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Table of Contents

Original Research Paper

Original Research

Impact of Biosynthesized Zinc Oxide Nanoparticles on the Kidneys Tissues of Male Mice Experimentally Infected with *Entamoeba histolytica*

Laith A. Yaaqoob [1], Mohannad Hamid Jasim [2], Lima Tariq Youash Lazar [3], Saade Abdalkareem Jasim [4*], Thaer Abdulqader Salih [5]

Abstract

Entamoeba histolytica has been detected in stool samples of patients attending the Children's General Hospital in Kirkuk City. Zinc oxide nanoparticles (ZnO NPs) are biosynthesized by *Pseudomonas aeruginosa*, which produces the pigment pyocyanin that acts as an agent for reducing the production of ZnO NPs. The purpose of this work was to ascertain how ZnO NPs might be used therapeutically to treat *E. histolytica*-infected mice's kidney tissues. We used scanning electron microscopy, atomic force microscopy, Fourier transform infrared spectroscopy, UV-visible spectroscopy and X-ray diffraction to characterize biosynthesized ZnO NPs. ZnO NPs were used to treat mice that had been experimentally infected with *E. histolytica*. Mouse kidneys were collected and divided into six groups for evaluation. Two groups served as negative and positive controls, respectively. Three other groups (four mice each) were treated with 30, 45, or 60 µg/mL ZnO NPs once daily for ten days by intraperitoneal injection. The final group was the uninfected group and treated with 30 µg/mL ZnO NPs. Mice treated with 30, 45, and 60 µg/mL ZnO NPs had kidney histological sections that resembled the negative control, demonstrating that the nanoparticles were effective against the parasite. The uninfected group treated with ZnO NPs showed no negative effects on the kidney tissue.

Keywords

Amebiasis; Zinc- oxide Nanoparticles; Entamoeba histolytica.

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Vpliv biosintetiziranih nanodelcev cinkovega oksida na ledvična tkiva mišjih samcev, poskusno okuženih z *Entamoeba histolytica*

Izvleček

Entamoeba histolytica je bila izolirana iz vzorcev blata pacientov v otroški splošni bolnišnici v mestu Kirkuk. Nanodelce cinkovega oksida (ZnO NP) biosintetizira Pseudomonas aeruginosa, ki proizvaja pigment piocianin, ki deluje kot reducent pri proizvodnji ZnO NP. Namen tega dela je bil ugotoviti, kako bi lahko ZnO NP uporabljali terapevtsko za zdravljenje ledvičnih tkiv miši, okuženih z E. histolytica. Za karakterizacijo biosintetiziranih NP ZnO smo uporabili skenirno elektronsko mikroskopijo, mikroskopijo atomskih sil, infrardečo spektroskopijo s Fourierjevo transformacijo, UV-vidno spektroskopijo in rentgensko difrakcijo. ZnO NP so uporabili za zdravljenje miši, ki so bile eksperimentalno okužene z *E. histolytica*. Mišje ledvice so bile zbrane in razdeljene v šest skupin za oceno. Dve skupini sta služili kot negativna in pozitivna kontrola. Tri druge skupine (vsaka po štiri miši) so bile deset dni zdravljene s 30, 45 ali 60 µg/mL ZnO NPs enkrat na dan. Končna skupina je bila neokužena skupina in zdravljena s 30 µg/mL ZnO NP. Miši, zdravljene s 30, 45 in 60 µg/mL ZnO NP, so imele ledvične histološke prereze, ki so bili podobni negativni kontroli, kar dokazuje, da so bili nanodelci učinkoviti proti parazitu. Neokužena skupina, zdravljena z ZnO NP, ni pokazala negativnih učinkov na ledvično tkivo.

Ključne besede

amebiaza, nanodelci cinkovega oksida, *Entamoeba histolytica*

Introduction

Intestinal parasites are problematic worldwide because of the increase in population density, lack of health awareness, and inadequate health care. The ingestion of water and food tainted with cysts of parasites causes this infection (Barzinji, 2023). *E. histolytica* causes amoebic dysentery and infection symptoms and ranks third among parasite-related deaths globally, after schistosomiasis and malaria (Yimer *et al.*, 2017). 10% of individuals with an *E. histolytica* infection exhibit symptoms of infection, according to a previous study, whereas 90% show no symptoms and act as only disease carriers (Al-Bayati *et al.*, 2023).

There is also growing attention towards metal NPs and their oxides with ability against microbes owing to the very tiny particle size (Raghunath and Perumal, 2017). In this perspective, such NPs are generated via a series of conventional techniques based on either chemical and physical ways but possibly accompanied with emergence of several issues regarding creation of by-products or these nanomaterials instability. Among these, the preparation of metal nanoparticles by microorganisms has been employed; these have been described as stable and without the generation of dangerous by-products (Baig et al., 2021). In this respect, zinc is known for its tendency to oxidize in the form of zinc oxide, due to its high reducing properties. Thus, these properties are used in the preparation of zinc oxide nanoparticles, ZnO NPs. ZnO NPs are synthesized by using the pigment Pyocyanin produced by certain bacteria as a reducing agent (Mandal et al., 2022).

ZnO NPs treatment leads to the leaching of proteins and nucleic acids, as well as being non-toxic. Nano-zinc oxide was categorized by the United States Food and Drug Administration as being safe, and it is considered suitable for appropriate use on humans as shown by Deka et al., 2020. Nano-zinc oxide has various forms of biological activities; it works as an antimicrobial. It is an antioxidant and stimulates immunity; it is used in a number of fields like agriculture, medicine and nutrition. (Mihailovic *et al.*, 2021). *Pseudomonas aeruginosa* produces the pigment pyocyanin, which is used in the biosynthesis of nanoparticles as an agent reducing (DeBritto *et al.*, 2020). This study aimed to determine the therapeutic impact of zinc oxide nanoparticles in the renal tissues of *Entamoeba histolytica*-infected mice.

Materials and Methods

Collection of Samples

Parasite samples were gathered from 20 infected patients under 5 years visiting the General Children's Hospital in Kirkuk who showed symptoms of infection represented by moderate or severe diarrhoea and, in some cases, the presence of bloody diarrhoea. The samples were examined immediately upon their arrival at the laboratory to prevent decomposition of the trophozoite phase. The samples were examined by the method of direct smear, by placing a 50 µl of the physiological solution on the glass slide and taking a small number of faeces with wooden chopsticks, mixing it with a drop of the physiological solution, placed the cover of the glass slide, and examined the sample under 40x magnification with an inverted light microscope (Fotedar et al., 2007b). The experiment protocol was submitted to the Ethical Committee of the College of Veterinary Medicine - Tikrit University and was approved according to the numbered document 27 on 26-10-2023.

Biosynthesis of ZnO NPs

Pseudomonas aeruginosa extract was used to biosynthesize ZnO NPs that produce the pigment pyocyanin as a reducing agent. UV visible spectroscopy and the size and form of the ZnO NPs were measured using transmission electron microscopy. Nano dimensions were ascertained by Fourier transform spectrum analysis employing infrared FTIR, X-ray diffraction (XRD) and atomic force microscopy (AFM) (Auda, 2023).

Preparation of culture media

The manufacturer's instructions were followed when preparing the solid and liquid culture media. The following media were used: nutrient agar, brain heart infusion broth, MacConkey agar, and brain heart infusion agar (MacFaddine, 2000).

Production of Pyocyanin

The isolate of P. aeruginosa, previously identified using the VITEK2 system (bioMeriux, Marcy-l'Étoile, France), was obtained from the Nanobiotechnology Laboratory at the Biotechnology Department, College of Science, Baghdad University. *Pseudomonas aeruginosa* was activated on brain-heart infusion agar as the culture medium and incubated for 24 h at 37 °C. To extract pyocyanin, 250 mL of brain-heart infusion broth medium was prepared in a 500 mL conical flask. The culture medium was inoculated with bacteria and incubated for 24 h at 37 °C., followed by incubation in a shaking incubator for 72 h at 37 °C at 200 rpm. The incubator was covered during incubation. The beakers were incubated in the dark to increase bacterial growth, competition, nutrient exhaustion, and dye production. Pyocyanins are secondary metabolites produced during the stationary phase of bacterial growth (Wijesinghe et al., 2019).

ZnO NPs were synthesised using pyocyanin from *P. aeruginosa*. After inoculating the culture medium with brain heart infusion broth containing bacteria, the culture medium was centrifuged. The liquid culture medium was distributed into new tubes and centrifuged for 20 min at 10,000 rpm to remove the precipitate and obtain the dye extract. The dye extract (50 mL), 5 g of zinc acetate, and a few drops of NaOH were added, and the pH of the reaction was measured during continuous shaking; NaOH was added gradually until the pH of the medium reached 8, and a white precipitate's creation was noticed. Following distribution into selection tubes, the mixture was centrifuged for 20 min at 10,000 rpm. The filtrate was separated from the precipitate and washed twice with deionised water. The sediment was collected, placed in petri dishes, and dried in an incubator. The obtained ZnO NPs in powder form were stored in a box and covered with a cell phone until analysis.

Characterization of ZnO NPs

The three-dimensional topography of ZnO NPs depends on the interactions and forces applied to the sample and the dimensions of the nanomaterial (Sun, 2018). The ZnO NPs' surface and shape were examined using an atomic force microscope. FTIR spectroscopy of the ZnO NPs was carried out at the Department of Chemistry - College of Science/ Baghdad University. Data were collected at wavelengths of 400–4000 wave number\cm (Mohamed et al., 2017). Baghdad's Scientific Laboratory conducted field-emission scanning electron microscopy. The shape and surface coverage of the ZnO NPs were determined. The objective lens was used to scan the sample's surface fully to project an electron beam onto it. As the electrons interfered with the atoms in the sample, signals were generated. Multiple models have

shown that surface topography provides information on the components present in a sample (Akhtar et al., 2018).

Laboratory animal infection

Twenty-four mature male mice weighing 30 ± 3 g and aged 7 to 9 weeks were acquired from Tikrit University's animal house. The mice were lodged in an animal house affiliated with the College of Veterinary Medicine at Tikrit University in plastic cages and provided sterile water and food throughout the housing period. The faeces of the mice were examined before starting the experiment to ensure that they were free of infection with intestinal parasites by combining the faeces with a drop of local iodine dye that had been placed on a glass slide. The samples were then covered with a glass slide and examined under a microscope. Four mice were euthanised and used as negative controls. The remaining 20 mice were divided into five groups, with four mice per group, and the *E. histolytica* suspension was administered orally to the four groups. After 48 h, the faeces of the mice were re-examined to confirm the infection by observing the trophozoite stage of the parasite.

Four of the infected mice were euthanised and used as positive controls. The remaining infected mice (4 in each group) were treated with 30, 45, or 60 µg/mL biosynthesized ZnO NPs once daily for 10 days. Uninfected control mice were treated with 30 µg/mL ZnO NPs only; the concentrations were determined based on the evaluation of the toxicological effect of the nanomaterial by exposing mice to different concentrations of the nanomaterial by intraperitoneal injection, and the concentrations were chosen based on the LD50 (Razooki and Rabee,2020).

ZnO NPs were injected under the peritoneal membrane rather than orally to avoid acid-base changes during their passage from the stomach to the intestine. Oral administration can cause NPs to lose their nano-properties and prevent them from crossing the cell membrane of the parasite. The mice were dissected, and the kidneys were collected for histological analysis. Tissue sections were prepared by fixation in 10% formalin for 1 day, followed by washing with alcohol and dehydration using a graded series of ethyl alcohol at 70%, 80%, 90%, and 99% for two hours each. The samples were placed in xylene for 20 min and then passed through paraffin wax, which was allowed to penetrate the cells. Later on, the samples were poured into moulds identical to the sample size and got frozen for 10min to solidify the wax. After that, they were cut into strips using a rotary microtome, and the strips were loaded onto glass slides and stained for the purpose of preparing them for microscopic examination to observe histological modifications in entire groups (Isaac *et al.*, 2023).

Results

Characterization of ZnO NPs

Atomically, atomic force microscopy substantially make up 2D and 3D images of ZnO NPs, as obviously illustrated in Figures 1a-b (Fadhil and Hadi, 2015). The average diameter of the NPs was meticulously calculated as 80.35 nm (Figure 1c). This aligns with Al-Taie's et al. (2022), who employed young leaves of aloe vera to produce ZnO NPs with a rate diameter of 44.45 nm.

FTIR analysis was done to help in the identification and specification of the active functional groups in pyocyanin, responsible for biosynthesis and the production of ZnO NPs. The average infrared spectra scanned for biosynthesis range from 400 to 4000 cm-1, which can be used to predict the presence of any chemical bonds and functional groups within the compound. Figure 1d presents the infrared spectra of the ZnO NPs biosynthesized using pyocyanin. The FTIR spectrum of the ZnO NPs showed expected bonds: the first one was around 3460.06–3438.84 cm-1, corresponding to O-H (alcohol and phenols) and N-H stretching of the amine group; the second one was approximately at 1633.59–1625.88 cm-1, related to the N-H bond of the amines group. The third bond that appeared around 582.46–547.75 cm-1 corresponds to a metal oxide (ZnO). These results have been reported by Alaa Alden and Yaaqoob (2022), too.

Using a field-emission scanning electron microscope, the surface topography of ZnO NPs was investigated. Figure 1e shows that the particles formed rods to spherical aggregates that were smooth and spherical in shape. This result is consistent with that of Al-Taie *et al.* (2022), who found that ZnO NPs have smooth, regular, spherical, and rod-shaped surfaces.

Histological analysis

The negative control group of kidney sections contained normal glomeruli, renal tubules, distal convoluted tubules,

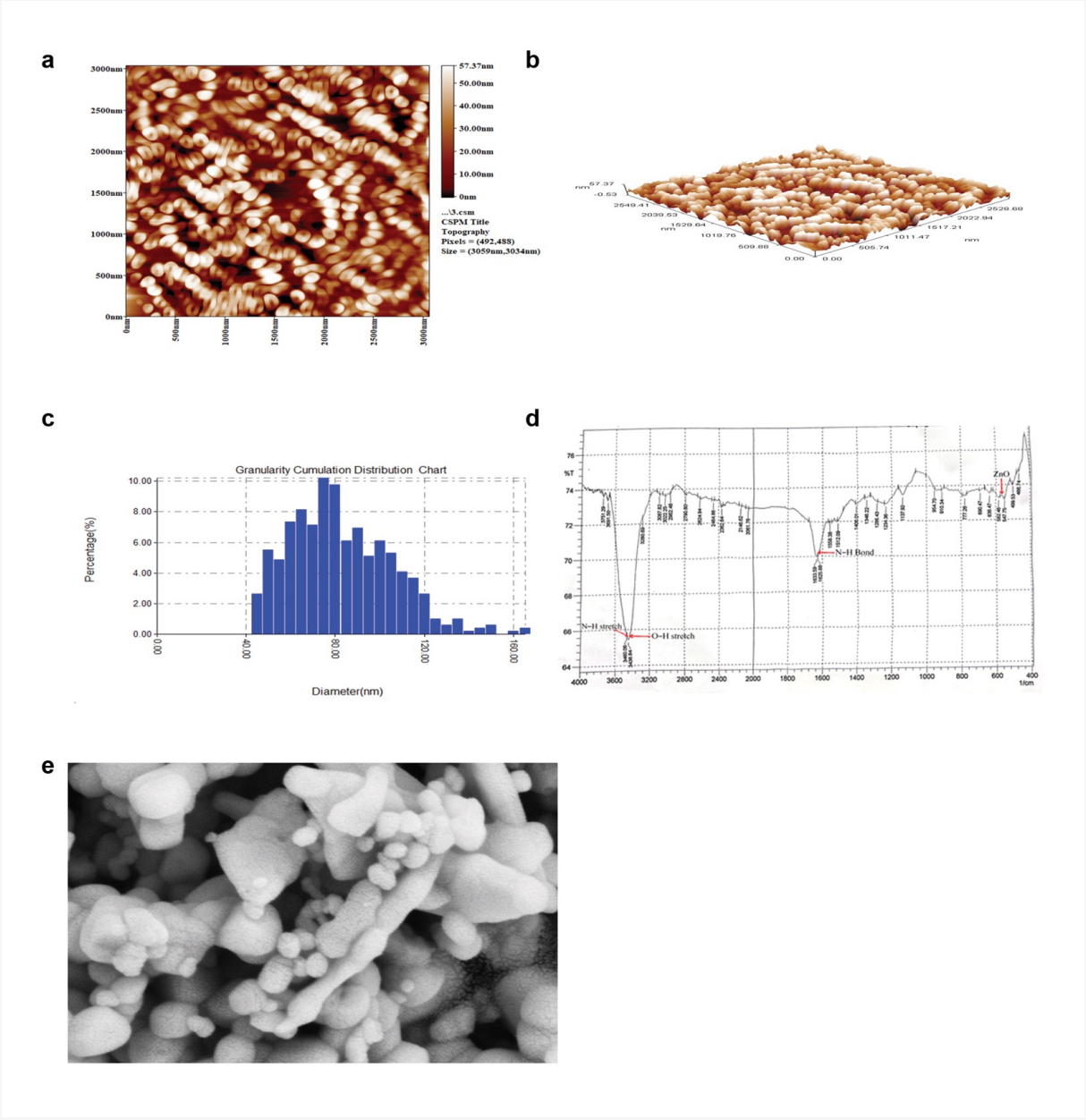

Figure 1. a) image of ZnO NPs (2D); b) image of ZnO NPs (3D); c) AFM result report of ZnO NPs; d) FTIR of ZnO NPs; e) FE-SEM image of ZnO NPs.

Slika 1. a) slika ZnO NP (2D); b) slika ZnO NP (3D); c) poročilo o rezultatih AFM ZnO NP; d) FTIR ZnO NP; e) slika FE-SEM ZnO NP.

proximal convoluted tubules and collecting tubules (Figures 2a and 2b). Sections of the cortex of renal in the positive control group (infection without treatment) showed focal cortical kidneys with a tubular matrix, tubular atrophy, necrosis, and tissue depletion, as shown in Figures 2c and 2d.

Sections treated with 30, 45, or 60 µg/mL ZnO NPs showed a normal appearance for the glomeruli, renal tubules, collecting tubules, proximal convoluted tubule, and distal convoluted tubule, as shown in Figures 3a-d. In the uninfected group treated with 30 µg/mL ZnO NPs, the kidney sections had a similar appearance as those in the negative control group. The glomeruli, collecting tubules, proximal convoluted tubule, and distal convoluted tubule had a normal appearance, as shown in Figure 3e.

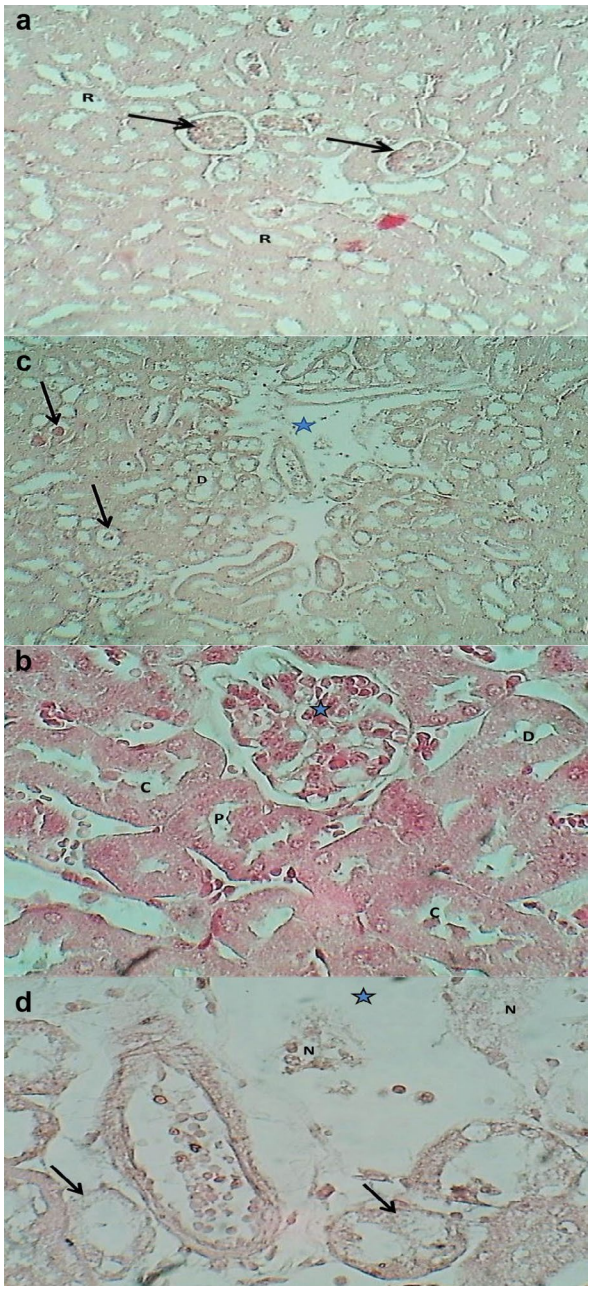

Figure 2. a) section of the renal cortex (Control negative) shows the normal appearance of glomeruli (arrows) & tubules of renal (R), 100x magnification; b) section of the renal cortex (Control negative) shows glomeruli (Asterisk), collecting tubule (C), distal convoluted tubules (D) and proximal (P), 400x magnification; c) section of renal cortex (Control positive) shows: focal cortical nephrosis with tubular cast formation (arrows), tubular degeneration (D), necrosis and depletion (Asterisk), 100x magnification; d) Section of renal cortex (Control positive) shows: tubular degeneration (Arrows), tissue depletion (Asterisk) and necrosis (N), 400x magnification. All figures were stained with H&E stain.

Slika 2. a) prerez ledvične skorje (kontrola negativna) prikazuje: normalen videz glomerulov (puščice) in ledvičnih kanalčkov (R), 100-kratna povečava; b) prerez ledvične skorje (kontrola negativna) prikazuje: glomerule (zvezdica), zbiralne kanalčke (C), distalne zvite kanalčke (D) in proksimalne (P), 400-kratna povečava; c) prerez ledvične skorje (kontrola pozitivna) prikazuje: (puščice), degeneracija tubulov (D), nekroza in izčrpanost (zvezdica), 100-kratna povečava; d) prerez ledvične skorje (pozitivna kontrola) kaže: degeneracijo tubulov (puščice), izčrpanost tkiva (zvezdica) in nekrozo (N), 400-kratna povečava. Vse slike so bile obarvane z barvilom H&E.

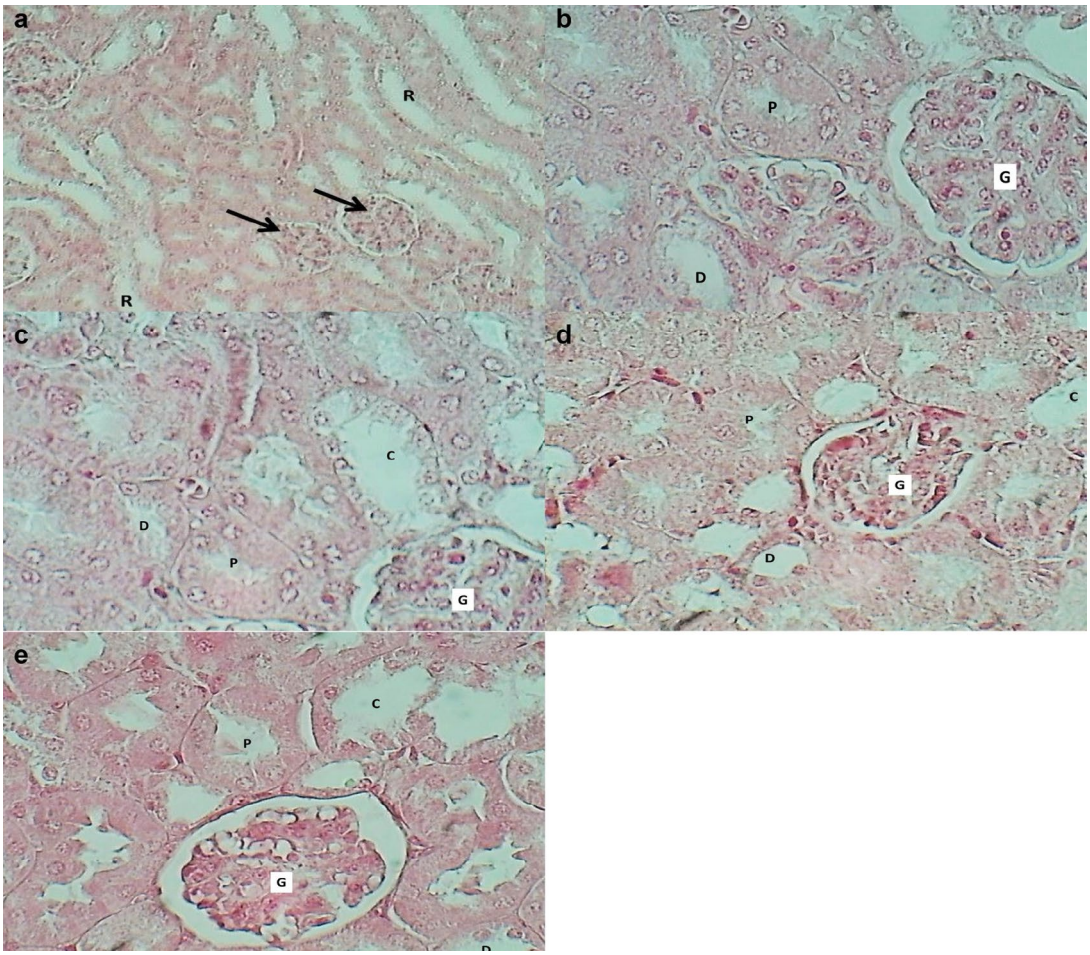

Figure 3. a) section cortex of renal (group 3) shows: normal appearance of glomeruli (arrows) and tubules of renal (R), 100x magnification; b) section cortex of renal (group 3) shows glomeruli (G), collecting tubule (C), distal convoluted tubules (D) and proximal (P), 400x magnification; c) section of renal cortex (group 4) shows: glomeruli (G), distal convoluted tubules (D), collecting tubule (C) and proximal (P); d) section of renal cortex (group 5) shows: glomeruli (G), distal convoluted tubules (D), collecting tubule (C), proximal (P), 400x magnification; e) section of renal cortex (group 6) shows: glomeruli (G), collecting tubule (C), proximal (P) and distal convoluted tubules (D), 400x magnification. All figures were stained with H&E stain.

Slika 3. a) prerez ledvične skorje (skupina 3) prikazuje: normalen videz glomerulov (puščice) in ledvičnih kanalčkov (R), 100-kratna povečava; b) prerez ledvične skorje (skupina 3) prikazuje: glomerule (G), zbiralne kanalčke (C), distalne zvite kanalčke (D) in proksimalne (P), 400-kratna povečava; c) prerez ledvične skorje (skupina 4) prikazuje: glomeruli (G), distalni zviti kanalčki (D), zbiralni kanalčki (C) in proksimalni (P); d) prerez ledvične skorje (skupina 5) prikazuje: glomeruli (G), distalni zviti kanalčki (D), zbiralni kanalček (C), proksimalni (P), 400-kratna povečava; e) prerez ledvične skorje (skupina 6) prikazuje: glomerule (G), zbiralni kanalček (C), proksimalni (P) in distalni zviti kanalčki (D), 400-kratna povečava. Vse slike so bile obarvane z barvilom H&E.

Discussion

Nanoparticles possess many unique chemical and physical properties due to their small size, large surface area and electrical charge, and because of their large surface area, they break the passive activity of parasites and enter cells more than other molecules (Mendes et al., 2022). ZnO interferes with the surface of the parasite, causing damage and destruction of glycoprotein molecules and phosphorylated lipopolysaccharides on the parasite's surface. ZnO also generates active oxygen species, which have the ability to kill pathogens (Siddiqui et al., 2024).

The results observed for the positive control were consistent with those of Shakir and Abdulwahhab (2018) in the presence of desquamation of the renal tubules, nuclear degeneration, congestion of the proximal renal tubules and Necrosis of the kidney cortex.

Kidney sections from infected mice treated with ZnO NPs showed a normal appearance, similar to that in the group of negative control. Nano-zinc oxide helpfully reduced the impacts of the parasite's virulence factors in the renal tissue. Attiah et al. (2023) sparsely discussed how ZnO NPs strikingly diminished the virulence factors of the parasite by determining the influence of ZnO NPs on Giardia lamblia trophozoites. The authors reported an extremely significant reduction in the number of the trophozoites after treatment with ZnO NPs.

In one of the earlier studies, mice were experimentally infected with a parasite causing cryptosporidiosis by the use of ZnO NPs. The results indicated that ZnO NPs greatly improved the lesions in the intestines, liver, and lungs. Cheraghipour et al. (2023) illustrated that ZnO NPs are antiparasitic to Toxoplasma gondii with minimal lethal toxic effects on host tissues and highly augmenting survival in infected mice.

The uninfected group treated with 30 µg/mL ZnO NPs had a similar appearance as the negative control group, demonstrating that ZnO NPs do not negatively affect mice at low concentrations. These findings are in line with those of Abdelnasir et al. (2020), who observed that NPs did not negatively affect the cells and haemolytic activity of mice.

Conclusions

According to the results obtained, treatment with zinc oxide nanoparticles is safe and does not exert any harmful effects on the tissues of the examined animals. This study also highlighted the role of the nanoparticles of zinc oxide playing the role of an antiparasitic agent against Entamoeba histolytica and decreasing the pathogenicity of the parasite in destroying and necrotizing the tissue of renal cortex, glomeruli, and renal tubules and the collecting tubules.

Author Contributions

Conceptualization, L. T. Y.; methodology, L. T. Y. and L. A. Y.; software, S. A. J.; validation L. A. Y.; investigation , Th. A. S.; writing—original draft preparation, M. H. J.; writing—review and editing, Th. A. S.; visualization , S. A. J.; supervision, M. H. J.; All authors have read and agreed to the published version of the manuscrip.

