) were detected in two samples. Horse age, gender, season, and housing were significantly associated with ascarid infestation (Tirosh-Levy et al., 2015).

A study was conducted in Northwest Iran, Urmia. Two hundred twenty-one working horses were gathered from 14 villages to provide feces samples to identify GIPs. Forty-six horses (20.8%) had feces samples, but none had parasite eggs or oocysts. One kind of parasite was present in 145 positive horses (48.9%), whereas multiple infections with two and three parasites were seen in 49 (22.2%) and 18 (8.1%) horses, respectively. Little *strongyles* had the highest incidence and intensity rates. *Strongyles*, which were present in the majority of the positive horses, were responsible for 72.9% of the intestinal parasite eggs and oocysts, followed by *Oxyuris equi*, 22.6%, *Parascaris equorum*, 12.2%, *Anoplocephalidae*, 6.3%, *Fasciola* spp., 3.2%, and *Eimeria leuckarti*, 0.5%. Little *strongyle* larvae were identified as being the most common (97.6%), followed by *Strongylus edentatus* (22.6%), *S. equinus* (18.5%), and *S. vulgaris* (6.5%) (Avassoli et al., 2010).

For Ardabil City, Shabazi et al. (2018) carried out a study to estimate the GIPs of horses. Flotation techniques examined a total of 50 fecal samples. GIPs encountered were *Strongyles* (34%), *Parascaris equorum* (20%), *Strongyloides westeri* (12%), *Anoplocephalidae* (6%), *Trichostrongylus* spp. (4%); *Ornithobilharzia turkestanicum* (4%) and *Dicrocoelium dendriticum* (2%). According to the results, no significant differences in infection rates were found between male and female horses. And to the most recent study in Iran, held in the province of Tehran. The Clinton Lane approach was used to find the parasite eggs in the EPG and feces. The presence of GIPs was assessed in 294 horses in total. 61 out of 294 people had GIPs, making up 20.7% of the population (48 from 294). Using an adhesive tape test, it was possible to identify *Parascaris equorum*, 4.42% (13 from 294) *Strongyle* spp., and 0.68% (2 from 294) *Oxyuris equi*. Horse feces often included 1–12 worm eggs, strongyles, and parascaris. The outcomes of stool cultures revealed that the third-stage larvae were from *tiny strongyles* (Mirian et al., 2019).

Most importantly, two studies in Saudi Arabia conducted the prevalence of GIPs in horses, starting from the capital; a study on the prevalence of non-strongyle GIPs of horses in the Riyadh region of Saudi Arabia conducted a necropsy for 45 horses. 39 out of 45 horses were infected with GIPs, with an infestation rate of (86.6%), and infestations with seven nematode species. The most abundant parasites were *Strongyloides westeri* (64.4%) and *Parascaris equorum* (28.8%), followed by *Habronema muscae* (22.2%) (Al-Anazi et al., 2011).

Later, Al-Qudary et al. (2015) aimed to document the prevalence of GIPs among horses in the Eastern Province of Saudi Arabia. Three hundred and two horses were tested. Fecal samples were examined using direct smear, flotation, and sedimentation techniques. The overall rate of infection was (30.46%). *Tritrichomonas equi*, *Parscaris quorum*, *Dictyocaulus urnfield*, Strongyle-type ova, and

Eimeria leukarti were detected. The geographic distribution of these parasites indicated higher prevalence in horses in the Al-Ahsa area and seasonal prevalence.

CONCLUSIONS

GIPs are a common problem in horses worldwide, leading to significant health problems if not managed. To prevent the spread of parasites, horse owners must follow regular deworming programs and conduct proper pasture management. Monitoring the horse's overall health and behavior can also aid in detecting any potential parasite-related concerns early.

Recommendations

We recommend using more accurate techniques to identify parasites and reduce unknown percentages. The results of this study can be used to inform parasite control and management strategies for horses in the region, which can help improve their health and welfare.

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MICROBIAL BIODIVERSITY AND BIO-MITIGATION ASSESSMENT – RECENT ADVANCES AND STRATEGIES

Author name and information

Hafiz M. N. Iqbal

Quimicos 10410-, Parque Tecnologico, Monterrey CP 64700, Nuevo Leon, Mexico.

Correspondence: hafizmniqbal@gmail.com

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Bioremediation; Hazardous contaminants; Water matrices; Mitigation strategies

ABSTRACT

The ever-increasing population growth and industrialization have stemmed the genesis of several environmentally related contaminants of emerging concern. Numerous of them influence the living ecosystem, including pharmaceutically bioactive residues, antibiotics, pesticide-based polluting agents, industrial-based synthetic dyes, etc. Indeed, several approaches, e.g., chemical and physical treatments and their possible combinations, have been proposed, established, and executed to treat and remove environmental pollutants from numerous matrices. However, the existing approaches are becoming inefficient due to the growing quantity and complex nature of environmental contaminants. Therefore, more effective and suitable measures are needed to sustain the green environment by remediating and mitigating the hazards of emerging concerns associated with environmentally related pollutants. Among other recent approaches, microbial biodiversity, bio-mitigation assessment, and removal of current pollution load using robust materials are of supreme interest with evident merits. In addition, microorganisms have an enormous capacity to adapt and survive in divergent environments and, once established, produce unique biocatalytic molecules that break down and transform the contaminating agents, thus making it possible to revive the polluted matrices. Likewise, nanozymes are nanostructured materials with enzyme-like activities that effectively catalyze the breakdown of contaminating agents and, thus, are other suitable candidates to upgrade modern bioremediation practices. This review thoroughly examines the most recent developments in novel bio-mitigation and nanozyme-based approaches. Finally, we highlight their current challenges to provide a perspective on potential future research directions.

INTRODUCTION

Environmental pollutants, e.g., antibiotics and pesticides, can directly or indirectly have harmful influences on humans and the entire living environment. These effects include respiratory problems, developmental issues, and cancer (Bilal et al., 2019; Saeed et al., 2022). In addition, pollution can cause damage to ecosystems, including the loss of biodiversity and changes in the behavior and survival rates of different animal and plant species. Hence, it is essential to eliminate and take preventive measures to reduce pollution to shield the living environment. There are many examples of environmental polluting agents, such as carbon dioxide emissions from cars and factories, oil spills in the ocean, chemical waste dumped into rivers, plastic pollution in the sea, and deforestation (Cárdenas-Alcaide et al., 2022). Other examples include hazardous and pharmaceutically active micropollutants, which can harm people and the environment (Bilal et al., 2022).

Modern mass production attempts have signified environmental contamination issues. By considering the adverse hazards of environmentally related polluting agents, suitable measures are needed to sustain the green environment by remediating and mitigating the dangers associated with environmentally related pollutants of emerging concerns (Liu et al., 2019). The emerging concerns related to environmental impacts and toxic effects of contaminants on humans are documented in the literature. For example, the improper disposal and misuse of antibiotics are related to antimicrobial resistance (González-González et al., 2022c; WHO, 2014). Endocrine-disrupting chemicals (EDCs) can cause adverse health effects as well – even at very low concentrations – due to their capacity to alter the endocrine system and bioaccumulate (González-González et al., 2022b). The global concern caused by these pollutants and others like dyes and pesticides is maximized due to their persistence, bioaccumulation, and frequent occurrence in environmental matrices. Examples of sources/routes of antibiotics and pesticide-based contaminants of high concern and their toxicological impacts are shown in Figure 1.

Many techniques have been proposed and categorized into three main groups: physical, chemical, and biological. Furthermore, novel approaches involving integrated systems involving more than one technique have demonstrated enhanced efficiencies (Al-Maqdi et al., 2021). The chemical and physical methods have shown good efficiencies and large-scale application, but limitations remain, like high energy demand, expensive operation, and formation of toxic by-products (Al-Maqdi et al., 2021; Bilal et al., 2019). In these circumstances, biological methods are greener, safer, less expensive, and more eco-friendly alternatives.

Microbial bioremediation is a process where microorganisms are used to clean up contaminated environments. This could include breaking down and removing harmful chemicals or pollutants from soil, water, and even air (Saeed et al., 2022). Microorganisms like bacteria, fungi, and algae can break down the pollutants into less harmful substances or even convert them into harmless forms that can be safely disposed of. This process is often used in environmental cleanup efforts or industries producing many waste products. It is a natural and eco-friendly alternative to traditional hazardous waste removal methods.