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Data Availability

The data is on request.

Conflicts of Interest

The authors declare no conflict of interest.

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Original Research

Insecticidal effect of Essential Oils of *Citrus limon*, *Cymbopogon citratus* and *Syzygium aromaticum* and their Synergistic Combinations Against *Anopheles* Mosquitoes

Tunde Ayobami Owolabi [1*], Philippine Chigozie Okubor [2], James Danga [3], Issa Onimisi Bello [4]

Abstract

While synthetic insecticides offer effective pest control, their many side effects necessitate exploring alternatives, this study investigates the insecticidal potential of essential oils from lemon (*Citrus limon*), lemongrass (*Cymbopogon citratus*), and clove (*Syzygium aromaticum*) against Anopheles mosquitoes, a prime vector of malaria, a deadly global disease. The oils were extracted via hydro-distillation, Gas Chromatography-Mass Spectrometry (GC-MS) was used to analyze the chemical profiles. The insecticidal activity was tested using a susceptibility test to evaluate the knockdown, mortality rates and time against adult mosquitoes following standard protocols. The extraction procedure yields of 0.5, 0.5, and 0.2% were obtained for lemon, lemongrass, and clove respectively. GC-MS showed limonene as the dominant compound in lemon oil (66.4%), while citral a (48.3%) and citral b (39.9%) were most abundant in lemongrass oil, and that of Clove is eugenol (69.0%). Adulticidal efficacy results put clove oil to have the best activity with a knockdown rate of 75 ± 0.75 at 14 minutes, a mortality rate of 45 ± 0.74 at 25 minutes, lemon oil exhibited the least activity with 30 ± 0.866 knockdown rate at 26 minutes, a mortality rate of 15 ± 0.47 at 42 minutes. However, the combination of Lemongrass and Clove (FM2) demonstrated an enhanced overall insecticidal efficacy beyond the capabilities of individual oils with a knockdown rate of 85 ± 0.25 at 7 minutes and a mortality rate of 60 ± 0.37 at 16 minutes. Despite mosquitoes' resistance to the individual oil, these findings suggest that these oils can be optimized for more effective insect control when combined, consequently, this research has contributed to the quest for a better, cheaper, safer and eco-friendly for mosquito control.

Keywords

Natural insecticides; Essential Oils; Mosquitoes; Gas Chromatography-Mass Spectrometry.

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Insekticidni učinek eteričnih olj *Citrus limon*, *Cymbopogon citratus* in *Syzygium aromaticum* ter njihovih sinergijskih kombinacij proti komarjem *Anopheles*

Izvleček

Sintetični insekticidi zagotavljajo učinkovit nadzor nad škodljivci, vendar imajo tudi številne nezaželene stranske učinke. S tem namenom smo v raziskavi testirali nekatere njihove naravne alternative. V študiji smo raziskali insekticidni potencial eteričnih olj limone (*Citrus limon*), limonske trave (*Cymbopogon citratus*) in klinčkov (*Syzygium aromaticum*) proti komarjem *Anopheles*, glavnemu prenašalcu smrtonosne svetovne razširjene malarije. Olja so bila ekstrahirana s hidrodestilacijo, za analizo kemijskih profilov pa je bila uporabljena plinska kromatografija z masno spektrometrijo (GC-MS). Insekticidno aktivnost smo testirali s testom občutljivosti, da bi po standardnih protokolih ocenili izločanje, stopnjo smrtnosti in čas delovanja na odrasle komarje. S postopkom ekstrakcije je bilo pridobljeni 0,5 %, 0,5 % in 0,2 % ekstrakti eteričnih olj za limono, limonsko travo in nageljnove žbice. GC-MS je pokazala, da je limonen prevladujoča spojina v limoninem olju (66,4 %), medtem ko je bilo v olju limonske trave največ citrala a (48,3 %) in citrala b (39,9 %), v olju nageljnovih žbic pa evgenola (69,0 %). Rezultati testov učinkovitosti delovanja na odrasle komarje so pokazali, da je imelo olje nageljnovih žbic najboljšo aktivnost, saj je bila stopnja izločanja 75 ± 0,75 v 14 minutah, stopnja smrtnosti 45 ± 0,74 v 25 minutah, limonino olje pa je imelo najmanjšo aktivnost, saj je bila stopnja izločanja 30 ± 0,866 v 26 minutah, stopnja smrtnosti pa 15 ± 0,47 v 42 minutah. Vendar je kombinacija limonske trave in nageljnovih žbic (FM2) pokazala večjo splošno insekticidno učinkovitost, ki presega zmogljivosti posameznih olj, s stopnjo izločanja 85 ± 0,25 v 7 minutah in stopnjo smrtnosti 60 ± 0,37 v 16 minutah. Kljub odpornosti komarjev na posamezno olje te ugotovitve kažejo, da je mogoče ta olja v kombinaciji optimizirati za učinkovitejše zatiranje insektov, zato je ta raziskava prispevala k iskanju boljšega, cenejšega, varnejšega in okolju prijaznega sredstva za zatiranje komarjev.

Ključne besede

Naravni insekticidi; eterična olja; komarji; plinska kromatografija - masna spektrometrija.

Introduction

Plants have long served as a source of natural remedies, and essential oils, extracted from various plant parts, represent a concentrated form of these bioactive compounds. These oils exhibit various properties including insecticidal, antibacterial, and antioxidant effects, as documented in research (Petrovska et al., 2012).

The environmental impact of synthetic pesticides is a growing concern. While these chemicals offer effective pest control, their persistence in the environment and potential toxicity to non-target organisms necessitates the exploration of alternative solutions (Park and Tak, 2016). Essential oils from renewable resources present a potentially more sustainable approach. Their mode of action often involves repelling insects rather than eradicating them, minimizing disruption to ecological balance.

Essential oils are plant products derived through hydro-distillation or other processes that retain the original fragrance and flavor of their source (Lutz et al., 2014). They are a diverse group of plant oils that are highly fragrant. The most typical places to find them are peels, seeds, bulbs, rhizomes, bark, wood, flowers, buds, leaves, twigs, and resinous materials. According to reports (Park and Tak, 2016; Ayvaz et al., 2010), many plants that possess essential oils can be used as pesticides or repellents. Within the higher plant kingdom, there are 17,500 species of aromatic plants.

Cymbopogon citratus, also known as lemongrass, has become widely naturalized and invasive in various regions. This versatile herb is renowned for its medicinal properties, traditionally employed to treat a range of ailments including arthritis, headaches, dyspnea, common wounds, and bacterial infections (Oniha et al., 2023).

Citrus limon, commonly known as lemon, belongs to the Rutaceae family and is native to tropical and subtropical regions. This citrus tree is characterized by its glossy,

evergreen leaves, fragrant white flowers, and yellow fruit with a sour taste. Lemon is widely utilized for its medicinal and culinary purposes (Moshood, 2023).

Clove, scientifically known as Syzygium aromaticum, is an aromatic spice native to the Maluku Islands in Indonesia and widely cultivated in tropical regions. Belonging to the Myrtaceae family, clove is renowned as a culinary and medicinal herb. It is particularly rich in essential oils, notably eugenol, which gives it its characteristic fragrance and therapeutic properties (Cortés-Rojas et al., 2014).

Mosquito is the principal carrier of malaria. Malaria, a potentially fatal disease, is transmitted through the bite of an infected female Anopheles mosquito carrying protozoan parasites from the genus Plasmodium. These parasites enter the bloodstream during mosquito feeding and migrate to the liver where they mature and multiply rapidly. Upon release back into the bloodstream, infected red blood cells cause recurrent bouts of fever and other symptoms. Diseases transmitted by adult female Anopheles mosquitoes that feed on humans are collectively termed mosquito-borne diseases. Given the millions of lives at risk annually from these illnesses, they pose a significant global health threat. Malaria is caused by various species of the Plasmodium parasite (Foster and Walker, 2019).

The objective of this study was to investigate the adulticidal potential of several combinations of *Citrus limon*, *Cymbopogon citratus* and *Syzygium aromaticum* against female adults of Anopheles mosquitoes.

Materials and Methods

Plant collection and identification

In November 2023, the aerial parts of *Cymbopogon citratus* (lemongrass), and the fruit of *Citrus limon* (Lemon) were collected from Okada settlements, except for the dried fruits of Syzygium aromaticum (clove) which were purchased from Okada market in Ovia-Northeast LGA, Edo State, Nigeria. The plants were identified and verified in the Dora Akunyili College of Pharmacy herbarium, herbarium numbers; IUO/16/205, IUO/11/104, IUO/19/391 for *Cymbopogon citratus Citrus limon* and *Syzygium aromaticum* and respectively and voucher specimens deposited in the herbarium.

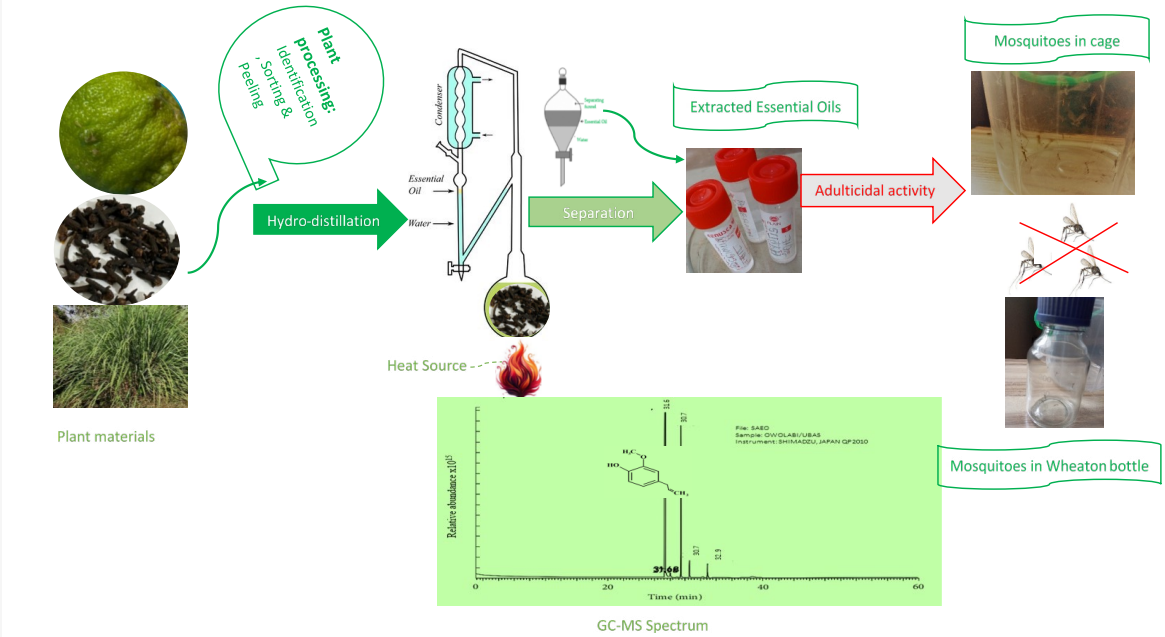

Figure 1. Flowchart of the methodologies.

Slika 1. Shema metodologij.

Extraction of the essential oils

The fresh parts of each plant (250 g) were individually subjected to steam distillation using a distillation setup comprising an electric heating mantle, a 2000 mL round-bottomed flask, a Clevenger, a condenser, and a chiller. The plant material was placed in the flask along with 1200 mL of distilled water. The mixture was heated using the electric mantle to approximately 100°C and maintained at this temperature for at least 3 hours until the distillation process was completed and no further essential oil (EO) could be extracted.

The collected distillate, consisting of a mixture of essential oil and water, was transferred to a glass-separating funnel for phase separation based on their differing densities. Sodium sulfate was added for the complete removal of the remaining water. The percentage yield of the essential oils extracted was determined using the formula;

$$\%\text{Yield} = \frac{\text{Volume of extracted Oil}}{\text{Weight of original plant material}} \times 100$$

Gas Chromatography-Mass Spectroscopy (GC-MS)

The essential oils were subjected to GC-MS analysis on a GC-MS instrument (SHIMADZU, JAPAN QP2010) with Elite – DB- 5M column and the GC-MS solution version 2.53 software. Initially, the oven temperature was maintained at 60°C for 3 minutes, and the temperature was gradually increased up to 250°C at 10.0/24.0 min and 4.0 µL of samples were separately injected for analysis. Helium gas 99.995% of purity was used as a carrier gas as well as an eluent. The flow rate of helium gas was set to 0.99 mL/min. The sample injector temperature was maintained at 250ºC and the split ratio is 40 throughout the experiment. The ionization mass spectroscopic analysis was done with 70 eV. The mass spectra were recorded for the mass range 50-600 m/z for about 24 minutes. The identification of components was based on a comparison of their mass spectra with that of the library. As the compounds separated on elution through the column, they were detected in electronic signals. As individual compounds eluted from the Gas chromatographic column, they entered the electron ionization detector where they were bombarded with a stream of electrons causing them to break apart into fragments. The fragments were charged ions with a certain mass. The m/z ratio obtained was calibrated from the graph obtained which was called as the mass spectrum graph which is the fingerprint of the molecule. The identification of compounds was based on the comparisons of their mass spectra with NIST Library 2021 WILEY8, FAME. Quantitative determinations were made by relating respective peak areas to TIC areas from the GC-MS.

Biological activity and synergism of essential oil samples

Essential oil combinations

Table 1. Individual essential oils and their combinations.

Tabela 1. Posamezna eterična olja in njihove kombinacije.

EOs and their combinations	Plant	Code	Formulation
Individual Eos	*Citrus limon*	CLEO	10% C. limon EO + 90% ethyl alcohol
	Syzygium aromaticum	SAEO	10% S. aromaticum EO + 90% ethyl alcohol
	Cymbopogon citratus	CCEO	10% C. citratus EO + 90% ethyl alcohol
Combinations		FM1	10%CLEO+ 10% SAEO + 80% ethyl alcohol
		FM2	10%SAEO+ 10% CCEO + 80% ethyl alcohol
		FM3	5% CLEO + 5% SAEO + 5% CCEO + 75% ethyl alcohol
	Positive control	LDT	10% LDT + 90% ethyl alcohol
	Negative control	ETAL	100% Ethyl alcohol

EO- Essential oil, CCEO- C. citratus essential oil, CLEO- Citrus limon essential oil, SAEO- Syzygium aromaticum essential oil, FM1- Formula 1, FM2- Formula 2, FM3- Formula 3

Mosquito Rearing

The eggs of the Anopheles Mosquitoes were collected within the Okada communities. The mosquito colony was kept under standard laboratory conditions and photoperiod of 12-h light and 12-h dark. The eggs of the mosquito were brought to hatch in a plastic tray containing 1000 mL of clean water. The larvae were fed with fish food pellets for 12–15 days until they pupated. The pupae were not fed with any food. One hundred new pupae were collected in a 300-ml plastic cup containing 200 ml of clean water, transferred into an insect cage (the size of 30 × 30 × 30 cm3), and left lying until developed into adults. Mosquito adults were provided with a 5% glucose solution as food, soaked in cotton sheets. Two-day-old female adults (not yet fed with blood meal) were collected as subjects for a World Health Organization susceptibility test (W.H.O, 2023).

World Health Organization Susceptibility Test

Five mosquitoes were taken to the Zoology laboratory for identification. Twenty-five 2-day-old female mosquitoes (not yet fed with blood meal) were exposed to 2 mL of each formulation, which was dropped onto a filter paper (Whatman® No.1) the size of 12 × 15 cm2 for 1 hr in a treatment tube (44 mm in diameter and 125 mm in length) then transferred to a non-treatment tube. The knockdown rate was recorded at 30 minutes, and the mortality rate was recorded at 1 hour after exposure. Each treatment was performed in three replicates. Ten percent (w/v) Lambdacyhalothrin and 90% v/v ethyl alcohol were used as positive control and negative control, respectively. The criterion for knockdown and mortality was no movement of any of the mosquito bodies. The distinction between knockdown and mortality was that knockdown was an occurrence recorded at 30 min after exposure while mortality was an occurrence recorded at 1 hr after exposure (W.H.O, 2023). Knockdown (KR%) and mortality rates (MR%) were calculated by the following formula:

$$\text{Knock down Rate (KR\%)} = \frac{NK}{NT} \times 100$$

$$\text{Mortality Rate (MR\%)} = \frac{ND}{NT} \times 100$$

where NK is the total number of knocked-down adults; ND is the total number of dead adults, and NT is the total number of treated adults. The means of these rates were analyzed and compared by analysis of variance (ANOVA) Susceptibility levels were classified according to WHO criteria: Susceptible (S) means 98–100% of mosquito mortality, Possible Resistant (PR) means 80–97% of mosquito mortality, and Resistant (R) means less than 80% of mosquito/housefly mortality.

Results

Percentage yield of Essential oil from the three selected plants

Essential oil obtained from several rounds of extractions of a total of 2kg of freshly collected plants for the individual selected plants is presented in Table 2 below. An average quantity of 1.2-1.3, 1.8-1.9, 0.8-1.0 mLs were separately extracted equivalent to average yields of 0.4-0.5, 0.1-0.2, and 0.5-0.6% respectively for *Cymbopogan citratus*, *Syzygium aromaticum* and *Citrus limon*. The colors oils are yellow, and white liquids respectively.

Chemical compositions of the evaluated Essential Oils

GC-MS studies on the chemical constituents of *Citrus limon, Cymbopogan citratus*, and *Synzygium aromaticum* oils revealed the presence of 19, 25, and 21 compounds, constituting 97.9, 91.1 and 97.4% of all the compositions, respectively (Table 3 – 5).

Results of GC-MS analysis of essential oil from *Citrus limon*

Table 3 below shows the mass spectrum of essential oil of *Citrus limon* peels obtained by GC-MS, it revealed that the oil contains nineteen (19) compounds. The most abundant compound is limonene with a peak area of 66.4% and retention time of 6.93. This is followed by γ-terpinene, with a retention time of 7.73, geranial with a peak area of 4.0% and retention time of 15.98, while trans-carveol is the least abundant compound with a peak of 0.1% and a retention time of 13.64. Table 3 shows the breakdown, while figure 2 shows the spectrum of the entire components in the essential oil from *Citrus limon* peels.

Table 2. Physical characteristics and percentage yield of the essential oils (EO).

Tabela 2. Fizikalne lastnosti in odstotni izkoristek eteričnih olj (EO).

Plant name	Family	Common name	Parts extracted	Colour of EO	Yield %
Cymbopogan citratus	Poaceae	Lemon grass	leaves	yellow	0.48
Citrus limon	Rutaceae	Lemon	Peel	white	0.5
Syzygium aromaticum	Myrtaceae	Clove	Dried buds	white	0.2

Table 3. GC-MS analysis of volatile oil composition of *Citrus limon*.

Tabela 3. GC-MS analiza sestave hlapnih olj *Citrus limon*.

No.	Retention Time (min)	Compound	% Concentration
1	4.25	α-pinene	2.09
2	4.83	sabinene	0.59
3	5.32	β-pinene	3.90
4	5.58	myrcene	2.5
5	5.84	n-octanal	0.14
6	5.94	α-phellandrene	0.13
7	6.30	α-terpinene	0.23
8	6.93	limonene	66.4
9	7.29	(E)- β-ocimene	0.12
10	7.73	γ-terpinene	6.97
11	8.65	terpinolene	0.55
12	9.03	linalool	0.97
13	12.03	terpinen-4-ol	2.01
14	12.18	iso-verbanol	0.61
15	12.71	α-terpineol	2.06
16	13.64	trans-carveol	0.1
17	15.24	geraniol	0.24
18	15.98	geranial	4.0
19	20.64	geranyl acetate	0.41
		Unidentified	5.98
		Total	100

Results of GC-MS analysis of essential oil from *Cymbopogan citratus*

Table 4 shows the mass spectra of *Cymbopogon citratus* (Lemon Grass) obtained by GC-MS analysis it revealed twenty-five (25) compounds found in the essential oil from the plant. The most abundant compound is Citral a with a peak area of 48.26% and a retention time of 20.41. This is followed by Citral b with a peak area of 39.9% and retention time of 18.92, while Geranyl Butyrate, Dihydroisocaryophyllene epoxide, trans-β-Ocimene are the least abundant compounds with peaks of 0.02% each and retention time of 31.98, 27.09, 10.01 respectively. Table 4 and figure 3 shows the entire components and the spectrum of the essential oil from *Cymbopogon citratus* (Lemon grass).

Table 4. GC-MS Analysis of volatile oil composition of *Cymbopogan citratus.*

Tabela 4. GC-MS analiza sestave hlapnih olj *Cymbopogan citratus.*

No.	Retention Time (min)	Compound	% Concentration
1	5.716	α-Pinene	0.05
2	8.412	δ-3-Carene	0.05
3	9.161	Limonene	1.70
4	10.006	*trans*-β-Ocimene	0.02
5	12.266	Linalool	0.31
6	13.133	*cis*-Verbenol	0.05
7	14.261	γ-Geranial	0.24
8	14.427	*trans*-Chrysanthemol	0.32
9	15.171	Isogeranial	0.82
10	16.004	Isogeranial	1.43
11	16.529	Lilac aldehyde	0.60
12	18.922	Citral b	39.85
13	20.405	Citral a	48.26
14	22.962	2-Hexenyl laurate	0.17
15	23.751	Eugenol	0.08
16	24.878	Geranyl acetate	0.42
17	26.193	*trans*-Caryophyllene	0.32
18	26.341	Chrysanthenone	0.10
19	27.086	Dihydroisocaryophyllene epoxide	0.02
20	27.596	α-Humulene	0.06
21	29.721	Cuparene	0.07
22	30.078	γ-Muurolene	0.52
23	30.795	*cis*-γ-Bisabolene	0.07
24	31.981	Geranyl Butyrate	0.02
25	32.742	Caryophyllene oxide	1.07
		Unidentified	3.38
		Total	100

Results of GC-MS analysis of essential oil from Syzygium aromaticum

The spectroscopy analysis of *Syzygium aromaticum* (Clove) GC-MS revealed the essential oil from the plant contains twenty-one (21) compounds (Table 5). The most abundant and major compound is Eugenol with a peak area of 69.0% and a retention time of 27.04, followed by Eugenyl acetate with a peak area of 13.5% and a retention time of 33.66, while α-Muurolene, Cubenol, Isocaryophyllene, and 2-Heptyl acetate are the least abundant compound with a peak of 0.02% and retention times of 32.47, 38.01, 30.88 and 12.23 respectively. Table 5 shows the breakdown of the entire components, while Figure 4 shows the spectrum of the essential oil from *Syzygium aromaticum* commonly called clove.

Table 5. GC-MS analysis of the volatile composition of *Syzygium aromaticum*.

Tabela 5. Analiza hlapne sestave *Syzygium aromaticum* z GC-MS.

No.	Retention time (min)	Compound	% Concentration
1	22.48	Chavicol	0.25
2	12.23	2-Heptyl acetate	0.02
3	19.13	Methyl salicylate	0.06
4	26.10	α-Cubebene	0.42
5	27.50	α-Copaene	0.28
6	29.47	β-Caryophyllene	13.23
7	27.04	Eugenol	68.98
8	30.45	α-Cubebene	0.05
9	30.88	Isocaryophyllene	0.02
10	31.39	β-Cadinene	0.06
11	31.51	γ-Muurolene	0.07
12	30.75	α-Humulene	1.50
13	31.74	D-Germacrene	0.11
14	32.16	β-Copaene	0.05
15	38.01	Cubenol	0.02
16	32.78	α-Farnesene	0.27
17	33.66	Eugenyl acetate	13.48
18	35.73	Caryophyllene oxide	0.27
19	32.47	α-Muurolene	0.02
20	36.76	Humuladienone	0.03
21	37.47	Epicubenol	0.05
		Unidentified	0.76
		Total	100

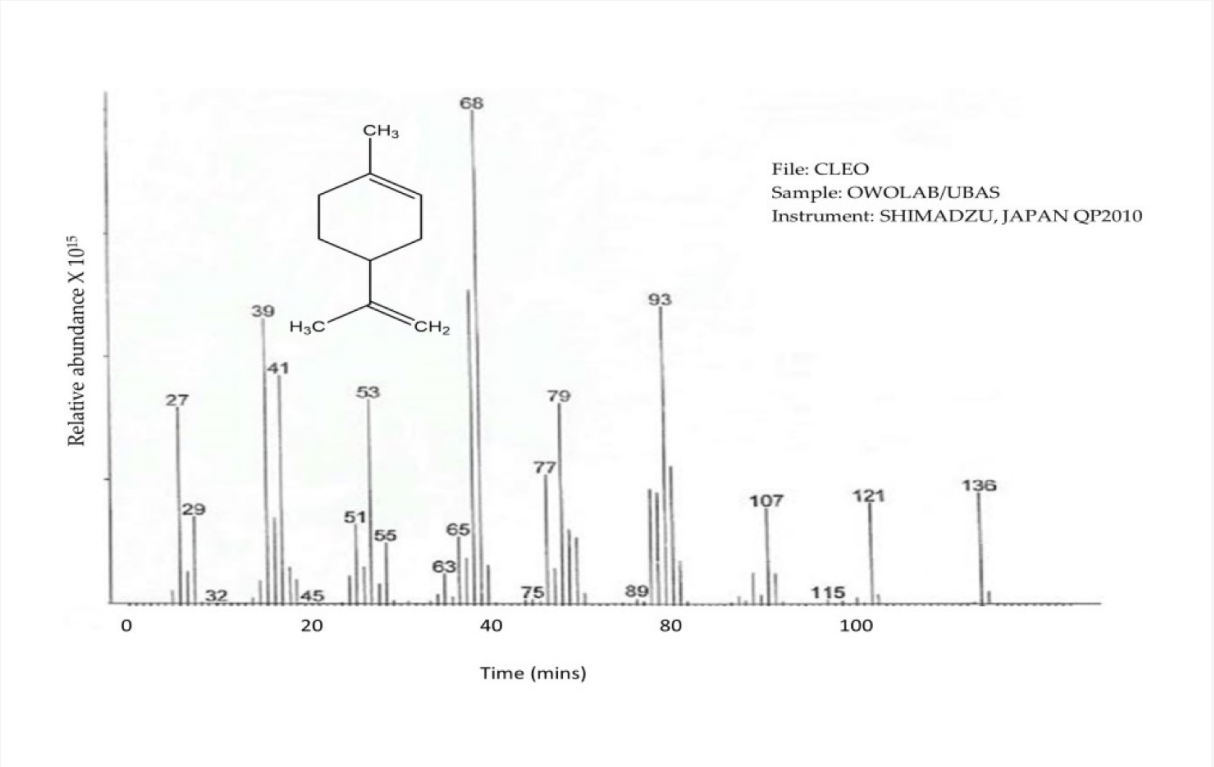

Figure 2. GC-MS Spectrum of *Citrus limon* essential oil.

Slika 2. GC-MS spekter eteričnega olja *Citrus limon*.

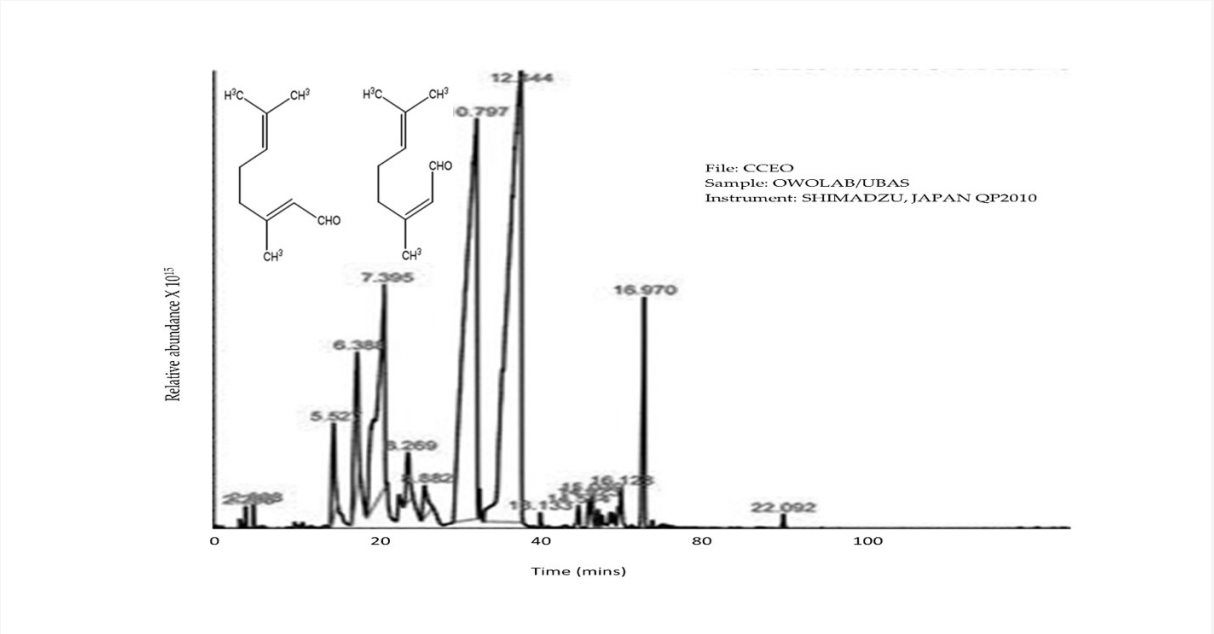

Figure 3. GC-MS Spectrum of *Cymbopogan citratus* essential oil.

Slika 3. GC-MS spekter eteričnega olja *Cymbopogan citratus*.