Traditionally, enzymes have been considered highly efficient biocatalysts with excellent selectivity. Thus, enzyme-assisted methods allow the enzymatic conversion of pollutants into smaller and less toxic molecules. However, enzymatic treatments for water decontamination have several drawbacks, such as reduced efficiency due to harsh environmental conditions and limited enzyme reusability (Al-Maqdi et al., 2021). This review examines the mechanism of action of microbial bioremediation by analyzing recent advances in the treatment of water contaminated by emerging contaminants such as antibiotics and pesticides. An in-depth discussion is also given regarding the function of nanomaterials as potential artificial enzymes for the degradation of dyes and phenolic compounds.

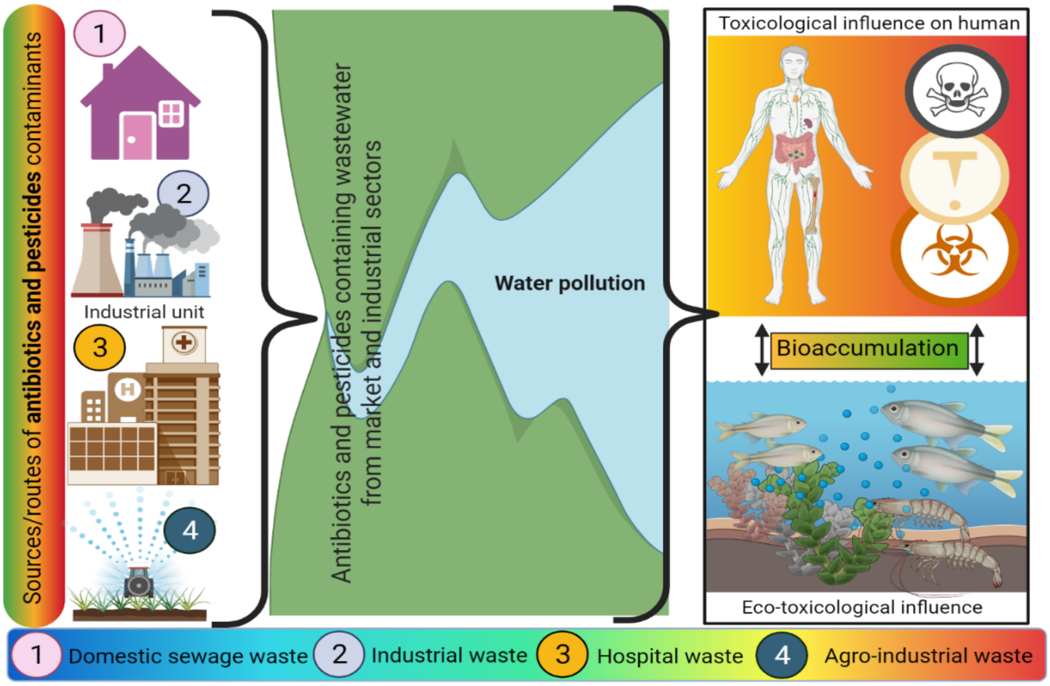

Figure 1: Sources/routes of antibiotics and pesticide-based contaminants and their toxicological impacts on humans and the environment. Figure was created with BioRender.com and extracted under premium membership.

Microbial-assisted degradation of antibiotics and pesticides

In natural environmental matrices, several microbial species thrive to naturally remediate the environment polluted by numerous hazardous polluting agents, e.g., antibiotics and pesticides based on active residues. Microbes transform any/many of these toxic residues into non-toxic forms through their natural and super-active enzyme system. In most cases, the end-products of microbial remediation include H_2O, CO_2, and other small metabolites, which microbes use as principal growth substrates throughout the reaction process (Saeed et al., 2022). Antibiotics belong to the important class of drugs used to fight bacterial infections in living beings. Regardless of the undeniable benefits of antibiotic drugs, any/many of them can also threaten the environment once disposed of in sewage systems. Once they enter the environment through wastewater discharges and agricultural runoff, antibiotics can bioaccumulate and thus pose harmful effects on living aquatic organisms (Bilal et al., 2019, 2020; Cruz-Cruz et al., 2022). Besides the free discharge of antibiotic residues in sewage matrices, the abuse and mismanagement of antibiotics in humans and animals have led to antibiotic-resistant bacteria. Thus, it is imperative to reduce the amount of antibiotics released into the environment by properly disposing of expired medications and investing in research to develop new antibiotics and alternative treatments.

In microbial-based bioremediation treatment of antibiotics, microorganisms such as bacteria and fungi act as the agents of degradation and perform an appropriate breakdown of the target antibiotic molecules effectively. These organisms produce novel enzymes and use them to break down antibiotics into more simple substances that are no longer harmful or at least less toxic to the environment. However, microbial degradation can prevent this problem by breaking down the antibiotic before it can cause harm (Singh Aditi and Saluja, 2021). Another antibiotic that is subject to microbial degradation is chloramphenicol. It can cause environmental problems if it accumulates in the soil or water. Microbial degradation can prevent this by breaking down the drug into safer, simpler substances. In conclusion, microbial degradation is crucial for reducing the environmental impact of antibiotics such as tetracycline and chloramphenicol. It is essential to focus on strategies to promote this process, such as optimizing environmental conditions and finding new microorganisms that can effectively degrade antibiotics. By doing so, the harmful effects of drugs can be minimized in the environment and safeguard a healthier planet for future generations.

Antibiotics can be removed by three primary mechanisms, including biodegradation, bioadsorption, and bioaccumulation, with biodegradation being the most common mechanism (Eheneden et al., 2023). An ample range of antibiotics have been removed by microbial approaches; for example, Gao et al. (2023) reported the removal of sulfadiazine by employing *Chlorella* sp. G-9 in a membrane photobioreactor. They reported the effective removal in synthetic wastewater, between 54 – 94 %. Moreover, they achieved simultaneous antibiotic removal and enhanced lipid production since sulfadiazine stress increased the lipid content of microalgae in the membrane photobioreactor (Gao et al., 2023). Microbial degradation has been employed for the removal of different antibiotics, such as sulfanilamide, cephalosporins, oxytetracycline, and ofloxacin, among others (Guo et al., 2016; Zhang et al., 2023; Zhang et al., 2022). Furthermore, antibiotics can be effectively removed by symbiotic systems such as microalgae-bacteria consortia, which have shown additional benefits like minimal operational costs and reduced carbon emissions (Wang et al., 2022).

There are many types of pesticides, including insecticides, herbicides, fungicides, and rodenticides. Regardless of the beneficial use of pesticides as pest-controlling agents, they also negatively influence environmental health and soil and adversely impact human health. Microbes can degrade many different types of pesticides through various metabolic processes. Microbes such as bacteria and fungi have developed the ability to break down these chemicals and effectively remove them from the environment. However, the removal effectiveness varies based on the type of pesticide and the environmental conditions in which it is found. In some cases, additional treatments may be required to remediate contaminated areas fully (Liu et al., 2019). *Stenotrophomonas* sp. G1 was evaluated for the degradation of eight organophosphorus pesticides. Among them, methyl parathion, methyl paraoxon, diazinon, and phoxim were completely degraded in 24h. Parathion also reached a good degradation efficiency of 95% within the same period. Authors reported that methyl parathion hydrolase, an intracellular enzyme presented in Strain G1 cells, is responsible for pesticide degradation (Deng et al., 2015). In another study, soil microbes sampled from different regions were tested for the degradation of imidacloprid.

Interestingly, the degradation efficiency varied according to the geographic location where the sample was recovered due to the different soil microbiota composition. It was reported that *Achromobacter* sp. could remove 100% of the pesticide in 20 days; However, its degradation activity was enhanced when combined with *Paracoccus* sp., reaching complete remotion within 15 days (Gao et al., 2021). Different microorganisms, such as Pseudomonas and Bacillus, have demonstrated excellent degradation activities for pesticides like chlorpyrifos and pyraclostrobin through metabolic pathways involving enzymes (Chen et al., 2018; Liu et al., 2023).

Emerging contaminants and traditional enzymatic techniques

Pharmaceutical products and their metabolites are a class of organic chemical compounds with a high presence in aquatic environments. Those pollutants can reach the environment by different pathways. The primary sources of these pollutants include hospital and domestic effluents with loads of pharmaceutical compounds excreted by feces and urine, inadequate disposal of expired/unused drug products, agricultural effluents with pharmaceutical content due to feces and urine of animals, and direct discharges from manufacturing plants in the pharmaceutical industry (González-González et al., 2022c). The processes currently implemented to mitigate pharmaceutical products in wastewater treatment plants (Stadlmair et al., 2018).