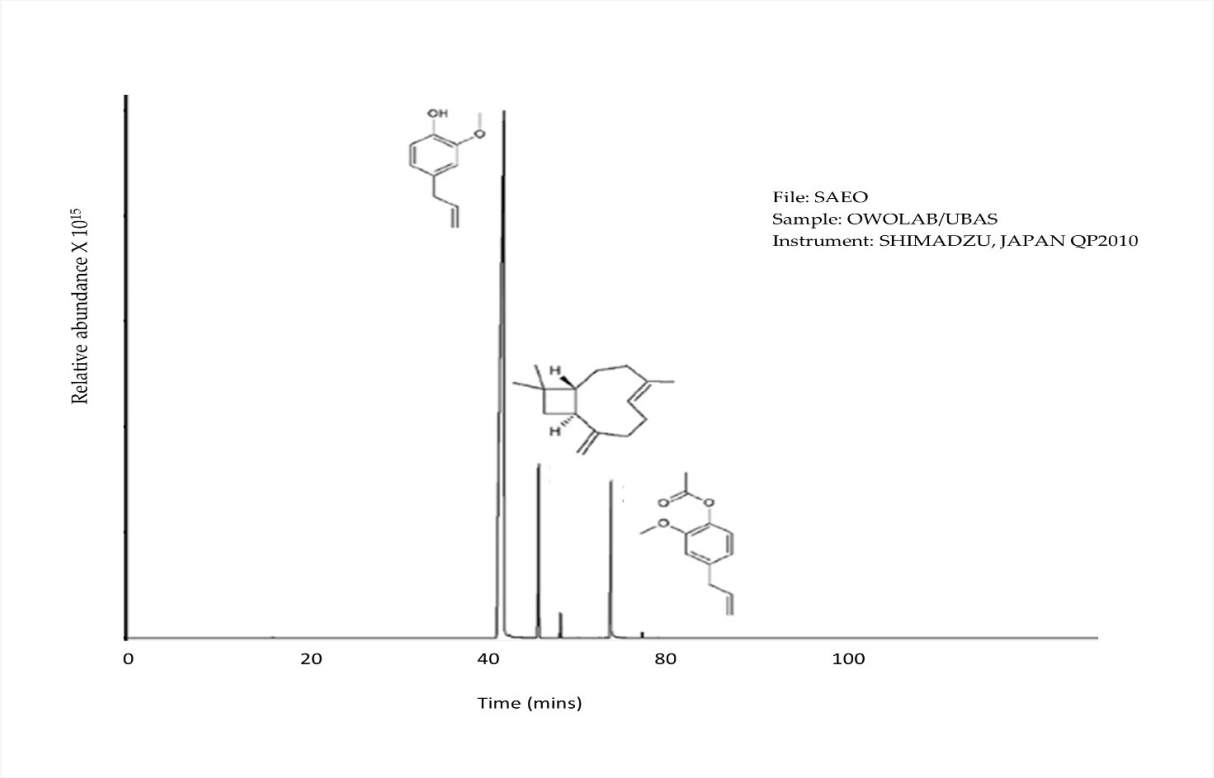

Figure 4. GGC-MS Spectrum of *Syzygium aromaticum* essential oil.

Slika 4. GC-MS spekter eteričnega olja *Syzygium aromaticum*.

Results of Knockdown, mortality, and time efficacy of EOs on Anopheles mosquitoes

Table 6 presents the knockdown rates and times of various essential oils (EOs) and their combinations on Anopheles mosquitoes. *Cymbopogan citratus, Syzygium aromaticum,* and *Citrus limon* oils were tested both individually and in combinations, among the individual oils, *Syzygium aromaticum* exhibited the shortest knockdown time at 14 minutes and knockdown rate of 75%, while *Citrus limon* showed the longest times at 26 minutes for knockdown time and 30% of the mosquitoes were knocked down. Combinations of these oils generally reduced both knockdown times and rates compared to individual oils, with the combination of *Cymbopogan citratus,* and *Syzygium aromaticum* (FM2) demonstrating the most rapid effects with a knockdown time of 7 minutes and rates of 85%. These findings suggest synergistic effects of the oil combinations in enhancing their effectiveness against *Anopheles* mosquitoes.

Discussion

While synthetic insecticides are effective, their widespread use raises environmental and health concerns. They not only harm target insects but also pose risks to animals, humans, and beneficial insects crucial for ecosystem health (Chrustek *et al.*, 2018; Khazri *et al.*, 2015; Liu, 2015). This current study aligned with the efforts to combat mosquito-related issues emphasizing the exploring natural insecticides against mosquitoes.

The GC-MS analysis of essential oils from *Citrus limon, Cymbopogon citratus*, and Syzygium aromaticum revealed diverse and significant chemical compositions that contribute to the observed biological activities. *Citrus limon* oil is predominantly composed of limonene (66.4%), followed by γ-terpinene (7.0%) and geranial (4.0%). *Cymbopogon citratus* oil primarily contains citral a (48.3%) and citral b (39.9%), with minor compounds contributing to its profile. *Syzygium aromaticum* oil is rich in eugenol (69.0%) and eugenyl acetate (13.5%). These results are closely similar to the previous

Table 6. Percentage mortality rates (MR), mortality time (MT), susceptibility status (S), knockdown rates (KR), and knockdown time (KT) of individual essential oil and their combinations against females *Anopheles* mosquito at 1 hour after exposure.

Tabela 6. Odstotki smrtnosti (MR), čas smrtnosti (MT), stanje občutljivosti (S), stopnja otrpnosti (KR) in čas otrpnosti (KT) posameznih eteričnih olj in njihovih kombinacij proti samicam komarja *Anopheles* 1 uro po izpostavljenosti.

Essential oils	Knockdown Time (minutes)	Knockdown Rates (%)	MR%	Status	MT (min)
ETAL	∞	0d	0d	R	∞
LDT	4	100d	100d	S	7
CCEO	18	60±0.866	30±0.25	R	33
SAEO	14	75±0.75	45±0.74	R	25
CLEO	26	30±0.866	15±0.47	R	42
FM1	9	45±0.66	60±0.5	R	16
FM2	7	85±0.25	50±0.37	R	29
FM3	17	65±0.5	57±0.5	R	13

LDT: Lambdacyhalothrin, ETAL: Ethyl alcohol, SAEO: Syzygium aromaticum Essential oil, CCEO: *Cymbopogon citratus* Essential oil, CLEO *Citrus limon* Essential oil, F1 = 10%CLEO+ 10% SAEO + 80% ethyl alcohol, F2 = 10%SAEO+ 10% CCEO + 80% ethyl alcohol, F3 = 5% CLEO + 5% SAEO + 5% CCEO + 75% ethyl alcohol, S = Susceptible is defined as 98–100% mortality, PR = Possible Resistant is defined as 80–97% mortality, R = Resistant is defined as < 80% mortality. Data represent mean ± SD, (n = 25); *p < 0.05

reports on the chemical composition of these oils with slight variations in the percentage concentrations but with complete deviations from physical characteristics such as color, and percentage yield (Kaur *et al.*, 2019; Dangol *et al.*, 2023; El Aboubi et al., 2023; Valková et al., 2022; Haro-González et al., 2021; Khan et al., 2023), for instance, in 2019, Kaur *et al.* reported 69.68 % of eugenol from *Synzygium aromaticum* obtained from Punjab in India with a pale yellow oil and a yield of 12.3 % (w/v) as opposed our findings which revealed 68.98% eugenol, with white oil and a yield of 0.2%. Some literatures (Ben Hsouna et al., 2017; Mortazavi et al., , 2010) also reported a yield of 3 % from *Citrus limon* peel and others reported it to contained a yellow color oil chiefly composed of limonene (60.7 %) (Kačániová et al., 2024) as opposed to ours with white colored oil and yield of 0.5%, although, with similar limonene concentration of 66.4%. The color of essential oils can vary considerably based on factors such as the plant species, extraction method, growing conditions, and processing techniques (Hüsnü et al., 2007; Karalija et al., 2022). Citrus limon oil is typically pale yellow or colorless due to its high limonene content, which is a colorless compound. Our research shows that the limonene content in our samples is higher than most previously reported ones (Ben Hsouna et al., 2017; Mortazavi et al., 2010) with pale yellow color, thereby resulting in an oil with a white colored oil we obtained. Additionally, essential oils from plants harvested at different growth stages (e.g., early bloom versus full maturity) may exhibit subtle color differences (de Sousa et al., 2023). Furthermore, the composition of the soil plays a role, with nutrient-rich soils leading to oils with more intense colors, particularly if they contain higher levels of pigment-producing compounds (Hüsnü et al., 2007).However, many researchers have postulated similar reports on the chemical compositions of *Citrus limon* (Gbolade et al., 2011; El Khoury et al., 2017; de Lima et al., 2024; Khan et al., 2024). Similarly, the results of the chemical composition of *Cymbopogon ctratus* are in line with the reports of many researchers (Bicchi et al., 2008; Nurmansyah et al., 2022; de Lima et al., 2024). These noted variations are mainly due to geographical, meteorological, and soil circumstances, as well as the plant's maturity during harvest time (Karalija et al., 2022).

The insecticidal efficacy tests demonstrated that these essential oils, both individually and in combination, are effective against Anopheles mosquitoes. Clove oil exhibited the quickest knockdown and mortality, while a synergistic blend of the oils of *Cymbopogan citratus*, and *Syzygium aromaticum* significantly enhanced these effects. An old study by Núñez and Aquinosupports this phenomenon(Núñez and Aquino, 2012) with a more recent study by Uçkun and Karakoyun, and Owolabi et al. which testified to the efficacy of essential oils in combating pests like mos-

quitoes (Uçkun and Karakoyun, 2023; Owolabi et al., 2024). The efficacy of Clove essential oil against insects has been elaborately reported by many researchers (Saini et al., 2024; Khan et al., 2023), good number of literature have reported the pesticidal activities of Lemon grass (Moustafa et al., 2021; Sharma et al., 2022), while other studies have also justified the efficacies of *Citrus limon* against pests (Zhang et al., 2023; Almeida et al., 2022; Ben Hsouna et al., 2017). Synergism in pharmacological activities refers to the phenomenon where the combined effect of two or more substances is greater than the sum of their individual effects. This is a common occurrence in plant constituents including essential oil, where multiple compounds work together to enhance therapeutic outcomes or reduce side effects. As noted in this current study, where combined oils resulted in better activity, many literatures have also demonstrated this phenomenon (Pezzani et al., 2019: Ye et al., 2011; Nagaprashantha et al., 2011).

Conclusions

In conclusion, this research demonstrates that the combination of essential oils (Lemongrass and Clove) significantly enhances their effectiveness against Anopheles mosquitoes, exhibiting both faster knockdown and mortality, despite the mosquitoes in the study area have developed resistance to the individual essential oil analyzed. The synergistic action of the combined oils was found to improve mosquito knockdown and mortality rates compared to individual oils, suggesting that these blends may offer a more potent alternative to conventional insecticides. The results also highlight the potential for developing environmentally friendly and sustainable mosquito control strategies, using essential oils as a natural remedy. Further studies are recommended to optimize the oil combinations, determine the most effective application methods, and assess their long-term efficacy and safety for both humans and the environment.

Author Contributions

Conceptualization, O.T.; Methodology, O.T., O.P.; Software, D.J., O.T.; Investigation, O.T., D.J.; Resources, O.P., O.T., I.O; Data curation, O.T.; Writing—original draft preparation, O.T.; writing—review and editing, O.P., D.J., and I.O; Supervision, O.T. All authors have read and agreed to the published version of the manuscript.

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Data Availability

Other data are available and can be accessed by mailing the corresponding author via owolabitunde1@gmail.com..

Conflicts of Interest

The authors declare no conflict of interest.

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ABS

Original Research

In Vitro Antioxidant and Phytochemical Evaluation of Methanolic Extracts from *Punica granatum* Peels in Northwestern Algeria

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Abstract

Natural antioxidants are becoming increasingly popular because of their importance in preventing and treating many diseases. This study investigates the antioxidant properties and chemical composition of methanolic extracts derived from the fruit peels of *Punica granatum* collected in Mostaganem, Algeria. Maceration was used for methanolic extraction of the dry peel powder, followed by both quantitative and qualitative chemical analyses. The antioxidant capacity of the extract was evaluated using three distinct assays: DPPH, ABTS, and FRAP. The results revealed a rich profile of bioactive compounds, including total phenolic content (187.13 ± 1.92 mg GAE/g dry extract), total flavonoid content (79.51 ± 0.83 mg QE/g dry extract), and total tannin content (48.09 ± 0.63 mg CE/g dry extract). Furthermore, the extract exhibited significant antioxidant activity, with IC50 values of 42.52 µg/mL for the DPPH assay, 47.49 µg/mL for ABTS, and 59.5 µg/mL for FRAP. Thus, very high antioxidant activity ratios were recorded, which were also statistically consistent with vitamin C and trolox at the highest concentration (200 µg/mL). These findings highlight the potential of *Punica granatum* fruit peels as a valuable source of natural antioxidants, contributing to the prevention of oxidative stress-related diseases. Given their notable biological activity, pomegranate peels warrant careful consideration for incorporation into functional foods and dietary supplements, offering promising avenues for enhancing health.

Keywords

antioxidant activity, *Punica granatum*, phytochemical screening, methanolic extract, IC50, fruit peels, and phenolic compounds.

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Vrednotenje antioksidativne aktivnosti in fitokemični pregled metanolnih izvlečkov iz lupin sadežev vrste *Punica granatum*, nabranih v severozahodni Alžiriji

Izvleček

Naravni antioksidanti postajajo vse bolj priljubljeni zaradi svojega pomena pri preprečevanju in zdravljenju številnih bolezni. Ta raziskava proučuje antioksidativne lastnosti in kemično sestavo metanolnih izvlečkov, pridobljenih iz lupin sadežev vrste *Punica granatum*, zbranih v regiji Mostaganem v Alžiriji. Metanolna ekstrakcija suhih lupin v prahu je potekala preko maceracije, temu pa so sledile kvantitativne in kvalitativne kemijske analize. S tremi različnimi analizami, DPPH, ABTS in FRAP, je bila ocenjena antioksidativna zmogljivost izvlečka. Rezultati so razkrili bogat profil bioaktivnih spojin, vključno s skupno vsebnostjo fenolov (187,13 ± 1,92 mg GAE/g suhega ekstrakta), skupno vsebnostjo flavonoidov (79,51 ± 0,83 mg QE/g suhega ekstrakta) in skupno vsebnostjo taninov (48,09 ± 0,63 mg CE/g suhega ekstrakta). Poleg tega je izvleček pokazal pomembno antioksidativno aktivnost z vrednostmi IC50 42,52 µg/ml za analizo DPPH, 47,49 µg/ml za ABTS in 59,5 µg/ml za FRAP. Pri najvišji koncentraciji (200 µg/mL) so bila zabeležena zelo visoka razmerja antioksidativne aktivnosti, ki so bila statistično skladna z vitaminom C in troloxom. Te ugotovitve poudarjajo potencial lupin sadežev vrste *Punica granatum* kot dragocenega vira naravnih antioksidantov, ki prispevajo k preprečevanju bolezni, povezanih z oksidativnim stresom. Glede na njihovo pomembno biološko aktivnost bi bilo potrebno lupine granatnega jabolka skrbno proučiti glede vključitve med funkcionalna živila in prehranska dopolnila, saj ponujajo obetavne možnosti krepitev zdravja.

Ključne besede

antioksidativna aktivnost, *Punica granatum*, fitokemični pregled, metanolni ekstrakt, IC50, lupine plodov, fenolne spojine

Introduction

Punica granatum (family *Lythraceae*), commonly known as pomegranate, is a deciduous shrub or small tree that can attain heights between 5 and 10 meters (Khan et al., 2020; Mendonca, 2020). This species thrives in subtropical and tropical climates, is prevalent in the Mediterranean region, and has been cultivated for over 4,000 years (Malik et al., 2020; Pfadenhauer et al., 2020; Nair et al., 2021). The pomegranate holds significant cultural and economic value, serving as a staple in various culinary traditions while symbolizing fertility and abundance across numerous cultures (Giesecke, 2023; Ramsay et al., 2023). The fruit is characterized by its distinctive red hue and sweet-tart flavour, comprising numerous edible seeds (arils) that are enclosed within a hard, thick rind (Mandal et al., 2021). Phytochemically, pomegranate is rich in polyphenols, particularly flavonoids and tannins, which are concentrated in both seeds and peels (Yassin et al., 2021; Yisimayili and Chao, 2022). Recent research has indicated that peels contain higher levels of certain bioactive compounds compared to the arils, contributing to their potential health benefits. These compounds exhibit a range of biological activities, including anti-inflammatory, antimicrobial, and anticancer properties (Kahlaoui, 2021). The utilization of pomegranate peels, often discarded as waste, presents an opportunity for valorization in the food, cosmetic, and pharmaceutical industries (Jimenez-Lopez et al., 2020; Nirmal et al., 2023).

Antioxidants play a crucial role in preserving cellular integrity by counteracting reactive oxygen species (ROS) and mitigating oxidative damage (Dumanović et al., 2021; Houldsworth, 2024). Oxidative stress occurs when there is excessive production of ROS that exceeds the body's antioxidant defences. This discord can result in cellular injury, which can be seen in the development of a variety of conditions, including cancer, cardiovascular diseases, diabetes, and neurodegenerative disorders, such as Alzheimer's disease (Demirci-Cekic et al., 2022). Natural antioxidants, especially those sourced from fruits and vegetables, have garnered attention due to their potential health benefits and safety profiles compared to synthetic alternatives (Gulcin, 2020; Wu et al., 2024). Compounds, such as flavonoids, phenolic acids, and vitamins (vitamins C and E), have demonstrated the ability to scavenge free radicals and

chelate metal ions, thereby preventing oxidative damage (Gulcin, 2020). Due to the growing consumer demand for natural products as a result of the expanding culture of bio-food consumption, exploring the antioxidant properties of plant extracts like those from *Punica granatum* fruit peels could lead to innovative applications in functional foods and dietary supplements (Benchagra et al., 2021).

The objective of this study is to investigate the chemical composition and antioxidant activity of methanolic extracts from *Punica granatum* fruit peels harvested from the Mostaganem region of northwestern Algeria. It includes qualitative identification of bioactive compounds; quantitative analysis of major phytochemicals, total phenolic content, flavonoids, and tannins using standard spectrophotometric methods; and evaluation of antioxidant potential through assays, such as DPPH radical scavenging, ABTS, and FRAP. By detailing the chemical profiles and biological properties of the *Punica granatum* peel extracts, this research seeks to enhance the understanding of their health benefits and promote their utilization, thereby increasing the value of this often overlooked agricultural by-product.

Materials and Methods

Sample Collection

P. granatum peels were collected from fully ripened fruits of pomegranate sourced from the Mostaganem region in northwestern Algeria (the coordinates are approximately 35.8° N latitude and 0.1° E longitude) (Fig. 1), which is characterized by a mild Mediterranean climate with moderate humidity, temperature and radiation, as well as soil fertility that is an essential source for nutrition, plant growth and the production of natural bio-active compounds.

The samples were harvested during the peak harvest season, in the second half of October 2023, to ensure optimal phytochemical composition. After collection, peels were carefully separated from the fruit (Fig. 2), washed thoroughly with distilled water to remove any contaminants, and air-dried in a dark, well-ventilated room, with a temperature not exceeding 40°C to prevent degradation of sensitive compounds. The dried husks were then ground into a fine powder using an electronic grinder and stored in

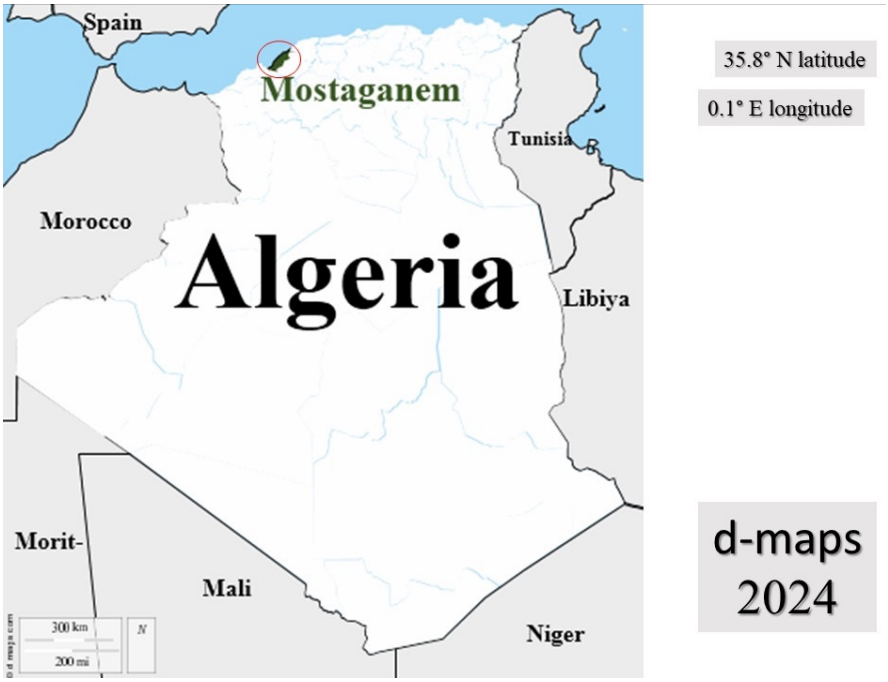


Figure 1. Geolocation of the pomegranate fruit collection area (Mostaganem, Algeria).

Slika 1. Geolokacija območja zbiranja plodov granatnega jabolka (Mostaganem, Alžirija).

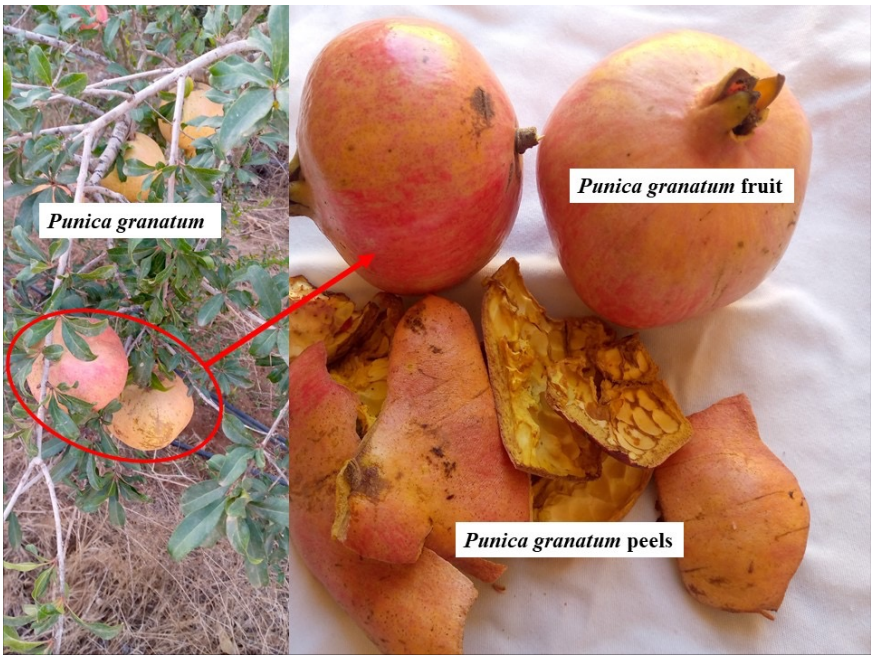


Figure 2. The *Punica granatum* fruit peels.

Slika 2. Lupine plodov *Punica granatum*.

dark, airtight glass containers at room temperature until further analysis. All sample handling is conducted by following standard laboratory protocols to minimize contamination and preserve the integrity of the extracts (Nida et al., 2021; Banza and Rutto, 2022).

Preparation of Methanolic Extract

The methanolic extracts of *P. granatum* fruit peels were prepared using the maceration method. 50 g of the dried and powdered fruit peels were placed in a clean, dry glass container and mixed with 250 mL of analytical-grade methanol. The mixture was allowed to macerate at room temperature for 72 hours, with periodic agitation every 12 hours to enhance the extraction efficiency. After the maceration period, the mixture was filtered through Whatman (N° l) filter paper to separate the liquid extract from the solid residues. The resulting filtrate was then concentrated using a rotary evaporator at a temperature not exceeding 40°C to eliminate the solvent. The concentrated extract was subsequently transferred to pre-weighed vials and stored at -20°C until further analysis (Samsuri et al., 2020; Yassin et al., 2021). In addition to minimizing thermal degradation, the maceration method was also chosen to maximize the extraction of bioactive compounds since the methanol molecule has the smallest molecular mass among alcohols, and it is richer in hydroxyls and more connected to the metabolite (Mali and Kumar, 2023; PM et al., 2024).

To determine the methanolic extract yield from *P. granatum* fruit peels, the following formula was followed:
Yield (% w/w) = ($W_{initial}$ / $W_{extract}$) * 100, where w indicates weight with the same unit (g) (Samsuri et al., 2020; Yassin et al., 2021).

Phytochemical Screening

The qualitative chemical analysis of the methanolic extract of *P. granatum* fruit peels was performed through a series of standardized phytochemical screening tests to identify the presence of various bioactive compounds. The following classes of phytochemicals were examined (Sutoyo et al., 2021; Maigoda et al., 2022):

Alkaloids: A total of 4 mg of ethanolic extract was dissolved in 3 mL of methanol and 5 mL of ammonia, adjusting the pH to 8-9. The resulting mixture was then filtered. Following filtration, 2 mL of a 2 M HCl solution was added to the filtrate. After thorough shaking, five drops of the upper layer were distributed into four test tubes. The solution in tube 1 served as a blank, while solutions in tubes 2, 3, and 4 received one drop each of Mayer, Wagner, and Dragendorff reagents, respectively.

Flavonoids: Flavonoid content was assessed through the Shinoda test. An aliquot of the extract was treated with several drops of concentrated hydrochloric acid (HCl) and magnesium turnings (Mg). The appearance of a pink or red hue indicated the presence of flavonoids.

Tannins: Approximately 1 mg of ethanolic extract was positioned on a drip plate, to which five drops of methanol were added and mixed using a spatula until complete dissolution occurred. Subsequently, 2-3 drops of a 1% $FeCl_3$ solution were incorporated. The emergence of a blue, green, or brownish-green colour signified the presence of tannins.

Saponins: Approximately 1 mg of ethanolic extract was placed in a test tube, followed by the addition of 5 mL of distilled water. The mixture was then agitated for 1 minute. In the presence of foam, two drops of a 1 M HCl solution were introduced. If the foam persisted at a height of 1-3 cm for a duration of 10 minutes, it was concluded that the sample contained saponins.

Terpenoids: The detection of terpenoids was carried out using the Liebermann-Burchard test. A small quantity of the extract was combined with chloroform ($CHCl_3$) and concentrated sulfuric acid (H_2SO_4). The emergence of a red or purple colour signified the presence of terpenoids.

Steroids: Steroids were identified via the Salkowski test. An aliquot of the extract was treated with chloroform ($CHCl_3$) and concentrated sulfuric acid (H_2SO_4). The appearance of red colour in the chloroform layer indicated the presence of steroids.

Glycosides: The presence of glycosides was assessed using the Keller-Kiliani test. A sample of the extract was treated with a few drops of glacial acetic acid (CH_3COOH), followed by concentrated sulfuric acid (H_2SO_4). The appearance of a reddish-brown ring at the interface indicated the presence of glycosides.

The qualitative screening provides a comprehensive overview of the phytochemical profile of *P. granatum* fruit peels and reveals the presence of bioactive compounds that may contribute to their antioxidant and therapeutic properties, forming a basis for further quantitative analysis and exploration of the biological activities associated with these phytochemicals.

Quantitative Chemical Analysis

The quantitative chemical analysis of the methanolic extract obtained from the fruit peels of *P. granatum* was conducted to ascertain the total phenolic content (TPC), total flavonoid content (TFC), and total tannin content (TTC). The initial concentration of 1 mg/mL of the methanolic extract in PBS (phosphate-buffered saline) was used as the base sample in the determination of the doses of metabolites in the extract (Hayat et al., 2020).

Total Phenolic Content (TPC)

Total phenolic content (TPC) was measured using the Folin-Ciocalteu method. An aliquot of the extract (0.5 mL) was mixed with 2.5 mL of the Folin-Ciocalteu reagent and allowed to react for 5 minutes. Following this, 2 mL of a 20% sodium carbonate solution (Na2CO3) was added, and the mixture was incubated in the dark for 30 minutes at room temperature. The absorbance was then measured at 765 nm using a UV-VIS spectrophotometer. TPC was expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight of the extract, using the same experimental conditions with a series of reductive concentrations of gallic acid (500, 250, 125, 62.5 and 31.25 µg/mL) (Barbosa and Minguillan, 2021; Molole et al., 2022).

Total Flavonoid Content (TFC)

Total flavonoid content (TFC) was determined using a colourimetric assay. 1 mL of the extract was mixed with 3 mL of distilled water, followed by the addition of 0.3 mL of a 5% sodium nitrite solution (NaNO2). After 5 minutes, 0.3 mL of a 10% aluminium chloride solution (AlCl3) was added, and the mixture was allowed to stand for another 6 minutes. Finally, 2 mL of 1 M sodium hydroxide (NaOH) was added, and the absorbance was measured at 510 nm. The results were expressed as milligrams of quercetin equiva-

lents (QE) per gram of dry weight of the extract, using the same experimental conditions with a series of quercetin reductive concentrations (500, 250, 125, 62.5 and 31.25 µg/mL) (Shraim et al., 2021; Sari et al., 2023).

Total Tannin Content (TTC)

Total tannin content (TTC) was assessed using the vanillin method. 0.5 mL of the extract was mixed with 2.5 mL of vanillin-HCl reagent. After incubation for 20 minutes at room temperature, the absorbance was recorded at 500 nm. TTC was expressed as milligrams of catechin equivalents (CE) per gram of dry weight of the extract, using the same experimental conditions with a series of catechin reductive concentrations (500, 250, 125, 62.5, and 31.25 µg/mL) (Padumanonda and Phontree, 2021; Maobe et al., 2022).

Antioxidant Activity

The antioxidant activity of methanolic extracts from *P. granatum* fruit peels was evaluated using three different assays: DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) and FRAP (ferric reducing antioxidant power). The extracts were tested at concentrations of 10, 20, 50, 100, 150, and 200 µg/mL to determine their capacity to scavenge free radicals and reduce oxidized substrates. Each assay utilized appropriate controls to ensure the reliability of the results.

DPPH Assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was performed to evaluate the free radical scavenging activity of the extract. A 0.1 mM DPPH solution was prepared in methanol (CH_3OH) and kept in the dark to avoid degradation. 50 µL of each concentration of the extract (10, 20, 50, 100, 150, and 200 µg/mL) was combined with 150 µL of the DPPH solution in a 96-well plate. The reaction was allowed to proceed for 30 minutes in darkness, after which the absorbance was recorded at 517 nm. Ascorbic acid (vitamin C) was included as a positive control in this assay, facilitating a comparative assessment of the antioxidant capacity of the extracts. The formula used to calculate the antioxidant activity in the DPPH assay is typically expressed in terms of the percentage of the DPPH radical scavenging activity (RSA). The basic formula is: RSA (%) = $[(A_0 – A_s) / A0] * 100$, where A_0 is the absorbance of the DPPH solution without the sample and A_s is the absorbance of the DPPH solution in the presence of the sample (Chen et al., 2020; Bashkin et al., 2021).

ABTS Assay

The ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) assay was conducted to assess the scavenging capacity of the extracts against the ABTS radical cation. An ABTS solution was prepared by combining seven mM ABTS with 2.45 mM potassium persulfate, allowing it to stand in the dark for a duration of 14 hours prior to use. This ABTS solution was subsequently diluted to achieve an absorbance of 0.700 ± 0.03 at 734 nm. A total of 50 µL of each extract concentration (10, 20, 50, 100, 150, and 200 µg/mL) was combined with 150 µL of the diluted ABTS solution within a 96-well plate and incubated for 6 minutes at room temperature. The absorbance was recorded at 734 nm. Ascorbic acid was used as a control for antioxidant activity benchmarking using the same experimental conditions. The antioxidant activity in the ABTS assay was determined by calculating the percentage of ABTS radical scavenging activity (RSA) with the following formula: RSA (%) = $[(A_0 – A_s) / A_0] * 100$, where A_0 is the absorbance of the ABTS solution without the sample and A_s is the absorbance of the ABTS solution in the presence of the sample (Gaber et al., 2021; Rumpf et al., 2023).

FRAP Assay

The ferric-reducing antioxidant power (FRAP) assay was employed to assess the reducing capacity of the extract. A freshly prepared FRAP reagent was formulated by mixing 10 mL of 300 mM sodium acetate buffer ($NaCH_3COO$ at pH 3.6), 1 mL of 10 mM TPTZ (2,4,6-tris(2-pyridyl)-s-triazine) dissolved in 40 mM hydrochloric acid (HCl), and 1 mL of 20 mM ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$). Extract concentrations were prepared in methanol at 10, 20, 50, 100, 150, and 200 µg/mL. 50 µL of each extract was combined with 150 µL of the FRAP reagent in a 96-well plate. The mixtures were incubated at 37°C for 30 minutes, after which the absorbance was measured at 593 nm. A control sample containing Trolox served as a comparison. The antioxidant activity in the FRAP assay was determined by calculating the percentage of inhibited iron ions (IFe) with the following formula: IFe (%) = $[(A_0 – A_s) / A_0] * 100$, where A_0 is the absorbance of the ABTS solution without the sample and A_s is the absorbance of the ABTS solution in the presence of the sample (Mwamatope et al., 2020; Rumpf et al., 2023).

Statistical Analysis

All experiments were performed in triplicate. All data are presented as means ± standard deviation (SD). Total polyphenols, flavonoids, tannins, and antioxidant activity were examined for statistical significance using analysis of variance (Excel test, two-factor ANOVA test with replication and post hoc test, which was ensured by IBM SPSS version 25). A p-value of 0.05 was considered a threshold to declare a statistically significant difference. This rigorous statistical methodology reinforced the robustness and scientific validity of the study's conclusions.

For the graphical extrapolation of IC50 values for the antioxidant power of *P. granatum* fruit peels, the range between 10 and 200 was chosen for the x values, unlike previous studies, to obtain a more accurate curve and correlation values.

Results and Discussion

Extraction Yield

The yield of methanolic extract by the maceration method from *P. granatum* fruit peels was determined to be 41.13 ± 0.28 (w/w), indicating a high efficiency in the recovery of bioactive compounds. This yield exceeds those reported in previous studies using different solvents, including ethanol and water, while it was largely consistent with studies using methanol as the solvent. In Table 1, these comparisons are shown.

Chemical Composition

Qualitative Analysis

Qualitative analysis of methanolic extracts from *P. granatum* fruit peels revealed the presence of several bioactive compounds known for their antioxidant and therapeutic properties. The analysis confirmed the presence of the following compounds: alkaloids, flavonoids, tannins, saponins, terpenoids, steroids and glycosides (Table 2). These results indicate that the peel of the *P. granatum* fruit is rich in diverse phytochemicals, which likely contributes to its antioxidant properties and potential health benefits (Gosset-Erard et al., 2021; Parisi et al., 2022).

Table 2. Qualitative analysis of methanolic extracts from the *Punica granatum* fruit peels, where + indicates presence detected and ++ indicates significant presence detected.

Tabela 2. Kvalitativna analiza metanolnih ekstraktov iz lupin sadežev *Punica granatum*, pri čemer + označuje ugotovljeno prisotnost, ++ pa znatno ugotovljeno prisotnost.

Compound	Presence
Alkaloids	+
Flavonoids	++
Tannins	++
Saponins	+
Terpenoids	+
Steroids	+
Glycosides	+

Table 1. Yield of methanolic extract from *Punica granatum* fruit peels by the maceration method.

Tabela 1. Pridobivanje metanolnega ekstrakta iz lupin sadežev *Punica granatum* po metodi maceracije.

Extraction Solvent	Yield (% w/w)	Reference
Methanol	41.13 ± 0.28	Current Study
80% Methanol/ Water	45.4 ± 5.3	Mutahar et al., 2012
Methanol	28.9 ± 0.5	Konsoula, 2016
Water	27.4	Mutahar et al., 2012
Ethanol	21.9 ± 0.6	Konsoula, 2016
Methanol	9.82	Yassin et al., 2021
Acetone	6.51	Yassin et al., 2021
Hexane	4.98	Yassin et al., 2021

Quantitative Analysis

Quantitative analysis of the methanolic extracts from *P. granatum* fruit peels was performed to determine the concentration of major bioactive compounds, specifically total phenolic content (TPC), total flavonoid content (TFC), and total tannin content (TTC). The results are summarized below.

Total phenolic content (TPC) was measured using the Folin-Ciocalteau method. A calibration curve was generated using gallic acid as a standard (Fig 3.a). The concentration of phenolic compounds in the methanolic extract was found to be 187.13 ± 1.92 mg GAE/g DE (milligrams of gallic acid equivalents per gram of dry extract), as shown in Fig. 4. This indicates a significant concentration of phenolic compounds known for their antioxidant properties. A study reported that the total phenolic content in water and acetone extracts of *P. granatum* flowers was approximately 230.8 ± 9.5 and 183.6 ± 15.5 mg GAE/g of extract (Petrova et al., 2020). According to a study by Tunisian scientists, total polyphenols in the pomegranate peel amounted to 85.60 ± 4.87 mg GAE/g (Walid et al., 2012). Another *Punica granatum* fruit peel study from the USA showed the highest total phenols (202.4 mg GAE/g DW) (Wang et al., 2011). *P. granatum* peel methanolic extract had the highest polyphenolic content (190 mg/g) compared to seeds (70 mg/g) and juice (34 mg/g) (Konsoula, 2016).

Total flavonoid content (TFC) was determined using the colourimetric aluminium chloride method using quercetin as a standard (Fig. 3.b). The quercetin equivalent in the methanolic extract was measured at 79.51 ± 0.83 mg QE/g DE (milligrams of quercetin equivalent per gram

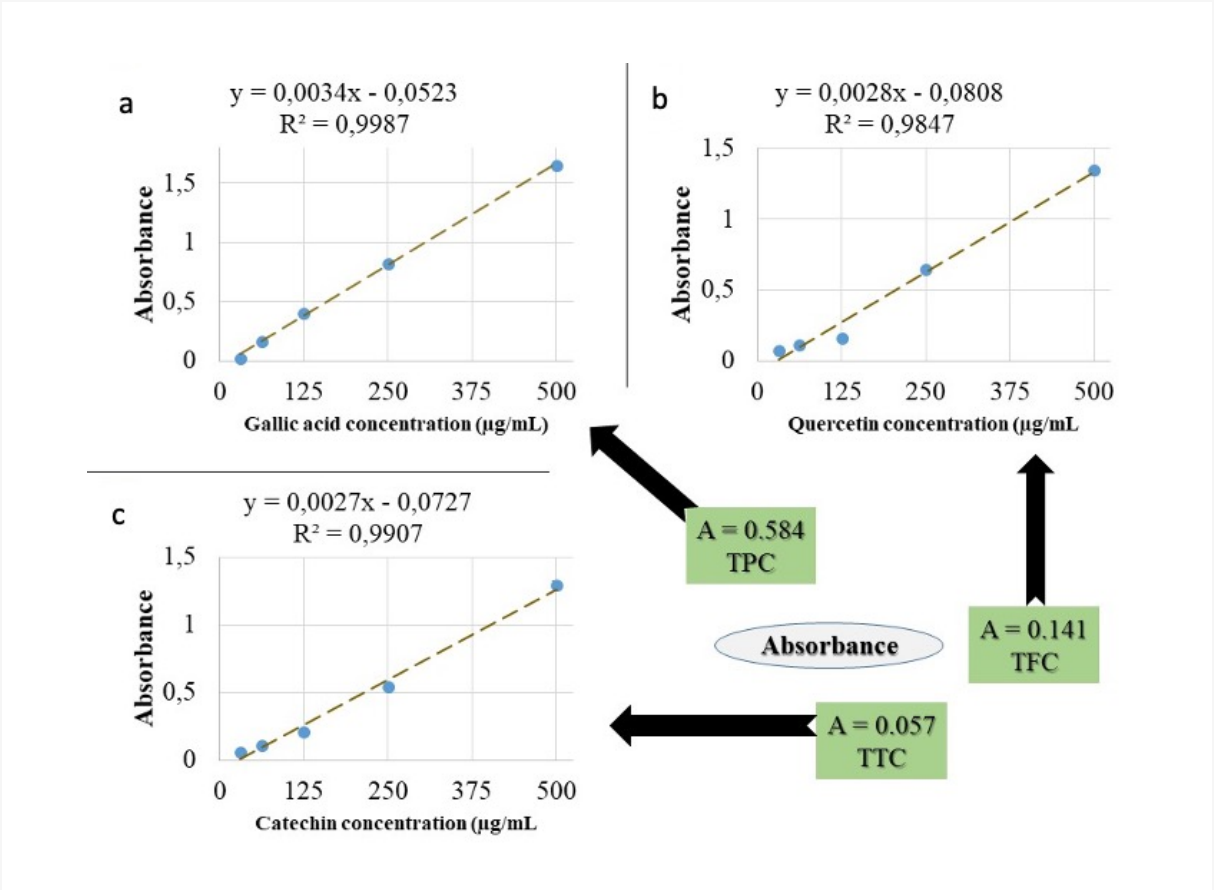


Figure 3. Linear representation of the reference reagents to determine the equivalent values of TPC, TFC, and TTC. a: gallic acid at 765 nm, b: quercetin at 510 nm, and c: catechin at 500 nm. TPC: Total phenolic content, TFC: Total flavonoid content, and TTC: Total tannin content.

Slika 3. Linearni prikaz referenčnih reagentov za določitev ekvivalentnih vrednosti TPC, TFC in TTC. a: galna kislina pri 765 nm, b: kvercetin pri 510 nm in c: katehin pri 500 nm. TPC: skupna vsebnost fenolov, TFC: skupna vsebnost flavonoidov in TTC: skupna vsebnost taninov.

of dry extract), a result shown in Fig. 4. This high level of flavonoids contributes to the overall antioxidant capacity of the extract. A study found that the extracts of *P. granatum* peel contained about 51.52 ± 8.14 mg quercetin equivalents (QE)/g of extract (Walid et al., 2012). Research indicated that the whole fruit peel extract in ethyl acetate contained about 39.2 mg QE/g of flavonoids (Wang et al., 2011). *P. granatum* peel methanolic extract had the highest polyphenolic content – the highest amount of flavonoids (21 mg/g), compared to seeds (9 mg/g) and juice (4 mg/g) (Konsoula, 2016).

Total tannin content (TTC) was assessed using the vanillin assay, which is based on the reaction of tannins with vanillin in the presence of hydrochloric acid, using the standard linear trace (Fig. 3.c). Total tannin content was estimated to be 48.09 ± 0.63 mg CE/g DE (milligrams of catechin equivalents per gram of dry extract), as shown in Fig. 4. This indicates the presence of a significant amount of tannins, which are known for their health-promoting properties. A study found that the extracts of pomegranate peel had a total tannin content of approximately 139.63 ± 4.25 mg tannic acid equivalents per g dry weight (mg TAE/g DW) (Walid et al., 2012). The authors of a Spanish study found that hydrolyzed tannin content in the extracts of pomegranate peel was approximately 21.25 mg tannic acid equivalents (TAE)/g (Viuda-Martos et al., 2013).

These quantitative results confirm that the methanolic extract of *P. granatum* fruit peels is rich in bioactive compounds, supporting its potential use in functional foods and food applications. The high concentrations of TPC, TFC, and TTC contribute to the overall antioxidant activity observed in the extract. There is a statistically significant difference between the amounts of metabolites studied, with 0.05 being the threshold for statistical significance.

Antioxidant Activity Results

The antioxidant activity of the methanolic extracts of *P. granatum* peels was evaluated using three assays: DPPH (2,2-diphenyl-1-picrylhydrazyl), FRAP (ferric reducing antioxidant power), and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)). The results are presented in Figs. 5, 6, and 7.

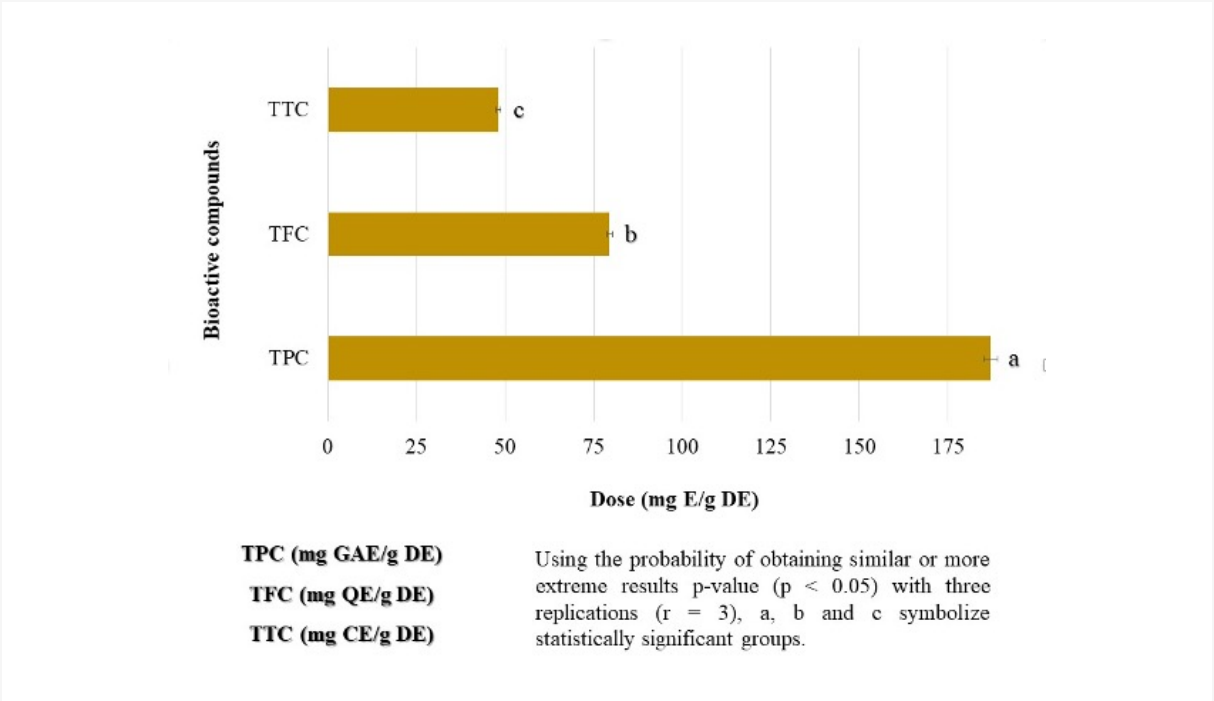


Figure 4. Quantitative analysis of bioactive compounds in the methanolic extracts of *Punica granatum* fruit peels. TPC: Total phenolic content, TFC: Total flavonoid content, and TTC: Total tannin content.

Slika 4. Kvantitativna analiza bioaktivnih spojin v metanolnih izvlečkih lupin sadežev *Punica granatum*. TPC: skupna vsebnost fenolov, TFC: skupna vsebnost flavonoidov in TTC: skupna vsebnost taninov.

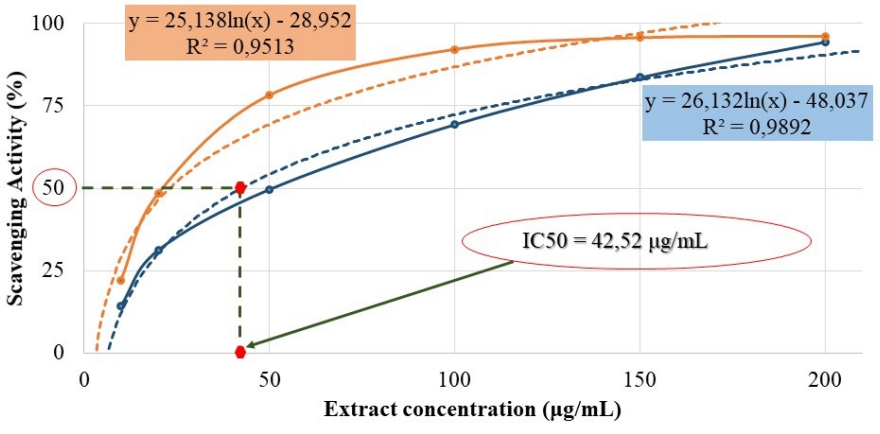
DPPH Assay

The DPPH assay conducted on the methanolic extract of *P. granatum* fruit peels demonstrated a significant radical scavenging activity across various concentrations. The results indicated a gradual increase in scavenging activity, with values reaching 94.39 ± 0.15 % at 200 µg/mL. The calculated IC50 value of the extract was 14.23 µg/mL, suggesting moderate antioxidant potential compared to ascorbic acid, which exhibited a lower IC50 of 42.52 µg/mL, affirming its status as a potent antioxidant (Fig. 5).

These findings align with previous research indicating the antioxidant properties of the *P. granatum* extracts. For instance, Benchagra et al. (2021) reported an IC50 value of 21.58 µg/mL for the *P. granatum* juice, highlighting its comparable effectiveness in scavenging free radicals. Similarly, Konsoula (2016) noted an IC50 value of 0.06 to 0.08 mg/mL for the methanolic extracts of *P. granatum* fruit peels, further supporting the notion that pomegranate-derived compounds possess substantial antioxidant capabilities.

The increasing scavenging activity with higher concentrations of the extract underscores the potential of *P. granatum* fruit peels as a source of natural antioxidants. The observed moderate activity of the methanolic extract can be attributed to the rich phytochemical profile identified in the qualitative analysis, including flavonoids and tannins, which are known for their radical-scavenging abilities (Khan et al., 2020).

Tannins, which are distinguished by their elevated molecular weight and astringent characteristics, act as antioxidants by creating complexes with proteins and other macromolecules. This interaction impedes oxidative enzymes, including lipoxygenase and cyclooxygenase, thereby diminishing the generation of pro-oxidant substances (Molino et al., 2023; Zeng et al., 2023). Additionally, tannins may stimulate endogenous antioxidant enzymes, thereby augmenting the body's inherent defence



Scavenging Activity (%)	10 µg/mL	20 µg/mL	50 µg/mL	100 µg/mL	150 µg/mL	200 µg/mL
Extract	14,23 ± 0,62 (h)	31,12 ± 0,59 (f)	49,58 ± 0,31 (e)	69,29 ± 0,26 (d)	83,59 ± 0,33 (b)	94,39 ± 0,15 (a)
Ascorbic acid	22,15 ± ,04 (g)	48,39 ± 0,37 (e)	78,26 ± 0,41 (c)	92,15 ± 0,12 (a)	95,68 ± 0,09 (a)	96,09 ± 0,05 (a)

Using the probability of obtaining similar or more extreme results p-value ($p < 0.05$) with three replications ($r = 3$), a, b, c, d, e, f and g symbolize statistically significant groups.

Figure 5. Antioxidant activity of the methanolic extract of *Punica granatum* fruit peels in the DPPH assay. Yellow color: Compatibility in antioxidant efficacy between the minimum concentration of the extract compared to vitamin C. Green color: Compatibility in antioxidant efficacy between the maximum concentration of the extract compared to vitamin C.

Slika 5. Antioksidativna aktivnost metanolnega izvlečka lupin sadežev *Punica granatum* v preskusu DPPH. Rumena barva: združljivost antioksidativne učinkovitosti med najmanjšo koncentracijo izvlečka v primerjavi z vitaminom C. Zelena barva: združljivost antioksidativne učinkovitosti med največjo koncentracijo izvlečka v primerjavi z vitaminom C.

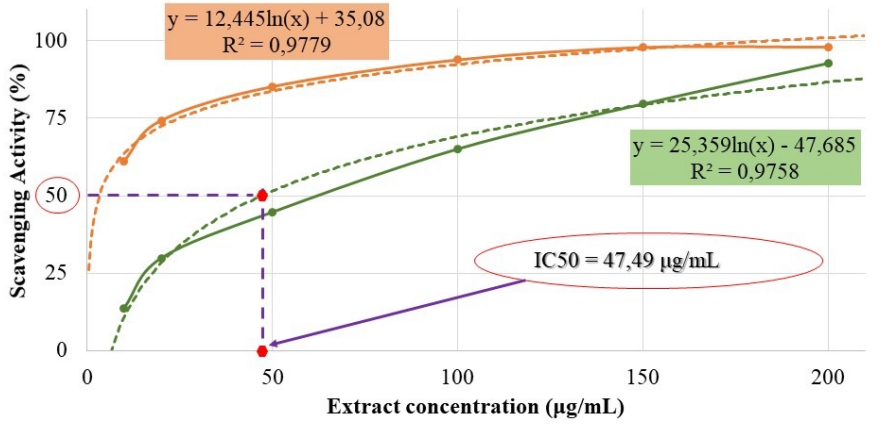
mechanisms against oxidative stress. Both flavonoids and tannins influence signalling pathways associated with oxidative stress, notably the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway, which results in the upregulation of antioxidant genes. This synergistic interaction of polyphenols highlights their promising therapeutic potential in the prevention of diseases linked to oxidative stress, including cardiovascular diseases, cancer, and neurodegenerative disorders, thereby enhancing overall health and well-being (Ciampi et al., 2020; Soldado et al., 2021). The methanolic extract of *P. granatum* fruit peels demonstrates promising antioxidant activity, reinforcing the fruit's status as a valuable source of bioactive compounds with potential health benefits. Future studies should focus on isolating specific phytochemicals responsible for this activity and exploring their mechanisms of action in oxidative stress-related conditions.

Statistically, with a p-value threshold of 0.05, there is a significant difference between all concentrations of the extract used in our study, while only the lowest concentrations between 10 and 100 µg/ml were found to be significant for vitamin C. On the other hand, we did not observe a significant difference between the 50 µg/mL concentration of the extract and the 20 µg/mL concentration of vitamin C. Statistically, the 200 µg/mL concentration of the pomegranate fruit peel methanolic extract is considered to be compatible with the 100 µg/mL concentration of vitamin C in terms of DPPH root scavenging power.

ABTS Assay

The ABTS test results indicate that the antioxidant activity of the *P. granatum* fruit peel extract increases with increasing concentration, with an IC50 value of 47.49 µg/mL (Fig. 6). This means that at this concentration, the extract exhibits 50% inhibition of the ABTS radical. In comparison, ascorbic acid, a known chemical antioxidant, shows a much lower IC50 value of 3.3 µg/mL, indicating stronger antioxidant activity per unit concentration.

The percentage inhibition data reveals a clear dose-response relationship for both pomegranate extract and



Scavenging Activity (%)	10 µg/mL	20 µg/mL	50 µg/mL	100 µg/mL	150 µg/mL	200 µg/mL
Extract	13,59 ± 0,6 (j)	29,81± 0,39 (i)	44,69 ± 0,61 (h)	65,09 ± 0,29 (f)	79,66 ± 0,53 (d)	92,81± 0,25 (ab)
Ascorbic acid	61,12 ± ,38 (g)	74,31± 0,41 (e)	85,29 ± 0,22 (c)	93,95 ±0,18 (ab)	97,98 ± 0,11 (a)	98,06 ± 0,03 (a)

Using the probability of obtaining similar or more extreme results p-value ($p < 0.05$) with three replications (r = 3), a, ab, c, d, e, f, g, h, i and j symbolize statistically significant groups.

Figure 6. Antioxidant activity of the methanolic extract of *Punica granatum* fruit peels in the ABTS assay. Green color indicates the compatibility in antioxidant efficacy between the concentration of the extract compared to vitamin C.

Slika 6. Antioksidativna aktivnost metanolnega izvlečka olupkov sadežev *Punica granatum* v testu ABTS. Zelena barva označuje združljivost antioksidativne učinkovitosti med koncentracijo izvlečka v primerjavi z vitaminom C.

ascorbic acid. At the highest concentration tested (200 µg/mL), the extract achieves 92.81 ± 0.25% inhibition, while ascorbic acid reaches 98.06 ± 0.03% (Fig. 6). This indicates that although the pomegranate extract is highly effective, it is less effective than ascorbic acid in terms of its ability to scavenge ABTS radicals.

The antioxidant activity of the *P. granatum* fruit peels can be attributed to their rich phytochemical profile, which includes metabolites. Polyphenols, encompassing flavonoids and tannins, are essential in countering oxidative stress through their diverse antioxidant mechanisms. Flavonoids, including quercetin and kaempferol, display notable free radical scavenging capabilities by transferring electrons to reactive oxygen species (ROS). This action effectively neutralizes ROS, thereby preventing cellular damage. The donation of electrons stabilizes the radical, diminishing its reactivity and lessening oxidative damage to lipids, proteins, and DNA. Furthermore, flavonoids possess the ability to chelate metal ions, such as iron and copper, which promote the formation of free radicals, consequently reducing oxidative reactions. Studies have shown that compounds, such as ellagitannins and anthocyanins, present in pomegranate contribute significantly to its antioxidant capacity (Benchagra et al., 2021). These compounds can donate electrons or hydrogen atoms to free radicals, effectively neutralizing them.

The IC50 values observed in this study for *P. granatum* are consistent with previous findings. For instance, a study by Sabraoui et al. (2020) reported IC50 values between 42.71 and 65.55 µg/mL for pomegranate peel extracts, which aligns well with our findings. Comparatively, Other plant extracts, such as those from *Satureja khozistanica, Withania somnifera* leaves, and *Cannabis sativa* flowers, have shown similar IC50 values, often in the range of 56-60 µg/mL (Venkatachalapathy et al., 2021; Al Khoury et al., 2022). This positions *P. granatum* fruit peels as a promising natural source of antioxidants, particularly when considering their availability and potential for incorporation into functional foods and supplements. The antioxidant activity of the *P. granatum* fruit peel extract, as evidenced by the ABTS assay, substantiates its potential as a significant source of natural antioxidants. Although it demonstrates considerable activity, it does not match the potency of ascorbic acid. Future investigations should prioritize the isolation and characterization of the active components responsible for the noted antioxidant activity, in addition to examining their synergistic effects with other dietary antioxidants.

Statistically, there is an overall significant difference between all extract concentrations and vitamin C concentrations below 200 µg/mL, with 0.05 being the significance threshold. On the other hand, statistical agreement was recorded between the highest studied concentration of the extract (200 µg/ml) and the 100 µg/ml concentration of vitamin C.

FRAP Assay

The FRAP assay was performed using a methanolic extract of *P. granatum* fruit peels. The absorbance measurements obtained from the assay allowed the calculation of the reducing power expressed in percentage compared with trolox as a potent inhibitor against metal ions that acts directly in the oxidation process and catalyzes the liberation of radicals. The results are summarized in Fig. 7. The evaluation reveals a promising profile of antioxidant efficacy. The extract demonstrated a clear dose-dependent increase in FRAP values, reaching 75.29 ± 0.14% at 200 µg/mL, which is comparable to the FRAP value of Trolox at the same concentration (75.39 ± 0.07%). Notably, the IC50 values indicate that the pomegranate peel extract (59.5 µg/mL) requires a higher concentration than trolox (36.8 µg/mL) to achieve a 50% inhibition of oxidative stress. This observation suggests that while *P. granatum* fruit peels possess significant antioxidant properties. Their potency may be slightly lower than that of synthetic antioxidants like trolox, which is consistent with findings of other studies that report varying levels of efficacy among natural extracts (Sihag et al., 2022; Saparbekova et al., 2023).

When comparing the antioxidant activities of *P. granatum* fruit peels with other plant extracts, the studied extract showed similar or even superior abilities to many fruits. The antioxidant activities of these extracts can be largely attributed to the diversity of their phytochemical properties, which are known for their strong free radical scavenging effect, as well as the inhibition of oxidation induced by metals, such as iron and copper. This comparative analysis highlights the competitive nature of the *P. granatum* fruit peels within the broader category of fruit extracts, making them a valuable source of natural antioxidants (Tiji et al., 2021; Nwoso, 2023; Radulescu et al., 2024).