EDCs are compounds of different origins that have gained great relevance due to their potential to interfere with the endocrine system and generate various health problems in humans and animals (Rodríguez-Hernández et al., 2022). The occurrence of these pollutants is highly problematic since their removal is difficult by current technologies in wastewater treatment plants. In addition, the high persistence of EDCs, their high bioaccumulation in living organisms, and the environmental conditions exacerbate the problem. The biological approaches involving microorganisms or enzymes outweigh the large diversity of methods due to their many advantages and capacity to degrade various emerging contaminants. Biological processes are biotechnologies considered green catalysis due to their lower cost, lower energy expenditure, high efficiency in the degradation of contaminants, even presented at low concentrations, and eco-friendly nature (Feng et al., 2021; Morsi et al., 2021).

A recent strategy to further increase the efficiency of biocatalysts is the immobilization of enzymes in nanostructured supports. Compared to free enzymes, the immobilized-enzyme approach results in systems with greater thermal stability, easier separation, increased reusability, and catalytic activity in broader reaction conditions (Wong et al., 2019). The genetic engineering strategy is a different approach, allowing "building" modified enzemerging contaminants effectively and at a lower cost. Thus, this strategy is suitable for improving biocatalytic performance and stability in different adverse media, such as wastewater (Bhatt et al., 2021; Feng et al., 2021). Finally, enzyme-mimicking nanomaterials or nanozymes have recently emerged with superior features to natural enzymes. For instance, they possess high catalytic activity and stability, functionalization ease, and cost-effective manufacturing processes (Lopez-Cantu, et al., 2022a, 2022b). In this manner, multiple applications can be achieved using novel nanozymes, which include the efficient degradation of pollutants.

Nanomaterials as potential artificial enzymes

Enzymatic remediation techniques have demonstrated their great potential, ample diversity, and capacity to degrade emerging contaminants. However, such biological approaches have significant challenges like enzyme reusability and enzyme stability (Al-Maqdi et al., 2021). Different alternatives have evolved, with nanozyme-based techniques emerging as innovative remediation approaches. Although nanomaterial-based artificial enzymes present significant structural differences from natural enzymes, they can imitate their catalytic activity and general principles. These alternative catalytic materials encompass a great diversity of nanostructured materials. Metal-based nanomaterials have been presented as potential candidates for mimicking the activity of several natural enzymes. Some examples are platinum-based, palladium-based, silver-based, and gold-based nanomaterials. Metal-based nanozymes are characterized by easy synthesis procedures and the requirement of few reagents. Gold nanoparticles have been extensively studied for their glucose oxidase activity, while platinum nanomaterials have shown peroxidase and catalase. The study by Zhang et al. (2019) is a representative example of using gold nanoparticles as nanozymes. The authors tested gold nanoparticles and five other common nanozymes for estradiol degradation in their study. The results demonstrate that gold nanoparticles can convert estradiol into estrone by dehydrogenase-mimicking activity. In addition, it was found that the particle size had a significant effect on the catalytic activity since smaller nanoparticles presented higher efficiency (Zhang et al., 2019).

Moreover, the enzyme-mimicking activity of bimetallic nanomaterials has also been explored. Interestingly, bimetallic nanozymes have shown significant improvement compared to monometallic nanomaterials due to a synergistic effect associated with their unique physicochemical properties. In this respect, Naveen Prasad et al. (2022) evaluated the performance of Cu nanoparticles combined with different metal ions such as silver, palladium, gold, and platinum. The Cu-Pt nanozyme exhibited the highest peroxidase-mimicking catalytic activity for detecting urine glucose (Naveen Prasad et al., 2022). Similarly, metal oxide-based nanomaterials such as ferric oxide, nanoceria, vanadium pentoxide, and ruthenium oxide nanoparticles, among others, have presented enzyme-like activity. In this respect, nanoceria is one of the most widely studied metal oxide nanozymes due to its excellent multienzyme-mimicking activity. Moreover, recent research has aimed to propose different strategies to regulate and enhance the catalytic activity of nanoceria nanozymes. For instance, Yue et al. (2021) developed a coordination chemistry approach to regulate the peroxidase-like activity of ceria nanorods. The authors observed a synergistic effect that significantly enhanced the catalytic activity of nanoceria nanozyme by chelation of metal ions (Yue et al., 2021). Iron oxide, cobalt oxide, copper oxide, manganese dioxide, and vanadium pentoxide are additional examples of metal oxide-based nanomaterials with enzyme-mimicking potential (Šálek et al., 2020; Wei and Wang, 2013).

Most of the reported nanozymes are composed of metallic elements; however, in recent studies, several carbon-based nanostructures have exhibited enzyme-like activity with reduced toxicity, increased biocompatibility, and eco-friendly properties. In this category, different carbon-based nanomaterials such as fullerene-like structures, carbon nanotubes, graphene, and carbon dots have present catalytic activity (Li et al., 2013; Lopez-Cantu et al., 2022a; Sun et al., 2013). Some research groups have further studied the mechanism involved to propose effective approaches to enhance their enzyme-like activities (Duan et al., 2019; Sun et al., 2013). In addition, metal-organic frameworks (MOFs) have been studied for their potential to imitate the enzymes' activities (Wang et al., 2020). Pores within MOFs provide a unique confined environment in which new physical properties and chemical reactions can be developed (Zhou and Kitagawa, 2014). These materials contain metal nodes, which might provide possible active sites for catalytic reactions (Niu et al., 2020). MOFs-based nanozymes present multi-enzyme activities under certain conditions (Wang et al., 2020). Overall, nanozymes offer multiple advantages over natural enzymes. For example, feasibility for low-cost and large-scale manufacturing, robustness to harsh conditions, which can be presented when environmental samples are involved, long-term storage, high stability, and tunable activity are among other advantageous features. Due to these advantages, a vast diversity of nanozymes have been established and employed to deplete emerging pollutants.

Dyes-based pollutants in the environment

Dyes have been used by many industries like textile, pharmaceutical, food, cosmetics, plastics, photographic, and paper (Al-Tohamy et al., 2022). An extensive list of dyes employed in industrial activities includes malachite green, reactive blue 19, congo red, methyl orange, rhodamine B, and methylene blue, among many others. Currently, the estimated production of dyes is around 800,000 tons/year; a significant percentage ends as waste discharged without further processing (Hassaan et al., 2017). Most of the production belongs to the textile industry, which has a significant impact mainly in developing countries such as India, Bangladesh, and Pakistan; however, many of their factories are equipped with inefficient wastewater systems (Gita et al., 2017). Effluents derived from dye production contain many contaminants, including surfactants, salts, heavy metals, oxidizing agents, and reducing agents (Madhav et al., 2018). An untreated effluent could cause hazardous consequences on organisms, showing diverse adverse effects, and many studies have reported that textile dyes may also cause respiratory affections in humans after exposure, such as asthma (Tang et al., 2018). Furthermore, dyes can affect water bodies and disturb the aquatic environment, causing turbidity, changes in pH, color, and temperature, and decreasing the dissolved oxygen (Varjani et al., 2021). Therefore, the relevance of finding an effective solution to these ecotoxicological threats has increased, leading to enzyme-based and nanozyme-based approaches. Some of their limitations are the excessive costs, low efficiency, and unsustainable nature of some conventional wastewater treatments (Selvaraj et al., 2021). Concerning biological therapies, there is enough evidence in the literature to prove the effectiveness of enzymes for degrading dyes. Despite the benefits of these natural tools, many disadvantages remain, such as operational stability, recovery, production cost, etc. A nanozyme-based strategy has been proposed as an innovative solution for degrading dyes, offered by the synergistic interaction between nanotechnology and biotechnology. The degradation of dyes through this approach consists of catalytic reactions that occur by employing artificial nanostructured enzymes (Al-Tohamy et al., 2022). Peroxidase is the most useful enzyme employed for degrading many emerging pollutants like dyes. This enzyme has the potential for bioremediation of wastewater, bioleaching in the paper industry, and textile dye degradation (Bansal and Kanwar, 2013). Another example is reported by Terres et al. (2015), who analyzed the peroxidase activity in the degradation of Indigo carmine dye (IC); their results showed the disappearance of coloration (Terres et al., 2014). Another study used Co-doped magnetite nanoparticles surrounded by the carboxymethylcellulose polymer shell; the nanozyme was termed a "Co-MIONzyme" and exhibited peroxidase-like activity. The Co-MIONzyme could efficiently degrade methylene blue (MB) with an efficiency of 95%. This result was obtained at pH 3, 40°C, within 10h of the reaction, and a dye dose of 22.5 μg/mL (Mansur et al., 2022). Laccase is another enzyme utilized to degrade dyes; it has been immobilized into pine needle biochar to degrade malachite green dye (MG). The study reported a dye removal efficiency of 85%, and only the 53% efficiency could be maintained after some cycles (Pandey et al., 2022). On the other hand, Ge et al. (2021) constructed hybrid composites of copper ions and tannic acid (Cu-TA) with laccase-like activity. In addition, after several cycles, the efficiency of degrading MG was 90%, with good reusability. The storage stability is another characteristic to highlight, which was superior to natural laccase (Ge et al., 2021). Similarly, other nanozymes have exhibited excellent results in removing a great diversity of dyes, as presented in Table 1. New methods for the degradation of dyes are of vital importance in the present days; nanozymes might become one feasible and cost-effective solution. This is because nanozymes exhibit many advantageous attributes compared to their natural counterparts; nanozymes can easily be adapted to current industries and environmental necessities. The published reports in the literature give insight into their incredible potential; however, research efforts are still required to design new nanomaterials through sustainable and optimized protocols to enhance their yield and large-scale performance. However, numerous dyes with combinations must be tested with the proposed nanozymes for further advancements, providing more data to compare results. In addition, toxicity tests need to be conducted in more studies.