Future research should focus on isolating specific bioactive compounds in the *P. granatum* peels and exploring their mechanisms of action. Furthermore, studies examining the synergistic effects of the *P. granatum* peel extracts with other plant sources may enhance antioxidant activity

and expand their use in functional foods (Sihag et al., 2022; Radan et al., 2024). Such studies will contribute to a more comprehensive understanding of how these natural products can be effectively harnessed to promote health.

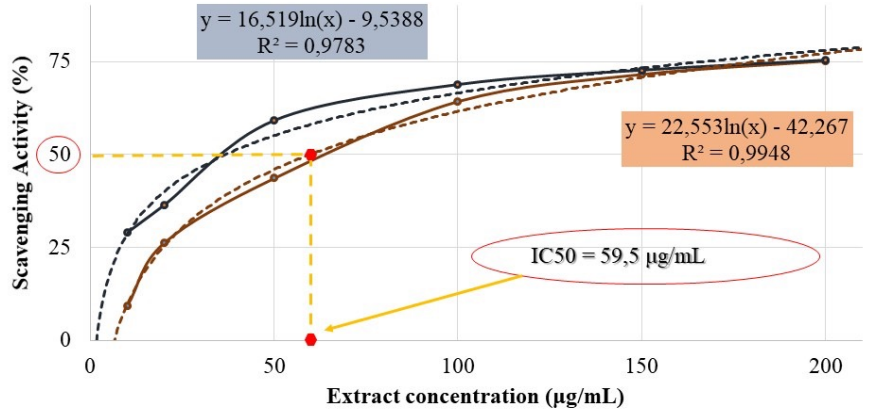
With a statistical threshold of 0.05, there is no significant difference between the concentrations above 150 µg/mL, where there is an agreement between the inhibitory activity of the methanolic extract and trolox. All concentrations equal to or less than 100 µg/mL showed a significant difference. There is a statistical agreement between the 20 µg/mL concentration of the extract and the 10 µg/mL concentration of Trolox.

Implications of Findings

The findings of this study have several implications. Firstly, the high antioxidant potential of *P. granatum* fruit peels suggests that they could be utilized as natural additives in food products to enhance shelf life and nutritional value by reducing oxidative damage. The presence of diverse bioactive compounds also points to potential health benefits, including anti-inflammatory, anticancer, and cardioprotective effects.

Moreover, the results indicate that *P. granatum* peels could serve as an economically viable source of natural antioxidants, particularly in the regions where pomegranate cultivation is prevalent. This could promote the valorization of agricultural waste, contributing to sustainable practices in the food industry.

Future research could explore the specific mechanisms through which these compounds exert their effects, as well as their bioavailability and pharmacokinetics in human health. Additionally, investigations into the synergistic effects of the various phytochemicals present in *P. granatum* peels could provide insights into their potential therapeutic applications.



Scavenging Activity (%)	10 µg/mL	20 µg/mL	50 µg/mL	100 µg/mL	150 µg/mL	200 µg/mL
Extract	9,29 ± 0,21 (h)	26,22± 0,46 (g)	43,66± 4 (e)	64,36± 0,22 (c)	71,66± 0,28 (a)	75,29± 0,14 (a)
Trolox	28,94 ± ,13 (g)	36,31± 0,26 (f)	59,14± 0,71 (d)	68,81±0,15 (b)	72,68± 0,11 (a)	75,39± 0,07 (a)

Using the probability of obtaining similar or more extreme results p-value ($p < 0.05$) with three replications ($r = 3$), a, b, c, d, e, f, g and h symbolize statistically significant groups.

Figure 7. Antioxidant activity of the methanolic extract of *Punica granatum* fruit peels in the FRAP assay. Yellow color: Compatibility in antioxidant efficacy between the minimum concentration of the extract compared to Trolox. Green color: Compatibility in antioxidant efficacy between the maximum concentration of the extract compared to Trolox.

Slika 7. Antioksidativna aktivnost metanolnega izvlečka lupin sadežev *Punica granatum* v testu FRAP. Rumena barva: Združljivost antioksidativne učinkovitosti med najmanjšo koncentracijo izvlečka v primerjavi s troloxom. Zelena barva: združljivost antioksidativne učinkovitosti med največjo koncentracijo izvlečka v primerjavi s Trolox.

Limitations of the Study

Despite the encouraging findings, this study presents several limitations. The antioxidant assays utilized were conducted *in vitro*, which may not completely mimic the complexities of biological systems *in vivo*. Consequently, while the findings suggest notable antioxidant activity, additional research is required to assess the bioavailability and metabolic pathways of these compounds in human subjects. Lastly, the study did not explore the potential toxicity or adverse effects associated with the consumption of *P. granatum* peel extracts. Investigating these factors is crucial for assessing their safety and efficacy for human use. This study establishes the antioxidant potential of *P. granatum* peels and their rich phytochemical profile. Further research is warranted to explore their application in functional foods and therapeutic settings.

Conclusion

At a time when the use of chemicals in the health and food industry has dominated, many researchers are seeking to reintegrate natural compounds as a healthier alternative, as research into antioxidants derived from natural substances has increased. The methanolic extract of *Punica granatum* fruit peels demonstrates significant antioxidant activity, as evidenced by robust results across three *in vitro* experimental methods: DPPH, ABTS, and FRAP. These findings highlight the potential of the methanolic extract of *P. granatum* fruit peels as a rich source of antioxidant compounds, which could play a crucial role in combating oxidative stress and associated diseases. This is because the metabolites in the peels can scavenge free radicals and inhibit oxidation with metal ions, such as iron. This was confirmed by this study.

Given the rising interest in natural dietary supplements, the incorporation of this extract into functional foods or as a standalone supplement presents an exciting opportunity for enhancing health and wellness. Future research should focus on optimizing the extraction processes, elucidating the specific antioxidant compounds responsible for these effects, and conducting clinical trials to validate the health benefits of *P. granatum* fruit peel extracts in the human population. By advancing our understanding of these compounds, we can pave the way for innovative dietary solutions that harness the full potential of the *Punica granatum* fruit peels.

Author Contributions

Conceptualization, B. B. and B. K.; Methodology, B. B., B. K., M. B., B. A.; Software, B. B., Verification, B. B., B. K., B. A. and M. B. Formal analysis, B. A. ; formal analysis, B. K.; Investigation, B. B. and B. A.; Sources, Scientific Research Laboratory of the Universities of Mostaganem, Mascara and Relizane, Algeria; Data organization, all authors. Preparation of the original draft, all authors; revision and editing of the manuscript, all authors; Conception, M. B.; supervision, B. B.; Project management, B. B. All authors have read and approved the published version of the manuscript.

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Declaration of Competing Interest

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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ABS

Original Research

Investigation of Phytochemical content, Antioxidant properties and Antibacterial Potential of Algerian *Bunium incrassatum*

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Abstract

The current study highlights the efficacy of tuber extracts from the Algerian medicinal plant *Bunium incrassatatum* for their phytochemical content, antioxidant and antimicrobial activities. Indeed, the ethanolic maceration method proved to be the most effective for phytochemical extraction, showing the highest yield extraction. Moreover, the ethanolic maceration displayed a higher polyphenol and flavonoid content compared to the methanolic extract. The antioxidant potentials of methanolic and ethanolic extracts of *Bunium incrassatum* were reported by the DPPH assay, revealing moderate efficacy of IC50 values of 380.53 µg/mL and 261.75 µg/mL, respectively, indicating that both of these extracts can be considered a good resource of natural antioxidants. In terms of antibacterial activity, the methanolic extract exhibited appreciable antimicrobial activities against different bacterial strains like *Staphylococcus aureus, Klebsiella pneumoniae, Bacillus cereus*, and *Escherichia coli*, with average zones of inhibition of 10.74 mm, 10.14 mm, 10.03 mm, and 9.45 mm, respectively. The present study underlined that the extracts from *Bunium incrassatum* tubers could be a rich raw material with an interesting profile in phytochemical content, antioxidant, and antimicrobial activities, deserving further studies for applications in drug medicine, therapy, and nutrition.

Keywords

Bunium incrassatum; tuber extracts; phytochemical content; antioxidant properties; antibacterial potential; ethnobotanical resources.

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Raziskava fitokemične vsebnosti, antioksidativnih lastnosti in antibakterijskega potenciala alžirske vrste *Bunium incrassatum*

Izvleček

Sedanja študija poudarja učinkovitost izvlečkov gomoljev iz alžirske zdravilne rastline *Bunium incrassatatum* zaradi njihove fitokemične vsebnosti, antioksidativnega in protimikrobnega delovanja. Dejansko se je izkazalo, da je metoda etanolne maceracije najučinkovitejša za fitokemično ekstrakcijo z največjim izkoristkom ekstrakcije. Poleg tega je etanolna maceracija pokazala večjo vsebnost polifenolov in flavonoidov v primerjavi z metanolnim ekstraktom. O antioksidativnem potencialu metanolnih in etanolnih izvlečkov *Bunium incrassatum* so poročali s testom DPPH, ki je pokazal zmerno učinkovitost vrednosti IC50 380,53 µg/mL oziroma 261,75 µg/mL, kar kaže, da se oba izvlečka lahko štejeta za dober vir naravnega antioksidanti. Kar zadeva protibakterijsko delovanje, je pokazal znatno protimikrobno delovanje proti različnim bakterijskim sevom, kot so *Staphylococcus aureus, Klebsiella pneumoniae, Bacillus cereus* in *Escherichia coli*, s povprečnimi conami inhibicije 10,74 mm, 10,14 mm, 10,03 mm oziroma 9,45 mm. Sedanja študija je poudarila, da bi lahko bili izvlečki iz gomoljev *Bunium incrassatum* bogata surovina z zanimivim profilom fitokemične vsebnosti, antioksidativnih in protimikrobnih aktivnosti, ki si zaslužijo nadaljnje študije za uporabo v medicini, terapiji in prehrani.

Ključne besede

Bunium incrassatum; izvlečki gomoljev; fitokemijska vsebnost, antioksidativne lastnosti; antibakterijski potencial; etnobotanični viri.

Introduction

Medicinal plants have been utilised for traditional medicine for many centuries. Their therapeutic value is presently more recognized, with many plant constituents being integrated into contemporary pharmacopoeia. Moreover, medicinal plants have consistently provided a rich source of bioactive compounds with significant therapeutic potential (Gurib-Fakim, 2006).

The use of plant extracts as antimicrobial drugs is becoming very popular and significant as microbes' resistance to the drugs increases rapidly (Banso et al., 2006; Khandal et al., 2012).

Singh and Kumar (2023) and Rasool et al. (2020) reported that ethnobotanical studies have hastened investigations into new natural compounds of vegetation that may answer great expectations in the development of novel therapies. However, safety and standardization with such medicinal herbs are still questioned; hence, the ability to integrate them into modern health systems is seriously challenged.

In addition, medicinal plants have played a very important active role in Algeria's traditional practices regarding the healing of several ailments. Ethnobotany has unravelled the richness and diversity of the plant species used by the local populations, underlining their importance in the health domain. Among them, Talghouda is considered synonymous with *B. incrassata* and *B. mauritanica*. *Bunium* is a medicinal plant belonging to *Apiaceae*, and it is widely distributed in several regions of Algeria (Quézel and Santa, 1963). It is found in several regions of Algeria, especially the northern part. Traditionally, Talghouda tubers are used to relieve bronchitis, thyroid disorders, inflammatory haemorrhoids, and antidiarrheals. Besides their usage in medicine, they are also consumed due to their nutritional value and as food (Bousetla et al., 2015). Interestingly, Talghouda (*B. incrassatum*) was one of the first medicinal plants documented in North Africa and Algeria. It has been used to treat asthma, cysts, thyroid disorders, and tonsillitis (Djahafi,2021).

The Talghouda plant, including varieties like *B. mauritanicum, B. incrassatum*, and *B. bulbocastanum*, has been reported on in earlier works on its chemistry (Bousetla et al., 2015; Bousetla et al., 2014; Dehimi et al., 2021). Further, Bousetla et al. 2015 reported that tubers of Talghouda in Algeria were consumed like potatoes. These dried and powdered medications serve as astringent and anti-diarrhoeal in action but anti-inflammatory in nature, especially in

conditions like haemorrhoids, besides bronchitis and cough.

According to Adoui et al. (2022), the chemical components of Talghouda are not yet fully described. In fact, only a limited number of studies have concerned the phytochemical characterization of Talghouda extracts and oils like Bousetla et al. (2014) on *B. incrassatum* fruits essential oil from Algeria and Bousetla et al. (2015) on *B. incrassatum* chloromethane methanol roots extract from Algeria; El Koli et al. (2017) on *B. incrassatum* aerial parts essential oils from Algeria; Karouche et al. (2020) reported on methanol and aqueous extracts of *B. mauritanicum* tubers; and Dehimi et al. (2021) reported on acetone and hexane extracts from *B. incrassatum* fruits. According to Adoui et al. (2022), the extracts of Talghouda, among other species of *Bunium*, possess remarkable biological activities. Therefore, the aim of the present study is to investigate the phytochemical composition, antioxidant activity, and antimicrobial properties of *B. incrassatum* tuber extracts, focusing on the quantification of key bioactive compounds and exploring their potential therapeutic application.

Materials and Methods

Plant extracts preparation

Bunium incrassatum, commonly called “Talghouda,” was collected in the region of Mila, in the east of Algeria. The tubers obtained were washed and homogenized with the help of a blender. Methanol and ethanol were employed to recover the phenolic compounds. Afterwards, 30 grams of the plant material was soaked with 300 ml of 80% methanol in a continuous stirring at room temperature, protected from light, together with 300 ml of 70% ethanol. After 72 h, the suspension was filtered, and the filtrate was collected before drying it in baking dishes at 40°C. The crude extract obtained was kept until use. Using the formula given by(Falleh et al., 2008), extraction yield was determined as follows:

Extractive yield = (Weight of extract obtained (g) / Weight of crude drug used for extraction (g)) × 100%.

Total Phenolic Content

The phenolic content was determined by using the Folin-Ciocalteu reagent assay (Singleton V et al., 1965). A standard calibration curve was plotted using gallic acid. Total phenolic content (TPC) was expressed as milligrams of gallic acid equivalents (mg GAE) per gram of extract.

Total Flavonoid Content

The content of flavonoids in the extracts of Bunium incrassatum was measured by an AlCl3 method, according to Lamaison and Carnet (1990). The amount of flavonoids was measured using a quercetin calibration curve and was presented as milligrams of quercetin equivalent per gram of dry weight of plant material- mg EQ/g Ps.

DPPH radical scavenging assay

The antioxidant activity was assayed by the method of DPPH radical scavenging (Kumarasamy et al., 2007). Briefly, 1 ml of the methanolic DPPH solution (0.2 mM) was added to 1 ml of different dilutions of plant extracts at concentrations ranging from 0 to 1 mg/ml. The mixture was kept at room temperature and in the dark for 30 minutes. Absorbance was measured against control at 517 nm of 1 ml DPPH solution and 1 ml methanol. The decrease in absorbance that occurred was monitored by a spectrophotometer, and the percentage inhibition PI was worked out using the following:

Percentage radical scavenged = [(A0 − A1/A0)] ∗ 100%

where A0 is the absorbance of the DPPH solution, and A1 is the absorbance of the sample.

The antibacterial activity

The antibacterial activity of the plant extracts was evaluated using the gel diffusion method (Treki et al., 2009), known as an antibiogram. The principle is based on the appearance of an inhibition zone in the culture medium (Chhetry et al., 2022). Mueller Hinton Agar (MHA) was prepared, sterilized, poured into Petri plates, and incubated at 37°C for 24 hours to check for contamination. Bacterial strains were streaked on the agar plates and incubated for 24 hours. Bacterial suspensions of *Escherichia coli* ATCC 11303, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus cereus* ATCC 10987, and *Klebsiella pneumoniae* ATCC 700603 were prepared in a nutrient medium and incubated for 24 hours at 37°C. Discs impregnated with different concentrations of the extract were placed on the agar. Gentamicin was used as

a positive control. Measurement of inhibition zones was performed after incubation - 24-48 hours at 37°C. Extracts were diluted in DMSO from 100 mg/mL stock to lower concentrations (T½, T¼, T⅛, etc.) for antibacterial testing.

Statistical Analysis

All the experiments were performed three times, and the data were presented as mean ± SD. Statistical significance of differences was calculated by one-way ANOVA and Tukey's test with significance set at $p < 0.05$.

Results

The extraction

Bunium incrassatum has been studied for its phytochemical composition and antioxidant properties. Research has shown that this plant contains significant amounts of polyphenols and flavonoids, which contribute to its antioxidant activities: total phenolics, flavonoids (Bousetla et al., 2015) using HPLC-DAD analysis; the presence of sterols, triterpenes, saponins, tannins, alkaloids and aglycone flavones using methanol and aqueous extract. The extraction yielded two types of extracts: ethanol extract (Et-OH) and methanol extract (Met-OH). Expressed as the percentage extract mass to the mass of the fresh plant, the highest yield was observed in the ethanol extract with a value of 12.266% compared to the methanolic extract. Results are represented in (Table 1).

Results of the Quantitative Study

Total phenolic content

The results obtained (Table 1) revealed that the ethanolic extracts contain a higher level of total polyphenols, with 19.25 mg Eq GA/g of extract, compared to the methanolic extracts, which registered 17.26 mg Eq GA/g. This suggests that ethanol is a more efficient solvent for extracting polyphenols from *B. incrassatum* tubers.

Total flavonoid content

The quantification of flavonoids was established through a calibration curve using quercetin as standard. Results are expressed in milligrams equivalent of quercetin per gram of extract (mg Eq Qu/g). Like the polyphenol content, flavonoid quantitative analysis revealed that the ethanolic tuber extracts of *B. incrassatum* showed higher flavonoid concentration compared to methanolic extracts, as shown in Table 1.

Biological Activity

Antioxidant Activity

The assessment of antioxidant activity using the DPPH assay revealed a progressive increase in free radical inhibition with increasing concentrations for both methanolic and ethanolic extracts of *B. incrassatum*.

The percentage of free radicals scavenged by ascorbic acid at different concentrations is shown in (Table 2). The methanolic extract achieved an inhibition rate of 20.76% at 100 µg/mL, while the ethanolic extract showed a slightly higher inhibition rate of 22.52% at the same concentration. These results indicate that both extracts exhibit moderate antioxidant activity, with similar performance at higher concentrations. However, ascorbic acid, used as a reference standard, demonstrated a clearly superior antioxidant activity, with an inhibition rate close to 89% at concentrations of 25 µg/mL and 50 µg/mL.

Our results also revealed that at low concentrations (1.5 µg/mL), ascorbic acid showed an inhibition of 24.39%, which is significantly higher than the methanolic and ethanolic extracts, which exhibited very low inhibition at this concentration.

Table 1. Results for the phytochemical screening of *B. incrassatum* extracted in different solvents.

Tabela 1. Rezultati fitokemičnega pregleda *B. incrassatum*, ekstrahiranega v različnih topilih.

Different samples	Yield %	TPC a mg Eq Qu/gE	TFCb Eq Qu/gE
Methanol (mg GAE/g)	9.29%	17.26 ±0.60	1.07 ±0.06
Ethanol (mg GAE/g)	12.26%	19.25 ±0.92	3.82 ±0.03

Note: Data were expressed as mean value ± standard deviation (n = 3). a: Total polyphenol content, b: Total flavonoid content.

These findings emphasize the potency of ascorbic acid as an antioxidant compared to *B. incrassatum* extracts, although the latter showed increasing activity with increasing concentrations, indicating a dose-dependent response.

Moreover, The IC50 values obtained from the DPPH analysis indicate a promising antioxidant activity for *B. incrassatum* extracts, with an IC50 of 261.75 µg/mL for the ethanolic extract and 380.53 µg/mL for the methanolic extract. Although these values are lower than those for ascorbic acid (IC50 of 3.41 µg/mL), they suggest that the plant extracts contain bioactive compounds capable of inhibiting free radicals.

Antibacterial Activity

The antimicrobial activity of the methanolic and ethanolic extracts was evaluated using the disc diffusion method, a qualitative technique based on measuring the diameter of the inhibition zones around the discs loaded with active substances. The table below (Table 3) presents the diameters of the inhibition zones obtained from extracts of *B. incrassatum* tubers.

The methanolic extract of *B. incrassatum* showed moderate antibacterial activity against *Staphylococcus aureus*, with an inhibition zone of 7.98 mm for the stock solution (100 mg/mL). Unexpectedly, a dilution to 50 mg/mL produced a larger zone of 10.74 mm, suggesting better diffusion of the bioactive compounds at a lower concentration. Although this inhibition is lower than that of the antibacterial standard (36.35 mm), it remains indicative of the presence of bioactive compounds capable of interacting with bacterial membranes. Further, the methanolic extract also moderately acted against the bacteria *Escherichia coli*, which was reflected as a 9.45 mm inhibition zone for a stock solution of 100 mg/mL. This activity is relatively constant, even at lower concentrations, but remains well below that of the antibacterial standard (30.8 mm). The extract appears to reach the highest level at a concentration of 12.5 mg/mL with an inhibition zone of 9.19 ± 0.25 mm. Such consistent activity, given the overall lower inhibition compared to the standard, emphasizes the potency of the extract for the target E. coli-a gram-negative bacterium known for its inherent resistance to various antibiotics.

The methanolic extract also exhibited moderate antibacterial activity against *Bacillus cereus*, with an inhibition zone of 10.30 mm for a stock solution of 100 mg/mL. In comparison, the antibacterial standard produced a much higher zone of inhibition, 33.58 mm. The observed activity with the extract could be considered a positive result. Knowing that *B. cereus* is known for its resilience and potential to cause foodborne illnesses, so even moderate inhibition from a natural extract like *B. incrassatum* highlights its potential as a valuable source of bioactive compounds.

Likewise, results also showed limited antibacterial activity against *Pseudomonas aeruginosa*, with an inhibition zone measuring 7.78 mm from a stock solution of 100 mg/mL of the extract. On the other hand, the antibacterial standard showed a rather larger inhibition zone measured at 37.77 mm. These findings signify that even though the crude extract had some activity against *P. aeruginosa*, its efficacy is much lower compared to the standard and needs further research to optimize its antibacterial potential. Finally, the methanolic extract presented very low activity against *Klebsiella pneumoniae*, with an inhibitory zone

Table 2. Percentage Inhibition of DPPH Free Radicals and IC50 Values by Methanolic and Ethanolic Extracts of *Bunium incrassatum*.

Tabela 2. Odstotna inhibicija prostih radikalov DPPH in vrednosti IC50 z metanolnimi in etanolnimi izvlečki *Bunium incrassatum*.

Concentration (µg/ml)	% Inhibition Methanolic Extract	% Inhibition Ethanolic Extract	% Inhibition ascorbic acid
0.3 µg/ml	4.81 ±0.08	0.12	4.16
1.5 µg/ml	6.14 ± 0.82	0.30 ±0.43	24.39
25 µg/ml	10.03 ± 0.41	6.24 ±0.17	89.09
50 µg/ml	13.28 ± 0.57	11.50 ±0.51	89.31
100 µg/ml	20.76	22.52 ±0.34	-
IC 50	380,53	261,75	3,41

Note: Values (mean of three replicates ± SD) represented in the same column by different letters are significantly different at $p<0.05$; IC50: concentration of the sample allowing 50% of the DPPH radical to be inhibited.

Table 3. The antibacterial activity of *B. incrassatum* tubers extracts was obtained by using different extraction solvents and four different concentrations.
Tabela 3. Antibakterijska aktivnost izvlečkov gomoljev *B. incrassatum*, pridobljenih z uporabo različnih ekstrakcijskih topil in štirih različnih koncentracij.

Bacterial strains	Concentration mg/mL					Standard
	Stock solution	1/2	1/4	1/16	1/18	
			Methanolic Extract			
S. aureus	7.98±0.027	10.74±0.28	-	-	-	36.35
P. aeruginasa	7.78±0.3	-	-	-	-	37.77
E. coli	9.45 ±0.05	8.59± 0.11	8.86±0.39	8.88±0.43	9.19±0.25	30.8
B. cereus	10.30±0.19	-	-		-	33.58
K. pneumonie	6.23±0.23	10.14±0.32	-	-	-	26.6
			Ethanolic Extract			
			N.S			

Note: Values are mean of inhibition in mm (mean ± SD) ; (-): No activity; NS: Not significative ; a : Gentamicin.

of 6.23 mm at stock solution concentration (100 mg/mL) improved to 10.14 mm at 50 mg/mL concentration. The antibacterial standard generated a larger diameter of inhibition of 26.6 mm. For the ethanolic extract's antibacterial activity, our results revealed that the zones of inhibition observed showed no statistically significant difference compared to the control, suggesting limited or insufficient antibacterial activity at these concentrations.

Discussion

As noted in previous literature, ethanol is an excellent solvent for plant maceration; it has been highly useful in many areas of plant research. Extensive studies were conducted on ethanol as an extraction solvent of bioactive compounds in various medicinal plants such as *Azadirachta indica* (Subapriya et al., 2005); *Moringa oleifera* (Sreelatha et al., 2011), and *Aloe vera* (Hamman, 2008) which was in agreement with our results which revealed that the extraction yielded higher ethanolic extracts from *B. incrassatum* tubers. Indeed, most research has proven that ethanol concentration is an important factor to consider during an extraction process to influence the yield of polyphenols, flavonoids, triterpenoids, and Vitamin C present in plants like *Clinacanthus nutans* (Qun et al., 2016).

Previous research on the same plant showed that the methanolic fraction contains the highest amount of total polyphenols extracted from the different parts: tubers of *Bunium mauritanicum*, seeds of *Bunium incrassatum*, and umbellules of *Bunium incrassatum* by Karouche et al. (2020), Toul et al. (2022), and Toul et al. (2023), respectively. These findings were not in agreement with our results, which showed oppositely higher total polyphenol concentrations from ethanolic maceration.

According to Dai et al. (2010), there is no universal extraction procedure suitable for the extraction of all plant phenolics. It is generally known that the solubility of polyphenols depends on the chemical nature of the plant sample, as well as on the polarity of used solvents (Toul and Djendar, 2023) and mostly on the extraction protocol conditions (extraction time, temperature, and pH) (Roselló-Soto et al., 2019).

Several studies reported the presence of flavonoids in extracts from different parts of *Bunium* species: flavonoids such as kaempferol and naringenin were identified from *Bunium incrassatum* Seeds (Toul et al., 2022); catechin, hesperetin, luteolin, and quercetin from *Bunium incrassatum* umbellules chloroform extract (Toul et al., 2023). Our results are in agreement with the findings of Karouche et al. (2020), who reported very low flavonoid contents in the tubers of *B. mauritanicum*. The results clearly show that the tubers of *Bunium* species are poor in flavonoids, which can be explained by the extraction method, concentration of solvent, environmental conditions, climate, collection period, genetic factor, and experiment conditions.

The quantitative study (extraction yield, polyphenol and flavonoids content) from *Bunium incrassatum* tubers

revealed that ethanolic maceration proved to be the most efficient extraction method, which is in accordance with the finding of (Sasidharan,2011), who reported that ethanol, a moderately polar solvent, is known to extract a wide range of compounds, including polyphenols, flavonoids, alkaloids, and saponins. Most plant phytochemical analyses include ethanol due to its ability to solubilize polar as well as weakly nonpolar compounds, thus offering more complete insight into the metabolic profile of plants.

In the current study, the methanolic extract of *B. incrassatum* exhibited moderate antioxidant; these results partially agree with El Kolli et al. (2017), who reported methanol extracts from *B. incrassatum* and *B. alpinum* to have the same activity, which is an indication that methanol is a suitable extracting solvent for these antioxidant compounds. However, our results disagree with Karouche et al. (2020), who obtained that water extracts of *B. mauritanicum* exhibited much stronger antioxidant activity (IC50 0.14 mg/mL), which supports that water can extract stronger antioxidants. Similarly, Dehimi et al. (2021) found that hexane and acetone fractions exhibited higher activities compared to their respective methanol fraction, further indicating that the possibility of bioactive compounds may be extracted with non-polar solvent extraction. Such findings suggest antioxidant extraction from *B. incrassatum* can be equally enhanced with other solvents like water or acetone. Further studies need to be undertaken on these solvents to optimize the plant's antioxidant potential.

In general, plant extracts tend to exhibit better inhibitory activity against Gram-positive bacteria compared to Gram-negative bacteria due to lipopolysaccharide composition in the multilayered cell wall of gram-negative strains (Chhetry et al.,2022; Essawi, 2000; Lin et al., 1999). The antibacterial quantitative study showed a positive response for both G-positive and G- G-negative bacterial strains used, which was in agreement with the findings of Karouche et al. (2020), who reported that tubers extract of *Bunium* reacted positively on the two reference strains: Gram-positive *Staphylococcus aureus* and Gram-negative *Pseudomonas aeruginosa*. Moreover, essential oils extracted from Algerian *B. incrassatum* and *Bunium alpinum* by El Koli et al. (2017) exhibited moderate antibacterial action against Gram-positive and Gram-negative bacteria, compared to gentamicin standard, which is in agreement with our results. Also, essential oils from *Bunium incrassatum* show promising antimicrobial activity, particularly against fungal strains, enhancing its value in natural medicine (Bousetla et al., 2014), which confirms the antibacterial properties of Talghouda tubers.

Since the present study underlines the benefits of *Bunium incrassatum*, further studies are necessary to explore its therapeutic application fully. Considering the antibacterial activity of *Bunium incrassatum* is promising, it remains relatively modest compared to conventional antibiotics. This points out the importance of continued research to enhance its efficacy and explore synergistic effects with other antimicrobial agents.