Phenolic compounds degradation

Phenolic compounds derived from the petrochemical, pharmaceutical, agricultural, and food industries play a relevant role in human life due to the negative impacts caused by their release and distribution in the environment. The environmental balance and human health might be seriously affected by problems such as endocrine disruption and carcinogenesis, among many other adverse effects (Wang et al., 2019). Certain phenolic compounds such as 4-nitrophenol and 4-chlorophenol are considered high-priority contaminants by the Environmental Protection Agency (EPA) of the United States.

The degradation of different phenolic pollutants can be performed by different processes, either physical, chemical, or biological. Typically, they present high degradation efficiency; however, disadvantages like high implementation and maintenance costs significantly hinder broad applicability. Thus, nanozymes are considered successful strategies for the degradation of phenolic compounds. Nanozymes have advantages over conventional catalysts or biocatalysts, showing superior and tunable catalytic efficiencies, great recyclability, high stability in different media, and simple and low-cost manufacturing (Liang et al., 2022). Furthermore, researchers have explored innovative strategies to enhance the performance of nanozymes, such as the design of nanomaterials with specific characteristics or the further modification of properties like size, morphology, and shape. Similarly, the obtention of higher catalytic activities and increased selectivity of nanozymes have been pursued by generating hybrid materials, adding coatings, and surface modifications (Xu et al., 2021). A recent study reported a new strategy for the preparation of a BSA-Cu-based laccase-mimicking nanozyme using ionic liquids; it showed enhanced stability and catalytic performance under extreme conditions of temperature, salinity, and pH values for many substrates, such as dopamine, 2, 4-dichlorophenol (2, 4-DP), guaiacol, epinephrine, and guaiacol (Huang et al., 2022).

Table 1. Recent nanozyme-based developments for the removal of dyes.

Nanozyme	Enzyme-mimicking activity	Dye	Degradation efficiency (%)	Reference
CNZ	Peroxidase	MO	93	(Geng et al., 2021)
Fe_3O_4 NPs	Peroxidase	IC	99	(Zha et al., 2022)
Co-MIONzyme	Peroxidase	MB	18	(Mansur et al., 2022)
Cu^{2+}-HCNSs-COOH	Peroxidase	MB	80.7	(Zhu et al., 2021)
$W_{18}O_{49}$ NSs	Peroxidase	MB	91	(Zhu et al., 2018)
$CoSe_2$ MS	Peroxidase	MG	99.45	(Khagar et al., 2021)
Fe_3O_4@Cu/GMP	Lacasse	OPD	90	(Zhang et al., 2020)
Cu/H_3BTC MOF	Lacasse	AB-10B	90	(Shams et al., 2019)
Cu-TA	Lacasse	MG	90	(Ge et al., 2021)
FeBi-NC SAzyme	Oxidase	RhB	100	(Chen et al., 2022)
Au-Au/IrO_2 nanocomposite	Peroxidase and glucose oxidase	RhB	98.16	(Zhong et al., 2021)
		MB	92.78	(Liu et al., 2020)
		MO	97.17	(Le et al., 2022)
Co_3O_4-g-C_3N_4	Peroxidase	RhB	90.2	(Liu et al., 2020)
H–Mn–Cu NFs	Laccase	CV	90	(Le et al., 2022)
		NR		
		RhB		