Conclusions

The present study revealed that the phytochemical composition of *Bunium incrassatum* tuber extracts indicated the presence of some bioactive compounds, such as polyphenols and flavonoids, which are likely responsible for their antibacterial activity. Also, the antioxidant activity of *Bunium incrassatum* extracts was evaluated through a DPPH assay, and it showed moderate efficacy. The tuber extract demonstrated significant activity against a wide range of pathogenic bacterial strains, suggesting its potential use in the treatment of microbial infections. These findings emphasise *B. Incrassatum* as a promising candidate in the development of plant-based antimicrobial agents, particularly in the context of Algerian medicinal plants. However, further in-depth investigations are necessary to identify the exact mechanisms of action involved and explore the full therapeutic potential of this plant, especially in its application in clinical practice.

Author Contributions

Conceptualization, S.B; H.B ; methodology, S.B; software, H.B.; N.B; Z.D; validation, H.B and S.B.; formal analysis, S.B investigation, N.B; Z.D; resources, H.B ; N.B and Z.D ; data curation, B.S.; writing—original draft preparation, S.B and H.B ; writing—review, editing, and paper communication, S.B; visualization, S.B; supervision, S.B and H.B ; All authors have read and agreed to the final version of the manuscript

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Conflicts of Interest

The authors declare no conflict of interest.

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Original Research

Antioxidant activity and metal content: a study on *Dictamnus albus*

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Abstract

Dictamnus albus is a perennial plant of warm, dry, and sunny habitats that have been traditionally used as a medicinal plant since ancient times. Our study focused on examining the phenolic compound content, antioxidant capacity, and concentrations of Fe, Cu, Zn, Mn, and Ni in the leaves of *D. albus*. The concentration of phenolic compounds in the leaves of *D. albus* was 5.40±0.09 mg GAE/g_{DW}. The leaf ethanol extract of *D. albus* exhibited significant antioxidant activity, as demonstrated by its ability to scavenge DPPH radicals, with an IC_{50} value of 38.20±0.46 µg/mL. The ethanol extract showed a slightly lower capacity to scavenge H_2O_2, with an IC_{50} value of 912±40 µg/mL. For the first time, we demonstrated that *D. albus* extract has a significant capacity to scavenge hydroxyl radicals (·OH). After adding *D. albus* extract, the residual ·OH radicals percentage was 39%, compared to 13% when using a 2 mM Trolox standard. In addition, the ethanol extract of *D. albus* showed the ability to reduce Fe^{3+} and Cu^{2+}, indicating the extract's ability to inhibit oxidative processes. Furthermore, *D. albus* extract can chelate Fe and thus prevent the Fenton reaction. The metal content in the leaves of *D. albus* was as follows: Fe 44.16±0.685 mg/kg, Cu 6.06±0.253 mg/kg, Zn 21.64±0.571 mg/kg, Mn 22.01±0.413 mg/kg, and Ni 1.21±0.112 mg/kg. Our results showed that the ethanol extract of *D. albus* has significant antioxidant capacity, that the concentrations of Fe, Cu, Zn, Mn, and Ni were below permissible doses for medicinal plants, and that the extract can contribute to the daily intake of these essential elements. These findings suggest that *D. albus* extract could be used in the therapy of diseases associated with oxidative stress.

Keywords

phenolic compounds; micronutrients; hydroxyl radical; oxidative stress

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Antioksidativna aktivnost in vsebnost kovin: študija na *Dictamnus albus*

Izvleček

Dictamnus albus je trajnica toplih, suhih in sončnih rastišč, ki se že od nekdaj tradicionalno uporablja kot zdravilna rastlina. V naši študiji smo se osredotočili na preučevanje vsebnosti fenolnih spojin, antioksidativne zmogljivosti in koncentracij Fe, Cu, Zn, Mn in Ni v listih *D. albus*. Koncentracija fenolnih spojin v listih *D. albus* je bila 5,40±0,09 mg GAE/gDW. Etanolni izvleček listov *D. albus* je pokazal pomembno antioksidativno aktivnost, demonstrirano z zmožnostjo odstranjevanja radikalov DPPH z vrednostjo IC_{50} 38,20±0,46 µg/mL. Etanolni izvleček je imel nekoliko manjšo sposobnost za odstranjevanje H_2O_2 z vrednostjo IC_{50} 912±40 µg/mL. Prvič smo dokazali, da ima izvleček *D. albus* pomembno sposobnost odstranjevanja hidroksilnih radikalov (·OH). Po dodatku izvlečka *D. albus* je bil odstotek preostalih ·OH radikalov 39 % v primerjavi s 13 % pri uporabi 2 mM standarda Trolox. Poleg tega je etanolni izvleček *D. albus* pokazal sposobnost redukcije Fe^{3+} in Cu^{2+}, kar kaže na sposobnost izvlečka, da zavira oksidativne procese. Izvleček *D. albus* kelatira tudi Fe in tako prepreči Fentonovo reakcijo. Vsebnost kovin v listih *D. albus* je bila naslednja: Fe 44,16±0,685 mg/kg, Cu 6,06±0,253 mg/kg, Zn 21,64±0,571 mg/kg, Mn 22,01±0,413 mg/kg in Ni 1,21±0,112 mg/kg. Naši rezultati so pokazali, da ima etanolni izvleček *D. albus* pomembno antioksidativno sposobnost, da so koncentracije Fe, Cu, Zn, Mn in Ni pod dovoljenimi odmerki za zdravilne rastline in da lahko izvleček prispeva k dnevnemu vnosu teh bistvenih elementov. Te ugotovitve kažejo, da bi se lahko izvleček *D. albus* uporabljal pri zdravljenju bolezni, povezanih z oksidativnim stresom.

Ključne besede

fenolne spojine; mikrohranila; hidroksilni radikali; oksidativni stres

Introduction

Medicinal plants have been used worldwide from ancient times to the present day for the treatment of various diseases, including cardiovascular disorders, inflammatory conditions, and cancer risk reduction. In addition to their role in traditional medicine, these plants are widely utilized in the pharmaceutical, cosmetic, and food industries (Škrovánková et al., 2012; Astutik et al., 2019; Salmerón-Manzano et al., 2020). One of the plants traditionally used for the treatment of various diseases is *Dictamnus albus* L. (Rutaceae). This is a bushy perennial plant species with a natural range that extends from Europe to southwestern Siberia and western Himalaya (Townsend, 1968; Diklić, 1973; Euro+Med, 2006-onwards; POWO, 2024). It grows in dry and sunny habitats, including rocky slopes, dry meadows, light thickets, openings in deciduous forests, and along roads from lowlands to lower mountainous areas (Diklić, 1973). The plant is characterized by a strong scent reminiscent of lemon. Since ancient times, plants referred to as "*Dictamnus*," which include several species from the Rutaceae and Lamiaceae families, have been used for medicinal purposes. However, since the 8th century, the most used species among these plants has been *Dictamnus albus*. Traditionally, all parts of the plant have been employed in medicinal treatments, with the root and root bark being the most commonly utilized components (Martínez-Francés et al., 2015). *D. albus* exhibits a wide range of biological activities, acting as an antipyretic, antiseptic, antispasmodic, cardiosedative, contraceptive, diuretic, emmenagogue, mutagenic, nervine, phototoxic, propeptic, sedative, stimulant, tonic, uterotonic, and vermifuge (Duke et al., 2002). For example, the leaves are commonly used in the form of tea to treat digestive issues and expel worms, while a tincture made from the leaves is applied externally to treat rheumatism (Gelenčir, 1989; Sarić, 1989; Popović et al., 2014). *D. albus* is also recognized for its use in veterinary care; shepherds, for example, have used fresh leaves to induce heat in goats (González et al., 2020). The bioactive compounds detected in *D. albus* have been classified into several groups, including quinoline alkaloids, limonoids, sesquiterpenes and their glycosides, flavones, and coumarins. Among these, quinoline alkaloids and limonoids are considered the most characteristic compounds (Lv et al., 2015).

Activated forms of oxygen, commonly known as reactive oxygen species (ROS), include free radicals such as hydroxyl ($^{\bullet}OH$), superoxide anion ($O_2^{\bullet-}$), alkoxyl ($RO^{\bullet}$), and peroxyl ($RO_2^{\bullet}$) radicals. These free radicals are highly unstable and reactive due to their unpaired electron in the outer orbit. While hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) are not free radicals, they can trigger free radical reactions (Jomova et al., 2023). Under normal physiological conditions, ROS are synthesized and play vital roles in energy production, detoxification, redox regulation of protein phosphorylation, ion channel activity, transcription factor regulation, and immune responses (Brieger et al., 2012; Škrovánková et al., 2012).

ROS-related diseases can result from either a deficiency (e.g., chronic granulomatous disease, certain autoimmune disorders) or an excess of ROS (Brieger et al., 2012). Overproduction of ROS accelerates oxidative processes, leading to cellular damage and, eventually, cell death. ROS have been linked to the development of numerous chronic and degenerative diseases, such as cancer, cardiovascular, respiratory, neurodegenerative, and digestive disorders (Brieger et al., 2012; Liu et al., 2018).

The human body has endogenous antioxidants that combat ROS, such as enzymatic antioxidants (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and glutathione-S-transferase) and non-enzymatic antioxidants (glutathione, urea, vitamin E, and melatonin). Exogenous antioxidants, derived from the secondary metabolites of medicinal plants, also play a crucial role in defending the body against oxidative stress (Brieger et al., 2012; Akanni et al., 2014; Liu et al., 2018).

Antioxidants from medicinal plants belong to different chemical classes, among which phenolic compounds are one of the most important. Phenolic compounds exert their antioxidant capacity through various mechanisms: they act as reducing agents, hydrogen-donating agents, singlet oxygen quenchers, and metal chelators (Vuolo et al., 2019; Tatipamula and Kukavica, 2021). Under certain conditions (high pH, high transition metal ions concentrations, and presence of O_2), phenolic compounds can exhibit pro-oxidative properties, which should be considered when using them for therapeutic purposes or treatment. Low molecular weight phenolic compounds (gallic acid) exhibit pro-oxidative properties more easily than phenolic compounds with higher molecular weight (condensed tannins), which have almost no pro-oxidative properties (Škrovánková et al., 2012). Antioxidant components may act independently or in plant extracts where it is possible to manifest synergism.

Micronutrients like iron (Fe), copper (Cu), zinc (Zn), manganese (Mn), and nickel (Ni) are essential for plant metabolism in optimal concentrations that differ depending on the plant species. Both lower concentrations (deficit) and increased concentrations (toxicity) negatively affect the plant's growth and development. Essential micronutrients for the human body are Fe, Mn, Cu, Zn, and selenium (Se). Iron is a key component of haemoglobin and an important cofactor for several human enzymes, such as catalase. It is found in high concentrations in erythrocytes and muscle cells. However, excessive iron intake can lead to hepatotoxicity and negatively affect the cardiovascular system, while iron deficiency results in anaemia. Copper is another essential element in the human body, playing a crucial role in transcription factors and enzymes. It regulates oxidation-reduction reactions, energy production, neurotransmitter synthesis, and iron metabolism. Excessive copper levels can lead to symptoms such as vomiting, dermatitis, abdominal pain, and diarrhoea. Chronic copper overload may result in Wilson's disease, characterized by the accumulation of free copper in the liver, brain, and kidneys. On the other hand, copper deficiency can lead to aneurysms, vascular damage, and impaired nutrient transport. Manganese is a component of metalloenzymes responsible for oxidizing cholesterol and fatty acids. A manganese deficiency can cause bleeding disorders, while excessive levels may lead to speech disorders, leg cramps, and encephalitis. Zinc is an essential element in human physiology, involved in transcription factors and enzymes like superoxide dismutase and carboxypeptidase. However, excessive zinc intake can result in toxic effects on blood lipoprotein levels and the immune system, induce copper deficiency, and lead to cell apoptosis. Zinc deficiency is also associated with suppressed immune function. Both zinc and copper play critical roles in glucose metabolism and cholesterol regulation as enzyme cofactors. Nickel is essential for iron metabolism but becomes toxic at elevated concentrations. Excessive exposure to nickel has been linked to an increased risk of hypertension, neurological deficits, cardiovascular disease, developmental delays in childhood, and lung cancer (Brima, 2018; Sulaiman et al., 2024).

The sources of metals available to plants can come from various origins, including natural sources (such as rocks, aerosols, and dust particles), domestic sources (biofuel combustion, electrical waste, and both natural and

chemical waste), agricultural waste (such as pesticides and chemical fertilizers), industrial sources (mine products, chemical waste, and thermal power waste), as well as other sources (fossil fuel combustion, open dump yards, and pharmaceutical waste) (Vinogradova et al., 2023).

The availability of these metals to plants and their accumulation in plant tissues can significantly impact the quality of medicinal plants, particularly by altering the concentration of active substances, such as phenolic compounds. Although the concentration of biologically active compounds in plants is species-specific, it is also influenced by environmental factors (Vinogradova et al., 2023). The uptake of metals from the soil is significantly influenced by soil properties such as water content, pH, organic matter content, and soil structure (Morgan and Connolly, 2013). The qualitative and quantitative composition of these compounds and micronutrients depends on the plant species' metabolic characteristics, habitat, and exposure to various abiotic, biotic, and anthropogenic factors.

Our work focuses on examining the antioxidant capacity of the ethanolic extract of *Dictamnus albus* leaves, with the aim of exploring its potential use in the prevention of diseases associated with oxidative stress. To the best of our knowledge, there are no previous studies reporting the concentrations of metals such as Fe, Cu, Zn, Mn, and Ni in *D. albus* leaves. Therefore, the present study aims to determine these metal concentrations, given their potential health benefits at optimal levels and the risk of toxicity at elevated concentrations.

Materials and Methods

Chemicals

DEPMPO (5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide, Focus Biomolecules, USA); H_2O_2 (Lach-ner, Czechia); $FeSO_4x7H_2O$ (Merck, Germany); dH_2O (Lonza, Belgium); $HClO_4$ (Lach-ner, Czechia); HNO_3 (Lach-ner, Czechia); Ethanol (MB IMPEKS d.o.o.); DPPH (2,2-diphenyl-a-picryl-hydrazyl,); TPTZ (2,4,6-tripyridyl-s-triazine, Sigma-Aldrich, Germany); Na_2CO_3 (Lach-ner, Czechia); Folin Ciocalteu reagens (Sigma-Aldrich, Germany); Na_2HPO_4 (Semikem, B&H); NaH_2PO_4 (Lach-ner, Czechia); Ferrozine (Sigma-Aldrich, Germany); $CuCl_2$ (ZORKA Pharma Šabac, Serbia); Neocuproine (Sigma-Aldrich, Germany); CH_3COOH (Lach-ner, Czechia); CH_3COONa (ZORKA Pharma Šabac, Serbia), Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Sigma-Aldrich, Germany); Gallic acid (Sigma-Aldrich, Germany).

Plant material

Leaves of the *Dictamnus albus* were collected during the flowering phase in May 2020 in the eastern Herzegovina region (Mosko, Bileća, Republic of Srpska, Bosnia and Herzegovina). The plants were growing in a sunny and dry site, on the edge of a rocky meadow, far from larger settlements and traffic roads. One fully developed middle stem leaf was collected from each of a total of 15 individuals. The specimens were identified using dichotomous keys (Townsend, 1968; Diklić, 1973; Nikolić, 2020). The nomenclature is in accordance with Euro+Med Plant Base (2006-onwards). A voucher specimen is stored in the herbarium collection of the Institute for Nature Conservation of Vojvodina province in Novi Sad (Herbarium Code: PZZP) (Thiers, 2024).

Preparation of plant extracts

The leaves were dried at room temperature in the shade in an airy place. For all analyses, we used dry plant material. After drying, the plant material was ground to powder with an electric mill and used for extraction. 80% ethanol was used as a solvent, and the extraction ratio was 10 g of plant tissue and 200 mL of 80% ethanol. The homogenate was then sonicated for 5 minutes and mixed for 30 minutes on a magnetic stirrer (750 rpm, VELP SCIENTIFICA). Then, the homogenate was filtered (filter paper, ALBET, 73 g/m^2, pore size 30-40 microns), and the filtrate was labelled as extract 1 (E1). The remaining residue was re-extracted with 100 mL of 80% ethanol, and the sonication and mixing were repeated. After filtering, extract 2 (E2) was separated. Extracts E1 and E2 were combined and evaporated (IKA Rotary Evaporator RV 8 V–C) at 45 °C to a total volume of 30 mL. The extracts were stored until analysis at -18 °C.

Determination of the content of total phenolic compounds

The content of total phenolic compounds (TPC) was determined according to Singleton and Rossi (1965) by measuring the absorbance at 724 nm (Shimadzu UV-1800). The reaction mixture contained 100 µL of plant extract and 450 µL of Folin-Ciocâlteu reagent (diluted 1:3 with distilled

water). After incubation for 5 minutes at room temperature (23°C), 450 µL of 1M Na2CO3 was added. The mixture was well vortexed (Velp Scientific Zx3) and then incubated for 60 minutes in the dark at room temperature. The quantification of TPC was calculated based on the calibration curve for gallic acid and expressed as gallic acid equivalent (GAE) per g_{DW} (dry weight).

Determination of the ability to remove DPPH radicals

The ability to remove DPPH (2,2-diphenyl-a-picrylhydrazyl) radicals was determined by the method given by Liyana-Pathiranana and Shahidi (2005). The sample contained 0.5 mL of 0.136 mM ethanol solution of DPPH radical and 0.5 mL plant extracts of different concentrations (2.5, 10, 25, 50, 75 µg/mL, in relation to the concentration of phenolic compounds). Different concentrations of plant extract were prepared in 80% ethanol. The sample was mixed and incubated for one hour in the dark at room temperature (23°C). The control contained 0.5 mL of 0.136 mM DPPH solution and 0.5 mL of 80% ethanol. After incubation, the absorbance was measured at 517 nm (Shimadzu UV-1800). The percentage of inhibition was calculated according to the equation:

$$\% \text{ inhibition} = \frac{A_C - A_S}{A_C} \times 100$$

Where AC is the absorbance value of the control and As is the absorbance value of the sample.

The IC_{50} was determined from a standard curve constructed as a dependence of the percentage of inhibition on the concentration of phenolic compounds in the extract and expressed in units of µg/mL.

Ability to scavenge H_2O_2

The ability of the extract to scavenge hydrogen peroxide was determined according to Ebrahimzadeh et al. (2010). The sample contained 920 µL of Na-phosphate buffer 0.067 M, 30 µL of 0.5 M H_2O_2 (prepared in 0.067 M Na-phosphate buffer pH 6.4), and 50 µL of plant extract of different concentrations (25, 500, 1000, 1250, 1500 µg/mL, in relation to the concentration of phenolic compounds). Different concentrations of plant extract were prepared in 80% ethanol. The samples were mixed and incubated at room temperature (23°C) for 30 minutes in the dark. After incubation, the absorbance was measured at 230 nm (Shimadzu UV-1800). The control contained 970 µL of Na-phosphate buffer 0.067 M pH 6.4 and 30 µL of 0.5 M H_2O_2. The sample blank (A_{Sb}) contained 50 µL of extract of different concentrations and 950 µL of Na-phosphate buffer.

The percentage of inhibition was calculated according to the equation:

$$\% \text{ inhibition} = \frac{A_C - (A_S - A_{Sb})}{A_C} \times 100$$

Where Ac is the absorbance value of control, As is the absorbance value of the sample, and A_{Sb} is the absorbance value of the sample blank.

The IC_{50} was determined from a standard curve constructed as a dependence of the percentage of inhibition on the concentration of phenolic compounds in the extract and expressed in units of µg/mL.

Ability to scavenge ·OH radicals

The scavenging ability of the extracts against hydroxyl radicals was assessed using a solution containing the extract, combined with the Fenton reaction and the spin trap 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide (DEPMPO) (Milutinović et al., 2023).

The reaction mixture (total volume 29 µL) consisted of the extract (1 µL, previously 1000 times diluted in water), deionized water (25 µL), H_2O_2 (2 µL, final concentration 0.35 mM), and DEPMPO (1 µL, final concentration 3.5 mM). This mixture was placed into a gas-permeable Teflon tube, followed by the immediate addition of $FeSO_4$ (1 µL, final concentration 0.15 mM). EPR spectra were recorded after 2 minutes at room temperature using a Bruker Biospin Elexsys II 540 spectrometer. The experimental setup employed the following operating parameters: centre field 3500 G, microwave power 10 mW, microwave frequency 9.85 GHz, modulation frequency 100 kHz, modulation amplitude 1 G. Control experiments were conducted by replacing the extract with an equivalent amount of solvent. The ·OH radical scavenging activity (AA) of the extracts was calculated using the following equation:

$$AA = \frac{(I_C - I_S)}{I_C} \times 100$$

Where Ic and Is are the double integral values for the control and sample, respectively, derived from the EPR spectra (using Xepr software).

Determination of Fe chelating ability

Determination of Fe chelating ability was performed using the method Carter (1971) gave, with certain modifications. The samples contained 937.5 µL of 80% ethanol, 12.5 µL of 1 mM $FeSO_4$, and 50 µL of plant extracts (concentrations of TPC 300 µg/mL). The samples were mixed and incubated for 10 minutes at room temperature (23°C), after which 50 µL of 2 mM ferrozine was added. The samples were then mixed and incubated for 10 minutes in the dark. The absorbance was measured at 562 nm (Shimadzu UV-1800). The control, instead of the plant extract, contained 80% ethanol. The percentage of Fe chelation was calculated according to the equation:

$$\% \text{ chelating} = \frac{A_C - A_S}{A_C} \times 100$$

Where Ac is the absorbance value of the control and As is the absorbance value of the sample.

Determination of Fe^{3+} reduction ability

FRAP (ferric reducing antioxidant power) was performed according to the method developed by Benzie and Strain (1996). The sample (concentration 500 µg/mL) with a volume of 0.2 mL was mixed with 1.8 mL of FRAP working reagent, incubated for 10 minutes at 37 °C, and the absorbance was measured at 593 nm (Shimadzu UV-1800). As a sample blank, 1.8 mL of FRAP working solution and 0.2 mL of distilled water were used. A calibration curve was constructed for $FeSO_4x7H_2O$ in a concentration range of 0.1-2 mM. The capacity of the samples to reduce Fe^{3+} results was presented as mmol Fe^{2+}/L of the sample.

Determination of Cu^{2+} reduction ability

The ability of ethanol plant extracts to reduce Cu^{2+} was determined using a method proposed by Apak et al. (2004). Samples were prepared by mixing 0.3 mL of 0.01 M $CuCl_2$, 0.3 mL of 10 mM acetate buffer (pH 7), 0.3 mL of 7.5 mM ethanolic neocuproine solution, and 0.05 mL of the ethanolic plant extract. The mixtures were vortexed and incubated at room temperature (23°C) for 30 minutes. After incubation, the absorbance was measured at 450 nm (Shimadzu UV-1800). A blank sample was prepared similarly, with 0.05 mL of ethanol replacing the plant extract. Trolox was used as a standard at concentrations ranging from 20 to 300 µg/mL, and the results are expressed as µg of Trolox equivalents per mL of extract.

Determination of metal contents in plant samples

Quantification of elements (Fe, Cu, Zn, Mn, and Ni) contents in the examined plant samples was done in the extracts obtained after the destruction of the plant tissues with concentrated nitric acid, supplemented with 30% H_2O_2 and 70% $HClO_4$ (Perquel et al., 1993) by atomic absorption spectrophotometry (AAnalyst 400, Perkin Elmer, USA). The selected wavelengths of elements to be determined were as follows: Fe: 248.33, Cu: 324.75, Zn: 213.70, Mn: 279.48, and Ni: 232.00. Standard solutions were prepared using a single element stock solution (1000 mg/L, Perkin Elmer, USA) and appropriate diluent extractant. All analytical procedures were done in three biological replicates and made with glassware prewashed with 10% HNO_3. Quality control of acid digestion and element quantification was performed by analyses of certified referent material (ERM®-CD218, ryegrass) and recovery values in the acceptable range (85-103%) for each element. Results are expressed as mg of metal content per kg of dry weight (mg/kg DW)

Data analyses

All data were analyzed using GraphPad Prism 8, employing One-way ANOVA and paired t-tests for statistical comparisons. Correlations between phenolic compound content, antioxidant activity (measured by various methods), and metal content were determined using Pearson's correlation coefficient (r), calculated in Microsoft Excel. The significance levels were defined as follows: *p<0.05, ***p<0.001 and ****p<0.0001.

Results and Discussion

Phenolic content and antioxidant properties of the plant extract

One of the major health problems in developing countries is malnutrition (deficiencies, excesses, or imbalances in an intake of energy and/or nutrients), which is the aetiology of various diseases (Liu et al., 2018; Liu et al., 2021). In their review paper, Liu et al. (2018) emphasized the connection

between nutrition and the occurrence of oxidative stress associated with cardiovascular diseases, neurodegenerative disorders, cancer, digestive diseases, and ageing. Antioxidants can mitigate or prevent oxidative processes through various mechanisms. They are defined as compounds that neutralize free radicals and other reactive molecules, halt chain reactions, and repair oxidative damage (Apak et al., 2007). Phenolic compounds, multifunctional antioxidants, as part of the diet or as supplements can be crucial for neutralizing ROS, reducing oxidative stress, and preventing the development of various diseases (Rahman et al., 2021). Numerous beneficial effects for human health of *Dictamnus albus* and its related species *D. dasycarpus* Turcz. (with a native range from southeastern Siberia to China and Korea) extracts are known such as neuroprotective, anti-allergy activity, protection of cardiovascular activity, improvement of gastrointestinal activity, and anti-inflammatory activity (Qin et al., 2021; İlgün and Karatoprak, 2022). The positive effects of the extract are based on the action of plant secondary metabolites, among which phenolic compounds play an important role. A total phenolic content (TPC) was measured in the ethanol extract of *D. albus*, and the results are shown in Table 1. The concentration of phenolic compounds in leaves of *D. albus* was 5.4±0.09 mgGAE/gDW. İlgün and Karatoprak (2022) measured TPC in the methanolic extracts of *D. albus* herb and root. Their results indicated a higher phenolic content in the herbal extract (77.13±5.73 mg GAE/g extract) compared to the root extract (43.81±9.49 mg GAE/g extract). The differences in phenolic compound concentrations may be due to variations in extraction methods, solvents used, and the units of measurement (e.g., fresh or dry plant mass, extract mass). Additionally, these results can be influenced by the geographical origin of the plants as well as the biotic and abiotic stresses to which the plants were exposed in their natural habitats (Shi et al., 2022; Bilgin and Şahin, 2023; El Kamari et al., 2024).

The two main mechanisms by which antioxidants inactivate ROS are via electron transfer (ET) and hydrogen atom transfer (HAT), although in some cases, the two mechanisms cannot be clearly distinguished. Through which mechanism the antioxidant will act depends on its structure, solubility, distribution coefficient, and the type of solvent (Çelik et al., 2010). Using different methods to evaluate antioxidant capacity gives us a clearer insight into the possibilities of plant extracts for the removal of ROS. Therefore, we evaluated the antioxidant capacity of *D. albus* using several different methods.

The electron donation ability of plant metabolites (e.g. phenolic compounds) can be measured by DPPH radical scavenging abilities (Akanni et al., 2014). The ethanolic extract of Dictamnus albus with an IC_{50} value of 38.20±0.46 µg/mL showed a great ability to inhibit DPPH radicals (Table 1). *D. albus* extracts from Serbia showed a lower ability to remove DPPH radicals compared to our sample with IC_{50} values of 59.80±1.53 µg/mL for the methanol extract and IC_{50} values of 76.48±2.30 µg/mL for the ethanol extract (Pavlović et al., 2018). The *D. albus* herbal extract from Turkey at a concentration of 4 mg/mL showed the ability to remove DPPH radical with a value of 56.86±2.4%, higher than root *D. albus* extract (34.56±2.77%). Another study showed a significantly higher antioxidant activity of *D. albus* leaf extract than the root. Cao et al. (2022) showed an IC_{50} for a leaf extract of 0.13±0.23 µg/mL, while for root extract, the measured IC_{50} value was 3.68±0.56 µg/mL.

Significant DPPH radical scavenging activity was demonstrated for the methanol extract of *Dictamnus dasycarpus* root bark from China for different years (Liu et al., 2021). The authors showed that DPPH radical scavenging activity was concentration-dependent from 1.6 to 14.4 µg/mL with IC_{50} values in the range of 1.287-9.758 µg/mL, whereby the scavenging activity was gradually enhanced with the prolonging of growth years. Differences in the anti-

Table 1. The phenolic content (TPC) and antioxidant activity of the ethanol extract of *Dictamnus albus* leaves were measured using different methods. Results are expressed as mean value ± SD (n = 3).

Tabela 1. Vsebnost fenolov (TPC) in antioksidativna aktivnost etanolnega izvlečka listov *Dictamnus albus*, izmerjena z različnimi metodami. Rezultati so izraženi kot povprečna vrednost ± SD (n = 3)

TPC	DPPH scavenging ability	H_2O_2 scavenging ability	Ability to reduce Fe^{3+}	Ability to reduce Cu^{2+}	Ability to chelate Fe
(mg GEA/g_{DW})	IC_{50} (µg/mL)	IC_{50} (µg/mL)	mmol/L Fe^{2+}	µg Trolox/mL	300 µg/mL (%)
5.40±0.09	38.20±0.46	912±40	975±52	164±8	53.62±2.84

oxidant abilities of the extract may arise from differences in the qualitative and quantitative composition of the extract (Ren et al., 2020).