Abbreviations: CNZ (cooper nanozyme); Fe_3O_4 NPs (polymer-coated Fe_3O_4 nanozymes); Co-MIONzyme (Co-doped iron oxide nanozymes); Cu^{2+}-HCNSs-COOH (Cu^{2+}-modified carboxylated hollow carbon nanospheres); $W_{18}O_{49}$ NSs ($W_{18}O_{49}$ nanospheres); $CoSe_2$ MS ($CoSe_2$ microsphere); Fe_3O_4@Cu/GMP (Fe_3O_4@Cu/GMP guanosine 5′-monophosphate nanozyme); Cu/H_3BTC (Copper and 1,3,5-benzene tricarboxylic acid nanozyme); Cu-TA (Copper and tannic acid hybrid composites single-atom nanozymes); FeBi-NC Sazyme (Fe-Bi bimetallic MOF-derived carbon single-atom nanozymes); g-C_3N_4 (graphite carbon nitride); H–Mn–Cu NFs (Manganese dioxide–copper phosphate hybrid nanoflowers); MO (methyl orange); IC (indigo carmine); MB (Methylene blue); OPD (o-phenylenediaminex); AB-10B (amido Black 10B); MG (malachite green); RhB (rhodamine B); CV (Crystal violet); NR (neutral red).

Similarly, a novel and not commonly explored strategy based on a series of multivalent Ce-MOFs, Ce-UiO-66 and Ce-MOF-808, showed great stability and enhanced efficiency for the degradation of 2, 4-DP; those results were significantly superior in comparison to those exhibited by laccase enzyme (Liang et al., 2022). Some other novel nanozymes can act in a reaction cascade, showing the activity of more than one enzyme with a higher response in terms of efficiency. For example, single Fe atom nanozymes (Fe SAEs) exhibited outstanding oxidase, peroxidase, and catalase activities. The nanozyme removed phenol from aqueous solutions with an efficiency of 83% within 30 min (Zhao et al., 2019).

Challenges and key recommendations

Regardless of current technological advancements in chemical and biological processes, microbial bioremediation is a sustainable method with excellent execution, low maintenance expenditures, exceptional process selectivity and adaptability, and no or minimal generation of byproducts. Nanotechnological implementation and functionalization or modification strategies are also recommended to improve pollution elimination. Such an approach can also facilitate microbial remediation of polluting agents or the induced production of remediating microbial enzymes (Benjamin et al., 2019; Khaliq 2023; Yagnik et al., 2023). Regardless of the manifestation of microorganisms in a water stream, their action is repeatedly perceived as ineffective. This lack can be directly or indirectly ascribed to a deficiency of nutrients such as phosphorus or nitrogen (Azubuike et al., 2016).

Although nanozymes represent effective alternatives for monitoring and degrading several emerging pollutants, either presented in wastewater or leachates, there is still a lack of data to compare results. In this respect, an ample and diverse range of emerging pollutants should be tested to degrade; complex water samples should be used in experimentation, in which a combination of different pollutants and co-existing substances can occur. Finally, the toxicity of nanozymes has received increased research attention recently due to their broad applicability in many fields and their potential adverse effects on the environment and the population's health. The biosafety and biocompatibility of nanozymes –even carbon-based nanozymes, which are typically biocompatible– should be carefully reviewed (Lopez-Cantu et al., 2022b). Applying nanozymes to the degradation of pollutants present in the environment involves the interaction of the nanozyme with natural systems and multiple organisms. Therefore, *in vivo* and *in vitro* toxicity tests should be performed using various concentrations and applied to different biological models. A detailed understanding of nanozymes in composition, shape, and size is required to define further their effect on toxicity, biodistribution, and *in vivo* uptake; which should be considered for future studies in the field. Nanomaterials also face these concerns whether they possess enzyme-like activities or not. Thus, significant advancements in this regard are expected in the near future.

CONCLUSIONS

In conclusion, microbial bioremediation-assisted environmental cleanup has become increasingly popular in recent years, providing a natural and sustainable alternative to traditional remediation techniques. In addition, microbial bioremediation is also considered a relatively low-cost method of environmental cleanup. Owing to the complete breakdown within the natural microbial process, it does not require removing and disposing of contaminated material, which is otherwise considered expensive and disruptive in other traditional techniques. In turn, this ultimately lowers the risk of further contamination and thus helps to promote and maintain a healthy and balanced ecosystem. Furthermore, the extensive use of harsh chemicals/reagents in chemical-based remediation measures can pose adverse side effects.

Conflict of interests

The authors declare no conflict of interest.

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