Hydrogen peroxide in a Fenton-type reaction with Fe produces a hydroxyl radical. The superoxide anion radical can also react with Fe^{3+}, whereby Fe^{2+} is regenerated, which can participate in the Fenton reaction. Hydroxyl radical is one of the most powerful ROS in biological systems. It reacts with cell membranes' polyunsaturated fatty acids, causing damage and "leakage" of membranes. The hydroxyl radical is capable of oxidative damage to every molecule of the biological system and contributes to carcinogenesis, mutagenesis, and cytotoxicity (Akanni et al., 2014). The mutagenic capacity of ·OH is a consequence of their interactions with DNA and, therefore, plays an important role in the development of cancer (Akanni et al., 2014). It is known that the reducing properties of an extract could serve as a measure of its antioxidant action by donating hydrogen atoms to break the free radical chain. In the ethanol extract of *Dictamnus albus*, the ability to remove H_2O_2 was with IC_{50} values of 912±40 µg/mL (Table 1).

In addition, the ethanol extract of *Dictamnus albus* showed the ability to remove ·OH radicals (measured as % residual hydroxyl radical after the addition of the extract) (Figure 1). After the addition of *D. albus* extract, the percentage of residual ·OH was 39%, indicating a lower ability to remove ·OH radicals compared to the 2 mM Trolox standard (13%). However, this removal capacity was significantly higher compared to the control (dH_2O), which represents 100% residual ·OH and is not shown in the figure. Our results indicate that *D. albus* extract has a significant ability to remove OH radicals, which may indicate its potential role in preventing oxidative DNA damage and lipid peroxidation (Laughton et al., 1989; Khennouf et al., 2010; Sevgi et al., 2015).

The determination of the antioxidant potential of the plant extract as the ability to reduce Fe^{3+} and Cu^{2+} is based on the theoretical assumption that any good reductant is a good antioxidant. Fe^{3+} reduction is used as an indicator of plant extract for its electron-donating capacity (Petkov et al., 2022). At a TPC concentration of 500 µg/mL, *Dictamnus albus* shows the ability to reduce Fe^{3+} with a value of 975±52 mmol/L Fe^{2+} and reduce Cu^{2+} with a value of 164±8 µgTrolox/mL at a TPC concentration of 300 µg/mL (Table 1). Literature data point out that the ability of plants to reduce Fe^{3+} ions varies greatly from species to species (Nwozo et al., 2023). The authors showed that the range of values of the FRAP method for different medicinal plants is very

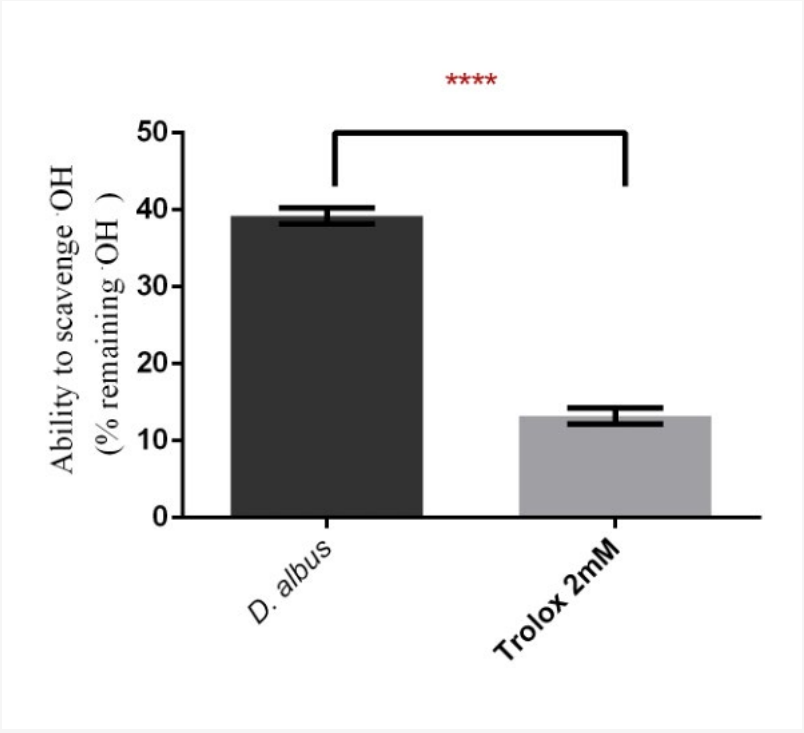

Figure 1. The ability of *Dictamnus albus* leaf extract to scavenge ·OH radicals. The ability was measured as a percentage of the residual ·OH radical after the addition of the extract and compared to the Trolox standard (2 mM). Results are expressed as mean value ± SD (n=3). Asterisks indicate statistical significance (p<0.0001).

Slika 1. Sposobnost izvlečka listov *Dictamnus albus*, da odstranjuje radikale ·OH. Sposobnost je bila izmerjena kot odstotek preostalega ·OH radikala po dodatku ekstrakta in primerjana s standardom Trolox (2 mM). Rezultati so izraženi kot povprečna vrednost ± SD (n=3). Zvezdice označujejo statistično pomembnost (p<0,0001).

wide (3.25-1225 µg/mL). Similarly, for 27 medicinal plants from the area of North Macedonia, the ability to reduce Cu^{2+} values was in the range of 53-1068 µmolTrolox/g_{DW} (Tusevski et al., 2014).

The extract of *Dictamnus albus* at a concentration of 300 µg/mL showed a good ability to chelate Fe with a value of 54±3% (Table 1). Gonçalves et al. (2014) showed that the ability to chelate Fe for different plants is very wide-ranging and that it depends on the plant species and the solvent used. Namely, for Mediterranean medicinal plants, it was shown that the ability to chelate Fe is in the range of 38-94% for a cold infusion and in the range of 40-67% for a hot infusion for a concentration of phenolic compound of 400 µg/mL. The antioxidant capacity of the extract is influenced by the plant's genetic potential, environmental factors in its natural habitat, the extraction process, and the choice of solvent used. Lipid peroxidation can be triggered by redox-active metals, particularly Fe. Gonçalves et al. (2014) demonstrated that the Fe^{2+}-chelating capacity of an extract plays a varying role in preventing lipid peroxidation, depending on the plant species. Consequently, the ability to chelate Fe emerges as a crucial property of the extract in combating lipid peroxidation.

This antioxidant property of medicinal plants evaluated by different methods usually correlates very well with the TPC, indicating that phenolic compounds contribute the most to the antioxidant potential of the extract (Petkov et al., 2022).

In the extract of *Dictamnus albus* leaves, we did not detect correlations between the concentration of phenolic compounds and the different antioxidant methods used in the research (Table 3). In contrast to our results, other authors have shown the existence of a correlation between the content of phenolic compounds and the antioxidant activity of plant extracts (Piluzza and Bullitta, 2011; Muflihah et al., 2021). Our results indicate that part of the antioxidative activity can be attributed to metabolites (terpenoids, ascorbate, glutathione). Among the antioxidant assays, a strong negative correlation (-0.998) with statistical significance ($p<0.036$) was observed between the Fe-reducing ability and H_2O_2 scavenging capacity, suggesting a shared neutralization mechanism in the *D. albus* extract.

Metal content

The importance of determining the metal content in medicinal plants is well documented in the literature (Kulhari et al., 2013; Vinogradova et al., 2023). The human body relies on various essential minerals, such as Fe, Cu, Zn, and Mn, for metabolic processes. These minerals are predominantly supplied by plants, which absorb them from the soil and transfer them into the food chain (Srivastava et al., 2017). However, plants are highly sensitive to environmental conditions and tend to accumulate metals, with the intensity of this uptake process directly affecting their overall elemental composition (Kulhari et al., 2013). This accumulation raises significant environmental concerns due to the potential adverse effects of metals on both animal and human health.

Metals, Fe, Cu, Mn, and Zn, are toxic only in higher concentrations (Nissar et al., 2020), but they can also cause serious consequences. For instance, Fe is one of the primary toxic substances contributing to deaths in children under six years old (Spanierman, 2011). Gastrointestinal toxicity can occur after ingestion of more than 20 mg/kg of Fe, while moderate intoxication is observed with elemental Fe ingestion exceeding 40 mg/kg. Serious toxicity, potentially fatal, arises at doses greater than 60 mg/kg (Spanierman, 2011). Nickel was identified as an allergen by the Contact Dermatitis Society of America in 2008, with its minimum risk level for inhalation set at 0.2 µg/m^3 for exposure durations of 15–364 days. However, no dietary limit for nickel has been established (Bhat et al., 2010; Kulhari et al., 2013).

The content of beneficial trace metals in medicinal plants generally decreases in the following order: Fe>Mn>Cu>Zn>Ni (Chizzola, 2012). Our results show that the content of micronutrients in *Dictamnus albus* leaves decreases in the order of Fe>Mn>Zn>Cu>Ni. Compared to the literature data, the Cu content was lower than Zn, while the Mn content was higher than the Zn content (Table 2). The Fe content in the leaves of *D. albus* was 44.16±0.685 mg/kg, Cu 6.06±0.253 mg/kg, Zn 21.64±0.571 mg/kg, Mn 22.01±0.413 mg/kg, and Ni 1.21±0.112 mg/kg (Table 2). The permissible limit for essential metals in medicinal plants is 50 mg/kg for Zn, 20 to 150 mg/kg for Cu, 200 mg/kg for Mn and Fe, and 5 mg/kg for Ni (WHO, 2007; Baba and Mohammed, 2021). The values of all measured metals in the leaves of *D. albus* were below the permissible doses for medicinal plants.

In the *Dictamnus albus* from the Kashmir Himalaya, the content of microelements in the leaves was as follows: Fe 1.130 mg/kg, Cu 0.023 mg/kg, Zn 0.055 mg/kg, and Ni 0.105 mg/kg (Nissar et al., 2020), which are significantly lower values for all metals compared to our results. The reason may be the specific conditions related to altitude

Table 2. Content of iron (Fe), copper (Cu), manganese (Mn), zinc (Zn), and nickel (Ni) in *Dictamnus albus* leaves. Results are expressed as mean value ± SD (n=3).

Tabela 2. Vsebnost železa (Fe), bakra (Cu), mangana (Mn), cinka (Zn) in niklja (Ni) v listih *Dictamnus albus*. Rezultati so izraženi kot povprečna vrednost ± SD (n=3).

	(mg/kg_{DW})				
	Fe	**Cu**	**Zn**	**Mn**	**Ni**
D. albus leaves	44.16±0.680	6.06±0.253	21.64±0.572	22.01±0.413	1.21±0.112

and temperatures in the Kashmir Himalaya. Differences also may stem from soil composition and various abiotic and anthropogenic influences.

According to Kulhari et al. (2013), there are varying concentrations of Mn, Fe, and Zn in leaf samples of medicinal plants from various sites in northwestern India, which were influenced by environmental pollution and soil composition. The study reported the highest Fe content in *Withania somnifera* (L.) Dunal leaf samples (17.44±0.0202 mg/kg) and the lowest in *Salvadora oleoides* Decne. Leaves (11.03±0.0497 mg/kg), with all Fe concentrations falling within permissible limits. The highest Zn content was found in *Bacopa monnieri* (L.) Wettst. (4.80±0.0907 mg/kg), while the lowest was in *Commiphora wightii* (Arn.) Bhandari (2.10±0.0173 mg/kg). Mn content ranged from 2.39±0.0218 mg/kg in *Withania somnifera* to 5.22±0.1040 mg/kg in *Acacia nilotica* (L.) Willd. ex Delile and Ni content ranged from 0.19±0.0371 mg/kg in *Withania somnifera* to 0.59±0.0202 mg/kg in *Acacia nilotica* (Kulhari et al., 2013). In our *Dictamnus albus* samples, higher concentrations of Fe, Mn, Zn, and Ni (Table 2) were measured compared to the levels reported in the plant samples from northwestern India (Kulhari et al., 2013). On the other hand, the concentrations of the metals (mg/kg) in the leaves of medicinal plants from the local markets in Nigeria were in the range 5.08-23.67 for Zn, 1.28-13.45 for Cu, 0.96-1.95 for Ni, and 20.58-108.50 for Fe (Sulaiman et al., 2024). Metal concentrations in plants from Nigeria were slightly higher than in *D. albus* (Table 2). These differences may be attributed to species-specific characteristics, substrate composition, and ecological conditions in the collected plants' habitats. The concentrations range for essential metals in plants *Annona squamosa* L., *Psidium guajava* L., *Anacardium occidentale* L., and *Ficus sycomorus* L. from Nigeria were: Zn (0.109-0.658 mg/kg), Cu (0.026-0.079 mg/kg), Mn (0.039-1.269 mg/kg), Fe (0.019-0.107 mg/kg), and Ni (0.054-0.144 mg/kg) (Baba and Mohammed, 2021), which are significantly lower compared to the values measured in *D. albus* (Table 2).

The ability of plants to absorb and accumulate microelements is influenced by various factors, such as soil type, climate, industrial pollution, and agricultural practices. Although trace elements are generally insoluble, plants have developed strategies to enhance the uptake of essential metal ions. One such mechanism involves the release of organic molecules and metabolites, including phenolic acids and flavonoids, which form water-soluble chelates with transition metal ions (Petkov et al., 2022). These metal-phenolic chelates enhance solubility, facilitating their transport into plant cells and movement across cellular compartments. Consequently, plants act as vital intermediaries, transferring trace elements from the soil to animals and humans (Petkov et al., 2022).

No correlations were detected between the metal content (Fe, Cu, Mn, Zn, and Ni) and the concentration of phenolic compounds in the *Dictamnus albus* extract (Table 3). Theuma and Attard (2020) reported a positive correlation between phenolic compound content and the concentration of Cu and Mn, suggesting that increased levels of phenolic compounds are associated with higher concentrations of Cu and Mn. This relationship can be explained by the ability of phenolic compounds to chelate Cu, while Mn complexes with condensed polyphenols (Theuma and Attard, 2020). Our results revealed a strong positive correlation (0.999) with statistical significance ($p<0.02$) between Fe concentration and the DPPH radical scavenging activity of the *D. albus* extract. Additionally, high correlation values were observed for Cu and Mn concentrations with DPPH radical scavenging activity (0.996 and 0.993, respectively), nearing statistical significance ($p=0.058$ for Cu and $p=0.077$ for Mn). The correlations between TPC, DPPH capacity, and Fe and Cu content were previously reported by Dobrinas et al. (2021), who suggested that TPC may serve as a reliable indicator of DPPH capacity and the influence of antioxidant compounds on mineral bioavailability.

Table 6. Percentage mortality rates (MR), mortality time (MT), susceptibility status (S), knockdown rates (KR), and knockdown time (KT) of individual essential oil and their combinations against females *Anopheles* mosquito at 1 hour after exposure.

Tabela 6. Odstotki smrtnosti (MR), čas smrtnosti (MT), stanje občutljivosti (S), stopnja otrpnosti (KR) in čas otrpnosti (KT) posameznih eteričnih olj in njihovih kombinacij proti samicam komarja *Anopheles* 1 uro po izpostavljenosti.

		Phenolic compound	DPPH IC_{50}	H_2O_2 IC_{50}	Fe Chelation	Cu^{2+} Reduction	Fe^{3+} Reduction	Scavenge $^{\cdot}OH$	Fe (mg/kg_{DW})	Cu (mg/kg_{DW})	Mn (mg/kg_{DW})	Zn (mg/kg_{DW})	Ni (mg/kg_{DW})
Phenolic compound	Pearson's r	—											
	p-value	—											
DPPH IC_{50}	Pearson's r	0.527	—										
	p-value	0.646	—										
H_2O_2 IC_{50}	Pearson's r	-0.909	-0.833	—									
	p-value	0.273	0.373	—									
Fe Chelation	Pearson's r	-0.049	-0.875	0.460	—								
	p-value	0.969	0.322	0.695	—								
Cu^{2+} Reduction	Pearson's r	0.060	-0.817	0.361	0.994	—							
	p-value	0.962	0.392	0.765	0.069	—							
Fe^{3+} Reduction	Pearson's r	0.884	0.863	-0.998	-0.510	-0.413	—						
	p-value	0.309	0.337	0.036	0.659	0.729	—						
Scavenge $^{\cdot}OH$	Pearson's r	-0.959	-0.266	0.755	-0.235	-0.339	-0.717	—					
	p-value	0.182	0.829	0.455	0.849	0.780	0.491	—					
Fe (mg/kg_{DW})	Pearson's r	0.500	0.999	-0.815	-0.890	-0.835	0.846	-0.235	—				
	p-value	0.667	0.020	0.394	0.302	0.371	0.358	0.849	—				
Cu (mg/kg_{DW})	Pearson's r	0.448	0.996	-0.779	-0.915	-0.866	0.814	-0.177	0.998	—			
	p-value	0.704	0.058	0.431	0.264	0.334	0.395	0.886	0.037	—			
Mn (mg/kg_{DW})	Pearson's r	0.421	0.993	-0.760	-0.927	-0.880	0.796	-0.148	0.996	1.000	—		
	p-value	0.723	0.077	0.450	0.246	0.315	0.414	0.905	0.056	0.019	—		
Zn (mg/kg_{DW})	Pearson's r	0.247	0.954	-0.628	-0.980	-0.953	0.671	0.037	0.963	0.977	0.983	—	
	p-value	0.841	0.195	0.568	0.128	0.197	0.532	0.977	0.174	0.137	0.118	—	
Ni (mg/kg_{DW})	Pearson's r	0.924	0.811	-0.999	-0.426	-0.325	0.996	-0.779	0.792	0.755	0.735	0.598	—
	p-value	0.249	0.397	0.024	0.720	0.789	0.060	0.431	0.418	0.455	0.474	0.592	—

Conclusions

Our study demonstrated that the leaves of *Dictamnus albus* contain significant amounts of phenolic compounds and exhibit strong antioxidant activity, including the ability to scavenge DPPH radicals, H2O2, reduced Fe3+, Cu2+, and chelate Fe. For the first time, we have demonstrated that *D. albus* extract possesses significant hydroxyl radical scavenging capacity, emphasizing its potential role in mitigating oxidative stress. The concentrations of metals such as iron, copper, zinc, manganese, and nickel in the extract were comparable to those in other medicinal plants, affirming its safety as a source of essential elements for human health. The pronounced antioxidant activity of *D. albus* extract indicates its potential as an adjunct in therapies for oxidative stress-related diseases. Further studies on its bioactive properties could contribute to the development of innovative therapeutic agents.

Author Contributions

Conceptualization, S.Š. and B.K.; methodology, S.Š., M.M., and B.K.; formal analysis, T.T., D.M. and Đ.N.; investigation, S.Š., T.T., D.M., Đ.N., M.M. and B.K.; resources, M.M. and B.K.; data curation, D.M., Đ.N., M.M. and B.K.; writing—original draft preparation, S.Š., D.M., Đ.N., M.M. and B.K.; writing—review and editing, S.Š., D.M., Đ.N., M.M. and B.K.; visualization, B.K.; supervision, M.M. and B.K.; funding acquisition, M.M. and B.K. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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ABS

Original Research

Terminalia glaucescens ethanol stem extract induces vasorelaxation via nitric oxide/guanylyl cyclase/ cyclic guanosine monophosphate pathway in the aortic smooth muscle of male Wistar rats

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Abstract

Blood vessels contain vascular smooth muscle that responds to various agonists and endothelial cells essential for maintaining vascular homeostasis. Studies have explored the vasorelaxant effects of *Terminalia* species but not *Terminalia glaucescens*. This study investigates the endothelium-dependent activities of *Terminalia glaucescens* ethanol stem extract (ESET) on the aortic smooth muscle (ASM) of male Wistar rats. ASM from male Wistar rats weighing 150-200 g was removed and mounted in 50-mL jacketed tissue baths containing physiological salt solution connected to a force transducer to measure the isometric contraction. The vasorelaxant effect of ESET after precontraction of the ASM by phenylephrine (PHE) and potassium chloride (KCl) was investigated. ASM was preincubated with ESET, and contractile response to PHE, KCl, acetylcholine and sodium nitroprusside (SNP) was observed. Furthermore, the vasorelaxant activities of ESET after mechanical removal of the endothelium, pre-incubation with L-NG-Nitro arginine methyl ester (L-NAME), methylene blue (MB), and indomethacin were determined. ESET significantly reduces ($p<0.05$) ASM contraction response to PHE and relaxed ASM precontracted with PHE and KCl. ESET improved relaxation (%) response to acetylcholine and SNP. The endothelium removal significantly reduced the ASM relaxation response to SNP in the presence of ESET. The vasorelaxant effect caused by the extract was significantly ($p<0.05$) reduced with pre-incubation of the ASM with MB, enhanced by indomethacin and not affected by L-NAME. The action of *Terminalia glaucescens* ethanol stem extract is endothelium-dependent and involves the NO/guanylyl cyclase/cyclic guanosine monophosphate pathway.

Keywords

Terminalia glaucescens, vascular smooth muscle, contractile response, vasorelaxant, endothelium-dependent.

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Etanolni izvleček stebla *Terminalia glaucescens* povzroča vazorelaksacijo po poti dušikovega oksida/guanil ciklaze/cikličnega gvanozin monofosfata v gladkih mišicah aorte samcev podgan Wistar

Izvleček

Krvne žile vsebujejo gladko mišičevje, ki se odziva na različne agoniste, in endotelijske celice, ki so bistvene za vzdrževanje homeostaze žil. Študije so preučevale vazorelaksantne učinke vrst *Terminalia*, ne pa tudi vrste *Terminalia glaucescens*. Ta študija raziskuje od endotelija odvisno delovanje etanolnega izvlečka stebla *Terminalia glaucescens* (ESET) na gladke mišice aorte (ASM) samcev podgan Wistar. ASM samcev podgan Wistar, težkih od 150 do 200 g, je bila odstranjena in nameščena v 50-mililitrske tkivne kopeli s plaščem, ki so vsebovale fiziološko raztopino soli in so bile povezane s pretvornikom sile za merjenje izometričnega krčenja. Preučevali smo vazorelaksacijski učinek ESET-a po predkontrakciji ASM s fenilefrinom (PHE) in kalijevim kloridom (KCl). ASM je bila predhodno inkubirana s ESET-om, opazovali pa smo kontraktilni odziv na PHE, KCl, acetilholin in natrijev nitroprusid (SNP). Poleg tega so bile določene vazorelaksacijske aktivnosti ESET po mehanski odstranitvi endotelija, predhodni inkubaciji z L-NG-nitro arginin metil estrom (L-NAME), metilen modrim (MB) in indometacinom. ESET pomembno zmanjša ($p<0,05$) odziv krčenja ASM na PHE in sproščeno ASM, predhodno skrčeno s PHE in KCl. ESET izboljša odziv sprostitve (%) na acetilholin in SNP. Odstranitev endotela je v prisotnosti zdravila ESET pomembno zmanjšala sprostitveni odziv ASM na SNP. Vazorelaksacijski učinek, ki ga je povzročil izvleček, se je pomembno ($p<0,05$) zmanjšal pri predhodni inkubaciji ASM z MB, povečal z indometacinom, L-NAME pa nanj ni vplival. Delovanje etanolnega izvlečka stebla *Terminalia glaucescens* je odvisno od endotelija in vključuje pot NO/guanilil ciklaze/cikličnega gvanozin monofosfata.

Ključne besede

Terminalia glaucescens, gladka muskulatura, ontraktilni odziv, vazorelaksant, odvisen od endotelija

Introduction

The blood vessels comprise several parts, such as adventitial tissues, vascular smooth muscle cells, and endothelial cells. The endothelium is the thinnest portion of the arterial wall and is critical for maintaining blood flow to tissues and organs (Wei et al., 2018). Endothelial cells dynamically react to stimuli to modify smooth muscle contractility. Their main function is to regulate blood flow, which ensures oxygen and nutrients supply to tissues (Drożdż et al., 2023). According to Félétou et al. (2008), endothelial cells interact with vascular smooth muscle cells and release substances that relax and contract blood vessels. They produce prostaglandins, nitric oxide (NO), and other relaxing agents (Krüger-Genge et al., 2019). As a gatekeeper for organ/tissue homeostasis and blood pressure regulation, a healthy endothelium offers anti-thrombotic, anti-inflammatory, and antioxidant properties (Eelen et al., 2015). Sodium nitroprusside, acetylcholine, and phytochemical substances from plants can all have an impact on endothelial cell activity.

Endothelial dysfunction (ED) is a common vascular phenotype in hypertensive individuals that aggravates atherosclerosis and arteriosclerosis (Perticone et al., 2001; Konukoglu & Uzun, 2017). The aetiology of hypertension involves endothelins, cyclooxygenase-dependent vasoconstrictors, and endothelium-derived hyperpolarizing factors (Ambrosino et al., 2021). Reduced nitric oxide bioavailability is strongly linked with ED-associated hypertension. Moreover, oxidative stress and vascular inflammation via aberrant vasoconstriction and relaxation may alter vascular resistance and lead to ED-associated hypertension (Zhao et al., 2015). In essential organs such as the heart, brain, and kidney, the close association between ED and hypertension might serve as the main pathogenic mechanism of small vessel disease (Gallo et al., 2021).

Substances with vasodilatory potential have been researched for their clinical relevance (Bartáková and

Nováková, 2021). Polyphenols in plants enhance vascular endothelial cell control and cardiovascular system protection (Yamagata, 2019; Li et al., 2019). They induce endothelium-dependent vasorelaxation, suppress vasocontraction, and have antihypertensive effects (Matsumoto et al., 2014). Medicinal plants such as *Tridax procumbens* (Salahdeen et al., 2012; Salami et al., 2021), *Hibiscus sabdariffa* (Zheoat et al., 2019), *Allium sativum* (Afzaal et al., 2021), *Theobroma cacao* (Irondi et al., 2019) have been shown to have vasorelaxant effects. Plants such as *Moringa oleifera* (Aekthammarat et al., 2020), *Alchemilla vulgaris* (Takir et al., 2014), *Morus alba* (Panth et al., 2018), and *Chenopodium ambrosioides* (Assaidi et al., 2019) show endothelium-dependent vasorelaxant activities. Studies have explored the vasorelaxant and cardioprotective effects of *Terminalia* species. *Terminalia arjuna* was revealed to reverse impaired endothelium function (Bharani et al., 2004) and possessed hypotensive properties by enhancing prostaglandin E2 (Dwivedi, 2007). The vasorelaxant mechanisms of *Terminalia fagifolia* rest on the endothelium and operate through the nitric oxide/soluble guanylyl cyclase/cyclic guanosine monophosphate (NO/sGC/cGMP) pathway (de Carvalho et al., 2019). The specific mechanism of *T. glaucescens* activity on the endothelium is unknown. This study, therefore, investigates the endothelium-dependent activities of *Terminalia glaucescens* ethanol stem extract on the ASM of male Wistar rats.

Materials and Methods

Plant preparation and extraction

The stem of *Terminalia glaucescens* was collected where present in abundance in Moko town, Ogun State, Nigeria. The plant was verified at the Department of Botany, Lagos State University. A specimen of the plant with the number LSH 001232 was deposited at the herbarium. The ethanol extract of the air-dried stems was prepared using the Soxhlets apparatus as described by Fotsing et al. (2022). The solvent-eliminated extract yield was 26 % w/v

Tissue preparation

The study was done using adult male Wistar rats weighing between 150 g and 200 g. The animals were bred at the Animal House of the Lagos State University College of Medicine, Ikeja Lagos, Nigeria. The rats were sacrificed by cervical dislocation under sodium pentobarbital (30 mg/kg). The aorta was quickly removed and freed of connective tissues. The aorta was cut into 2-mm rings and mounted in a 50-ml jacketed tissue bath containing physiological salt solution (PSS). The PSS was continuously bubbled with 95 % O_2 and 5 % CO_2 gas mixture. A 37°C temperature was maintained in the bath. The aortic ring was suspended using a fine stainless steel rod coupled to a force transducer (model 7004; Ugo Basile, Varese, Italy) to capture isometric contractions. The tissue was stimulated three times with PHE (10^{-7} M) at 30-minute intervals after being allowed to stabilize in the physiological solution for ninety (90) minutes.

Concentration-response studies with PHE and KCl

After incubating the aortic rings with the vehicle (0.2 ml distilled water) for 15 min, the rings were subjected to a cumulative addition of PHE (10^{-9} to 10^{-5} mol/L), and the contractile responses were noted. The aortic rings were washed in PSS, and the tissues were incubated with 0.81 mg/ml of ESET for 15 minutes. A concentration-response study was conducted with KCl (10–50 mmol/L) with and without the ESET in a different set of stabilized aortic rings, using the same methodology as the concentration-response study with PHE.

Aortic smooth muscle was precontracted with either PHE (10^{-7} M) in a different set of new aortic rings. A cumulative concentration-response of the ESET (0.1-1.25 mg/ml) was added from the lowest dose after the contraction was given time to peak. This procedure was described by Salahdeen et al. (2012).

Concentration-response of ESET in endothelium intact and denuded aortic rings

Aortic smooth muscles were exposed to cumulative concentrations of *Terminalia glaucescens* (0.25 – 1.25 mg/ml) after precontraction with PHE (10^{-7} mol/L) in endothelium intact and denuded aorta. The endothelium was removed with gentle abrasion and a cell scraper. A lack of relaxation in the aortic ring after treating precontracted ring segments with acetylcholine (1 mM) was used to confirm the absence of endothelium. The vasorelaxant effect of ESET was determined on contractions of endothelium-denuded aortas induced by phenylephrine (Salahdeen et al., 2012).

Role of ESET in the relaxation response of aortic smooth muscle to acetylcholine (ACH) and sodium nitroprusside (SNP)

The ASM relaxation response to ACH (10^{-9} – 10^{-5} M) and SNP (10^{-9} – 10^{-5} M) was measured after the ASM was precontracted with phenylephrine (10^{-7} M). This was done with or without preincubating the aortic ring with ESET (0.81 mg/ml). The relaxation response to SNP was carried out in the presence and absence of the endothelium. ACH and SNP were used to determine the role of NO-dependent pathways on the mechanism of action of ESET (Salahdeen et al., 2015).

Role of ESET on the responses of the aortic smooth muscle after preincubation with different blockers

1. The ASM was incubated with methylene blue (10^{-4} M) for 15 minutes before adding cumulative doses of the ESET (0.25 – 1.25 mg/ml) to determine the activity of endothelial guanylyl cyclase.

2. The role of prostacyclin in the extract's mechanisms of action was determined using indomethacin. After 15 minutes of incubation of the ASM with indomethacin (10^{-4} M), the ASM response to cumulative doses of the ESET (10^{-9} - 10^{-5} M) was measured.

3. The role of endothelium-derived NO was studied by incubating the ASM with L-NG-Nitro arginine methyl ester (L-NAME) (10^{-4} M) for 15 minutes. Then, cumulative dosages of ESET (10^{-9} - 10^{-5} M) were administered, and the response was measured.

Each response was allowed to stabilize before adding more doses, and ASMs were washed three times before using a separate drug, as described by Salahdeen et al. (2015) and Salami et al. (2023).

Results

Concentration-response studies with PHE and KCl

The concentration response of the aortic rings to PHE and KCl with or without *Terminalia glaucescens* ethanol stem extract (ESET) is shown in Figure 1A and 1B, respectively. The contraction obtained in the presence of the extract was significantly ($p < 0.05$) attenuated compared to the control for the PHE. ESET reduced the maximum (%) contraction response to PHE from 76.50 ±0.73 to 48.08 ±0.20 (Figure 1A). Preincubation of the aortic rings with the ESET had no significant difference ($p > 0.05$) in KCl-induced contraction. At successive doses, the relaxation response was significantly increased by ESET after precontraction with KCl (60 mM) as compared to precontraction with PHE (10^{-7} M) (Figure 1C).

Effect of ESET on the relaxation response in endothelium intact and denuded aortic ring

As shown in figure 2. There was no significant difference in the relaxation response of the endothelium intact and denuded aortic ring ($p > 0.05$). The cumulative maximum (%) relaxation in endothelium intact was (58.07 ±3.48), and the endothelium-denuded aortic ring was (50.00 ±3.23).

Relaxation responses of aortic rings to cumulative doses of acetylcholine (10^{-9} M to 10^{-5} M) and sodium nitroprusside (10^{-9} M to 10^{-5} M)

As shown in Figures 3A and 3B, preincubation of aortic rings in ESET (0.81 mg/ml) significantly ($p < 0.05$) increased ACH and SNP-induced maximum relaxation. However, the SNP-induced relaxations were significantly ($p < 0.05$) reduced in the endothelium denuded aortic rings (Figure 3C).

Effect of ESET on the maximum relaxation of aortic rings preincubated with inhibitors of nitric oxide, prostacyclin and guanylyl cyclase.

It was observed that L-NAME, a nitric oxide inhibitor, was only able to significantly ($p < 0.05$) reduce the maximum relaxation effect of ESET at lower doses but had no significant effect ($p > 0.05$) on the maximum relaxation effect of ESET at the highest dose (Figure 4A). Indomethacin (a prostacyclin inhibitor) significantly increased ($p < 0.05$) the ESET-induced relaxation response in aortic rings, as shown in Figure 4B. Methylene blue (a guanylyl cyclase inhibitor) significantly reduces ($p < 0.05$) ESET-induced aortic ring relaxation (Figure 4C).

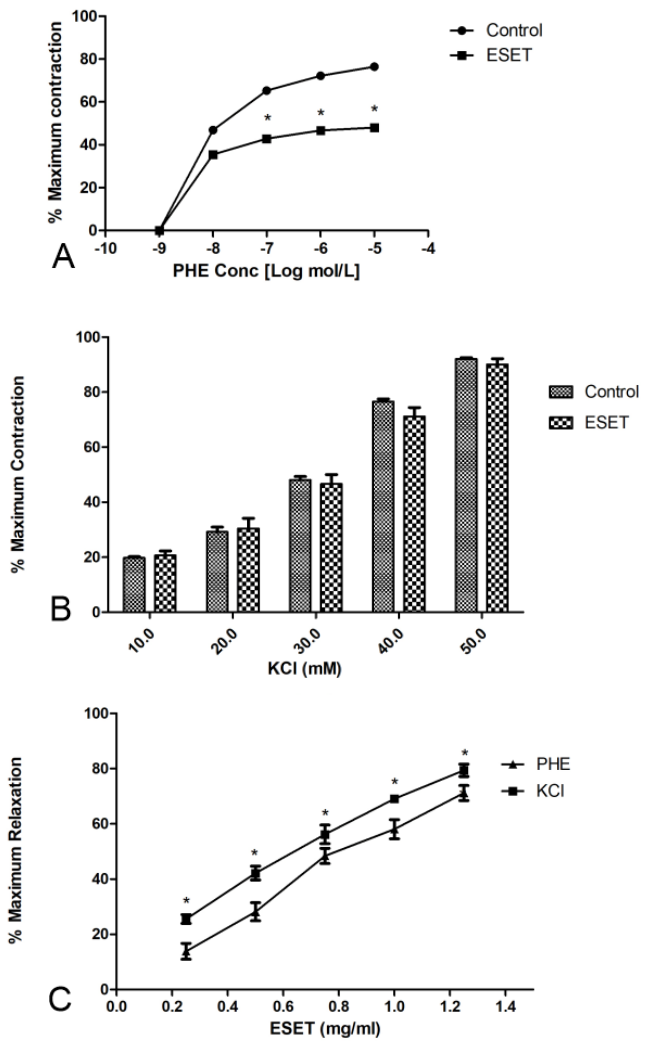

Figure 1. A). Contraction-response to PHE (10^{-9} – 10^{-5} M) in aortic rings with or without the preincubation with extract (0.81 mg/mL). B). Contraction-response of aortic rings to KCl (10 – 50 mM) with or without the preincubation with extract (0.81 mg/mL). C). Relaxation response to cumulative addition of ESET (0.25 – 1.25 mg/ml) after precontraction with PHE (10^{-7}) and KCl (60 mM). Each point represents the mean±SEM (n = 6), *: $p < 0.05$, PHE: Phenylephrine, KCl: Potassium chloride, ESET: *Terminalia glaucescens* ethanol stem extract.

Slika 1. A). Odziv kontrakcije na PHE (10^{-9} - 10^{-5} M) v aortnih obročkih z ali brez predhodne inkubacije z ekstraktom (0,81 mg/ml). B). Odziv aortnih obročkov na KCl (10 - 50 mM) z ali brez predhodne inkubacije z ekstraktom (0,81 mg/ml). C). Relaksacijski odziv na kumulativni dodatek ESET-a (0,25 - 1,25 mg/ml) po predhodni kontrakciji s PHE (10^{-7}) in KCl (60 mM). Vsaka točka predstavlja povprečje ± SEM (n = 6), *: $p < 0,05$, PHE: fenilefrin, KCl: Kalijev klorid, ESET: *Terminalia glaucescens*: etanolni izvleček stebla *Terminalia glaucescens*

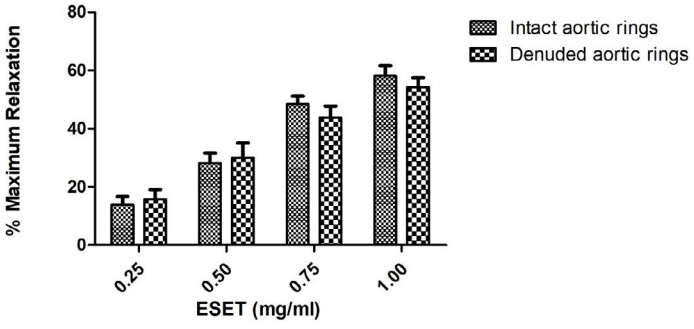

Figure 2. Maximum relaxation response of endothelium intact and denuded aortic ring to ESET after precontracted with PHE (10^{-7}). PHE: Phenylephrine, ESET: *Terminalia glaucescens* ethanol stem extract.

Slika 2. Največji sprostitveni odziv endotelija nepoškodovanega in denudiranega aortnega obroča na ESET po predhodni kontrakciji s PHE (10^{-7}). PHE: fenilefrin, ESET: *Terminalia glaucescens*: etanolni izvleček stebla.

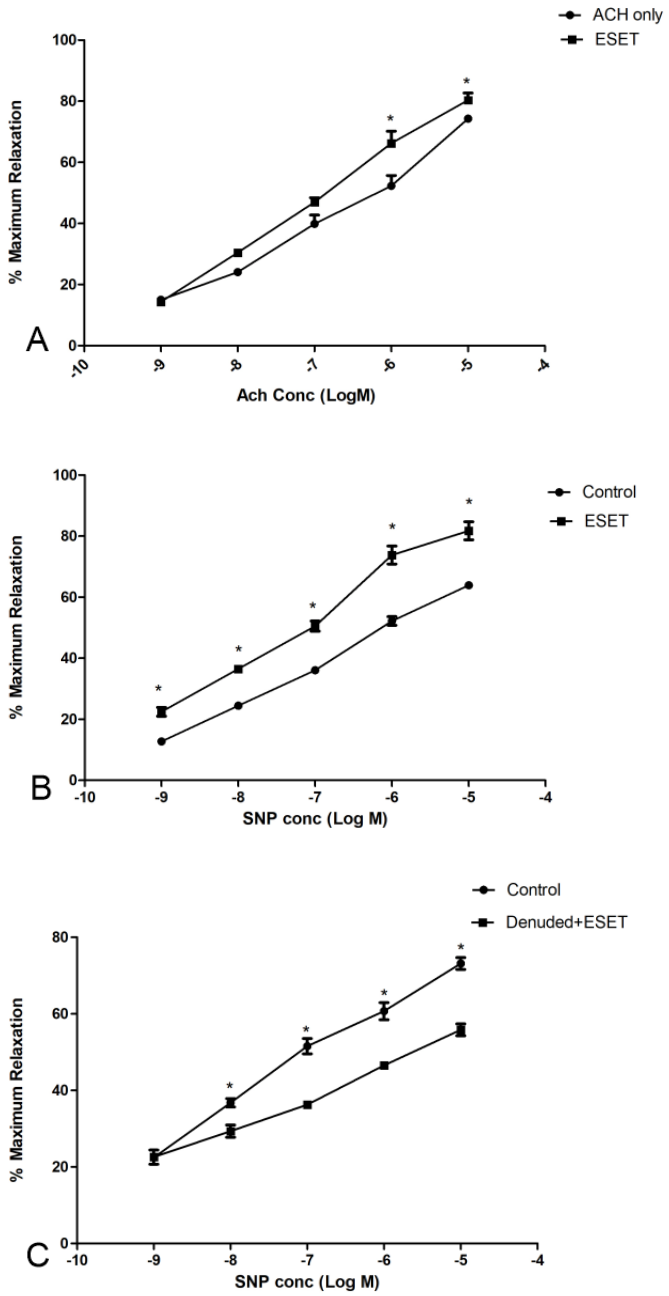

Figure 3. Maximum relaxation response (%) of aortic rings preincubated with ESET (0.81 mg/ml) to A) Acetylcholine (10^{-9} M to 10^{-5} M) and B) Sodium nitroprusside in intact endothelium (10^{-9} M to 10^{-5} M) C) Sodium nitroprusside with denuded endothelium. Each point represents the mean ±SEM (n = 6), *: Significant difference between each successive dose. ESET: *Terminalia glaucescens* ethanol stem extract, ACH: acetylcholine, SNP: sodium nitroprusside.

Slika 3. Največji sprostitveni odziv (%) aortnih obročkov, predhodno inkubiranih z ESET (0,81 mg/ml), na A) acetilholin (10^{-9} M do 10^{-5} M) in B) natrijev nitroprusid v intaktnem endoteliju (10^{-9} M do 10^{-5} M) C) natrijev nitroprusid z denudiranim endotelijem. Vsaka točka predstavlja povprečje ± SEM (n = 6), *: Pomembna razlika med vsakim zaporednim odmerkom. ESET: *Terminalia glaucescens*: etanolni izvleček stebla, ACH: acetilholin, SNP: natrijev nitroprusid.

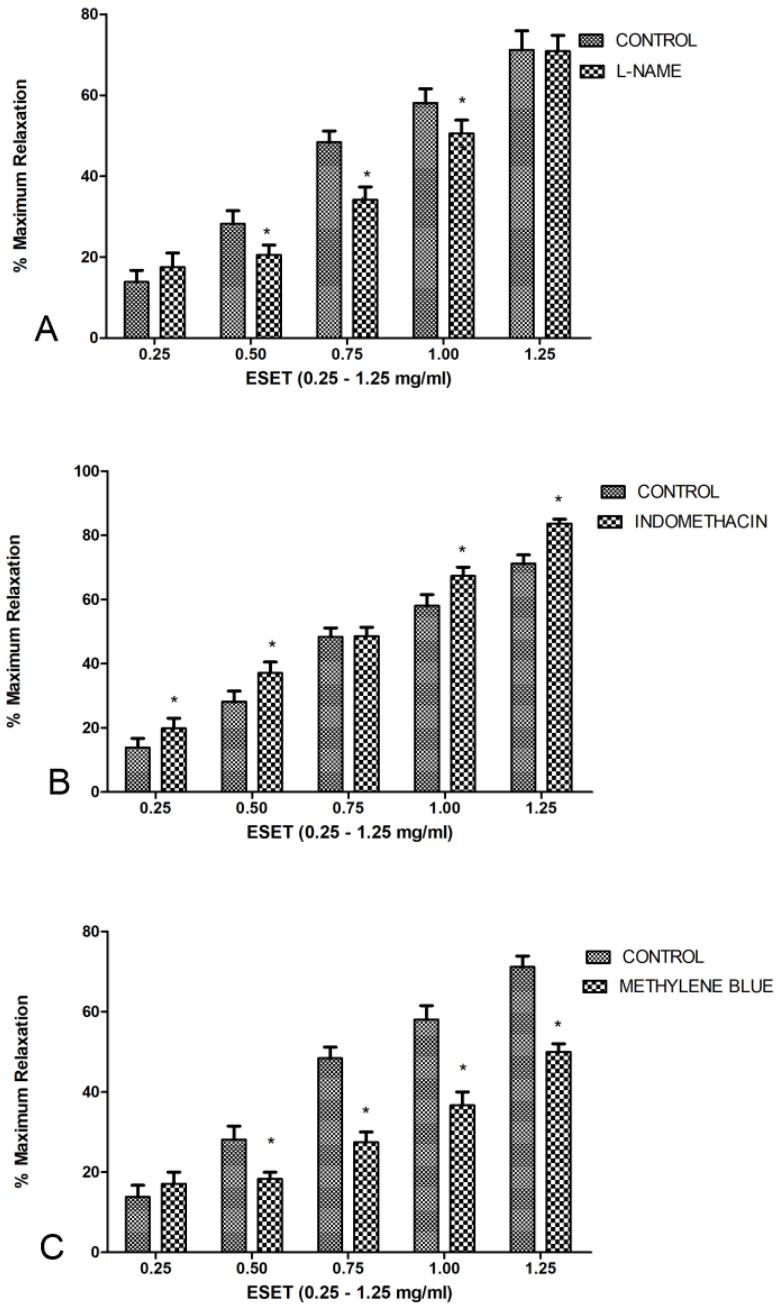

Figure 4. Maximum relaxation (%) response of aortic rings to ESET-induced (0.25 mg/ml – 1.25 mg/ml). A) After preincubation with L-NAME (10^{-4} M). B) After preincubation with indomethacin (10^{-4} M). C) After preincubation with methylene blue (10^{-4} M). *: Significant difference between each successive dose. ESET: *Terminalia glaucescens* ethanol stem extract, L-NAME: L-N^G-Nitro arginine methyl ester.

Slika 4. Odziv aortnih obročkov na največjo relaksacijo (%) ob ESET-u (0,25 mg/ml - 1,25 mg/ml). A) Po predhodni inkubaciji z L-NAME (10^{-4} M). B) Po predhodni inkubaciji z indometacinom (10^{-4} M). C) Po predhodni inkubaciji z metilen modrim (10-4 M). *: Pomembna razlika med vsakim zaporednim odmerkom. ESET: *Terminalia glaucescens*: etanolni izvleček stebla *Terminalia glaucescens*, L-NAME: L-N^G-Nitro arginin metil ester.

Discussion

The mechanism of smooth-muscle contraction through PHE is a result of direct stimulation of α-adrenergic receptors of the G protein, which, through phospholipase C, increases the levels of inositol 1,4,5-triphosphate (IP_3) and causes the release of intracellular Ca^{2+}, that leads to muscular contraction (Marconi et al., 2021 Richards et al., 2023). In the current study, ESET significantly reduces phenylephrine-induced ASM contraction (Figure 1A). This can be done by inhibiting any of the pathways involved in the PHE-induced contraction. Though the preincubation of the ASM with ESET did not significantly inhibit its vascular response to KCl (Figure 1B), it was, however, discovered that the relaxation response of ASM precontracted with KCl to ESET was more significant ($p<0.05$) compared to that of PHE (Figure 1C). KCl-induced contraction depolarizes the ASM, opening voltage-gated calcium channels (Kirschstein et al., 2009). It can be suggested that ESET relax the contraction of ASM stimulated by receptor-mediated (PHE) contraction and K+-induced depolarization.

The endothelium regulates vascular tone by producing endothelium-derived hyperpolarizing factor (EDHF) in addition to nitric oxide (NO) and prostacyclin (Félétou & Vanhoutte, 2006; de la Bastida-Casero et al., 2024). There was a reduction in relaxation (%) endothelium-denuded aortic rings (50.00±3.23). The reduction was not significantly different to endothelium-intact aortic rings (58.07±3.48) Figure 2. This suggests that aside from the endothelium-dependent mechanism through the NO/cGMP pathway, the extract might be using other mechanisms that are independent of intact endothelium. It was observed that preincubation of ASM with *Terminalia glaucescens* ethanol stem extract potentiated the acetylcholine-induced relaxation (77.26±4.46) compared to the acetylcholine-only (66.22±6.89) (Figure 3A). The stem bark and roots of *Terminalia glaucescens* have been reported to contain terpenoids and flavonoids (Adeeyo et al., 2018; Khan et al., 2019), both of which have been reported to increase the endothelial release of nitric oxide (NO) (Alves-Silva et al., 2016; Wani, 2017). The observed significant maximum relaxation (%) due to the synergistic effect of the ESET in SNP-induced aortic ring relaxation in endothelium-intact compared to denuded (Figure 3B) aortic rings in the current study might suggest the extract's ability to induce the release of NO oxide, which is abolished by the removal of the endothelium (Figure 3C), thereby reducing this effect.

ESET-induced maximum relaxation (%) response in the aortic rings shows variable response at the highest dose in the presence of L-NAME (70.88±3.88), indomethacin (83.69±1.35) and methylene blue (50.00±2.00) compared to the control (71.19±4.71) as shown in figure 4A-4C. The no significant effect of L-NAME on the maximum effect of ESET-induced aortic rings relaxation suggested that ESET relaxation properties might not be directly affected by the inhibition of NO synthase enzyme as this does not significantly abolish the vasorelaxant response similar to what was reported by Salahdeen et al. (2015) for *Tridax procumbens*. This further suggested that the *T. glaucescens* mechanism might not entirely be dependent on the intact endothelium to release NO and can be said to act similarly to common nitrovasodilators (e.g. Nitroprusside, nitroglycerin, hydroxylamine) that are capable of producing a free radical, which in turn activates the soluble cytosolic form of guanylyl cyclase (Rapoport & Murad, 1983). The relaxation (%) response significantly increased with incubation in indomethacin. Indomethacin has been shown to potentiate acetylcholine-induced vasodilation by increasing free radical production and, subsequently, the production of NO (De Angelis et al., 2004). This is similar to the potentiating effect of the extract on acetylcholine, which is not affected by NO synthase inhibitors, as reported earlier in this study. This finding is corroborated by a study that one of the fatty acids in *Terminal glaucescens*, linoleic acid, can induce relaxation and was unaffected by the cyclooxygenase inhibitor indomethacin (Pomposiello et al., 1998). Another plausible explanation for this is the anti-inflammatory roles of *T. glaucescens*, as reported by Dannana et al. (2019). Indomethacin is an anti-inflammatory drug that has also been shown to promote nitric oxide function in the ductus arteriosus in the mouse (Sodini et al., 2008). Although the exact method by which indomethacin (along with its concomitant suppression of cyclooxygenase) enhances endothelial nitric oxide synthase (eNOS) function is unknown, certain conclusions can be drawn. In controlling vascular tone, prostaglandin E2 (PGE2) and nitric oxide (NO) function separately and interact differently based on the size and condition. According to several studies (Gobeil et al., 2002; Namkoong et al., 2005; Hristovska et al., 2007), NO may actually act as a messenger for PGE2, or it may form a reciprocal relationship with PGE2 and become more effective when it is suppressed (Tetsuka et al., 1994; Shimpo et al., 2000; Ribeiro et al., 2004). The extract in the presence of indomethacin can be suggested to promote nitric oxide

functions in ASMs by directly activating the endothelial nitric oxide synthase. Methylene blue (MB) significantly reduce the vasorelaxant effect of the *Terminalia glaucescens* ethanol stem extract. Methylene blue is a potent guanylyl cyclase/cyclic guanosine monophosphate (cGMP) inhibitor (Evora, 2016). This suggested that one of the mechanisms of action of the *Terminalia glaucescens* ethanol stem extract is possibly through guanylyl cyclase, which is similar to what was reported by Salahdeen et al. (2015) for *T. proccumbens* vasorelaxant mechanism for superior mesenteric arteries.

Conclusion

The study shows that the *Terminalia glaucescens* ethanol stem extract can relax the aortic smooth muscle precontracted with PHE and KCl. Although it is suspected that one of the mechanisms involves the release of NO, the extract's ability to stimulate cGMP through guanylyl cyclase is similar to that of a nitrovasodilator. It is important to note that the extract's vasorelaxant mechanisms can be both endothelium-dependent and independent, as the endothelium's inability to release NO does not eliminate the relaxation induced by ESET.

Author Contributions

Conceptualization: H.M., S.A., and A.M.; Methodology: H.M., S.A., and A.M.; Software: H.M., S.A., and A.M.; Validation: S.A. and H.M.; Formal Analysis: A.M. and S.A.; Investigation: A.M. and B.A.; Resources: H.M., S.A., and A.M.; Data Curation: A.M., S.A., H.M., and B.A.; Writing – Original Draft: A.M.; Writing – Review & Editing: S.A., A.M., and B.A.; Supervision: S.A. and H.M.

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Ethical Approval

The experimental protocols and procedures employed in this work followed the National Institutes of Health Guidelines (1985) for Laboratory Animal Care. Ethical approval for this research was granted by the Lagos State University College of Medicine's Animal Ethics Committee with the reference number AREC/2021/021.

Conflict of Interest.

The authors declared no conflict of interest

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ABS

Original Research

Improving Soil Health and Potato Productivity through Lime and Nitrogen Management in Northern Ethiopia

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Abstract

Field research was conducted with the aim of evaluating the effect of liming and nitrogen fertilizer rate on soil properties and yield components of potato varieties in acidic soil. The treatments contained three levels of lime [un-limed (0 kg ha^{-1}), farmer practised (1000 kg ha^{-1}), lime recommended (2000 kg ha^{-1})], three levels of fertilizer [control (0 kg ha^{-1}), farmer practised (100 kg ha^{-1}), recommended (165 kg ha^{-1})] and two potato varieties [Belete and Gudenie]. A combination of the levels of those three factors was laid out in a randomized complete block design (RCBD) with three replications. Data such as the number and weight of different-sized tuber yields and soil chemical properties like CEC, PH, Nitrogen, phosphorus, and exchangeable potassium were collected and analyzed using SAS software version 9.2. Variety Belete performed well in all yield parameters compared to Gudenie. Liming increased soil pH values, as well as calcium and exchangeable potassium content in the soil. However, liming had no significant effect on most yield parameters except for the weight of medium and small tubers. The application of nitrogen fertilizer improves the yield obtained per hectare. Based on this experiment, it can be concluded that the Belete variety is beneficial for farmers, as it yields better per hectare and produces a considerable amount of marketable yield. Applying nitrogen at the recommended level can further enhance the marketable yield.

Keywords

Potato varieties, Lime, Nitrogen, soil properties, soil acidity

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Izboljšanje zdravja tal in produktivnosti krompirja z upravljanjem apna in dušika v severni Etiopiji

Izvleček

Na polju je bila izvedena raziskava, katere namen je bil oceniti vpliv apnenja in odmerka dušikovih gnojil na lastnosti tal in sestavine pridelka sort krompirja v kislih tleh. Obdelave so vsebovale tri stopnje apnenja [neapnjen (0 kg ha^{-1}), kmetova praksa (1000 kg ha^{-1}), priporočeno apnenje (2000 kg ha^{-1})], tri stopnje gnojenja [kontrola (0 kg ha^{-1}), kmetova praksa (100 kg ha^{-1}), priporočeno (165 kg ha^{-1})] in dve sorti krompirja [Belete in Gudenie]. Kombinacija ravni teh treh dejavnikov je bila določena v randomiziranem popolnem blokovnem načrtu (RCBD) s tremi ponovitvami. Podatki, kot so število in masa različno velikih gomoljev ter kemijske lastnosti tal, kot so CEC, PH, dušik, fosfor, izmenljivi kalij, so bili zbrani in analizirani z uporabo programske opreme SAS različice 9.2. Sorta Belete se je v primerjavi s sorto Gudenie dobro izkazala pri vseh parametrih pridelka. Apnenje je povečalo vrednosti pH tal ter vsebnost kalcija in izmenljivega kalija v tleh. Vendar apnenje ni imelo pomembnega vpliva na večino parametrov pridelka, razen na maso srednjih in majhnih gomoljev. Uporaba dušičnih gnojil izboljša hektarski pridelek. Na podlagi tega poskusa je mogoče sklepati, da je sorta Belete koristna za kmete, saj daje boljše pridelke na hektar in proizvede precejšnjo količino tržnega pridelka. Uporaba dušika v priporočeni količini lahko še poveča tržni pridelek.

Ključne besede

Sorte krompirja, apno, dušik, lastnosti tal, kislost tal

Introduction

Potato (*Solanum tuberosum* L.) belongs to the nightshade family Solanaceae, having chromosome number (2n=4X=48), and it is the fourth most important food crop in the world, following rice, corn and wheat (FAO., 2016). Potato was introduced to Ethiopia in 1858 by the German Botanist Pankhurst R. (Pankhurst, 1964). Since then, potatoes have become an important garden crop in many parts of the country. About 70% of the available agricultural land is suitable for potato production, which is located at an altitude of 1500 to 3000 m a.s.l with annual rainfall between 600 and 1200 mm (Gebremedhin et al., 2008).

The total area under potato production had reached 69,610.81ha, and the production was estimated to be more than 9,689,696.44 quintals (CSA, 2017/18). The current national average yield of potatoes is 13.92 t/ha (CSA, 2017/18), which is lower than the world's average 19.85 t ha^{-1} (FAO, 2016). This is attributed to factors such as depleting soil fertility, poor agronomic practices, and lack of sustainable supply of improved planting material, growing of local and very old varieties, diseases and pests.

As potato is predominantly grown in the highland and mid-altitude areas, it suffers from soil acidity effects. Since soil of high altitude areas receives high rainfall to leach down soluble salts and/or basic cations appreciably from the surface layers (root zone) of the soils. The potentiality of acid soil for crop production is limited due to low pH, deficiencies in organic matter content, less availability of P, Ca, Mg, K and high content (toxicity) of Fe, Al (Khandakar et al. 2004). Apart from its toxicity, the effect of soil acidity extends to limiting the availability of other soil nutrients and making the application of fertilizer less effective. Soil acidity causes adsorption of P to colloidal fractions and also deficiencies of calcium, magnesium, potassium and molybdenum (Wilkinson et al., 2000; Hocking, 2001; Brady and Weil, 2008). A pH less than 5.5 severely limits the availability of potassium, nitrogen, phosphorous, sulfur, calcium and magnesium while availing excessive levels of aluminium, manganese, boron, iron, copper and zinc (Roques et al., 2013; Ochapa, 1984). Liming is an age-old agricultural practice to alleviate soil acidity problems and has had positive effects on the yield, protein content, ash, starch, and calcium of potato tubers (Rahman et al., 2014; Lalljee and Facknath, 2002).

Low soil fertility is one of the contributing factors to low potato yield in most parts of the world. The deficiency of nitrogen and phosphorous in most Ethiopian soils is the most important constraint limiting potato production

(Zewide et al., 2012). In Ethiopia, the amount of fertilizer applied by the farmer is below the recommended rate, which is because of a lack of technical knowledge (Legese et al., 2009; Ketema and Bauer, 2011), high costs (Ali et al., 2011; Tufa et al., 2015) and geographical conditions. A certain amount of the applied fertilizer is also lost through volatilization and leaching due to improper time and method of application. Therefore, the rate of fertilizer application could be optimum to satisfy the demand of the crop for the nutrients given that the potato is a heavy feeder of the major soil nutrients, removing estimated amounts of 90 to 120 kg N/ha, 13.8 to 25.8 kg P/ha, and 150 to 250 kg K/ha from the soil (Sikka, 1982).

The other major constraint to increased potato production is the lack of improved and good-quality seed tuber. Access to improved seed tuber adapted to local conditions is the key to achieving sustained efforts towards food security. Plant species and even varieties of the same species may differ in nutrient efficiency, which determines their ability to produce dry matter or yield. According to MoA (2018), in Ethiopia, about 36 different varieties of potato were released, which also differ in nutrient use efficiency and could have different optima of balanced macronutrient requirements for maximum yield of good quality seed tubers. Therefore, the objective of this study is i) to evaluate the main and interaction effect of liming and nitrogen fertilizer on soil properties and yield components of potato varieties and ii) to evaluate the performance of different potato varieties in the study area.

Material and Methods

Experimental site

The experiment was conducted at Kino Kebele in the Debark district. Debark is located in the northern part of Ethiopia, 830 km north of Addis Ababa and 90 kilometres north of Gondar. It has a latitude and longitude of 13°08′N 37°54′E and an elevation of 2850 meters above sea level.

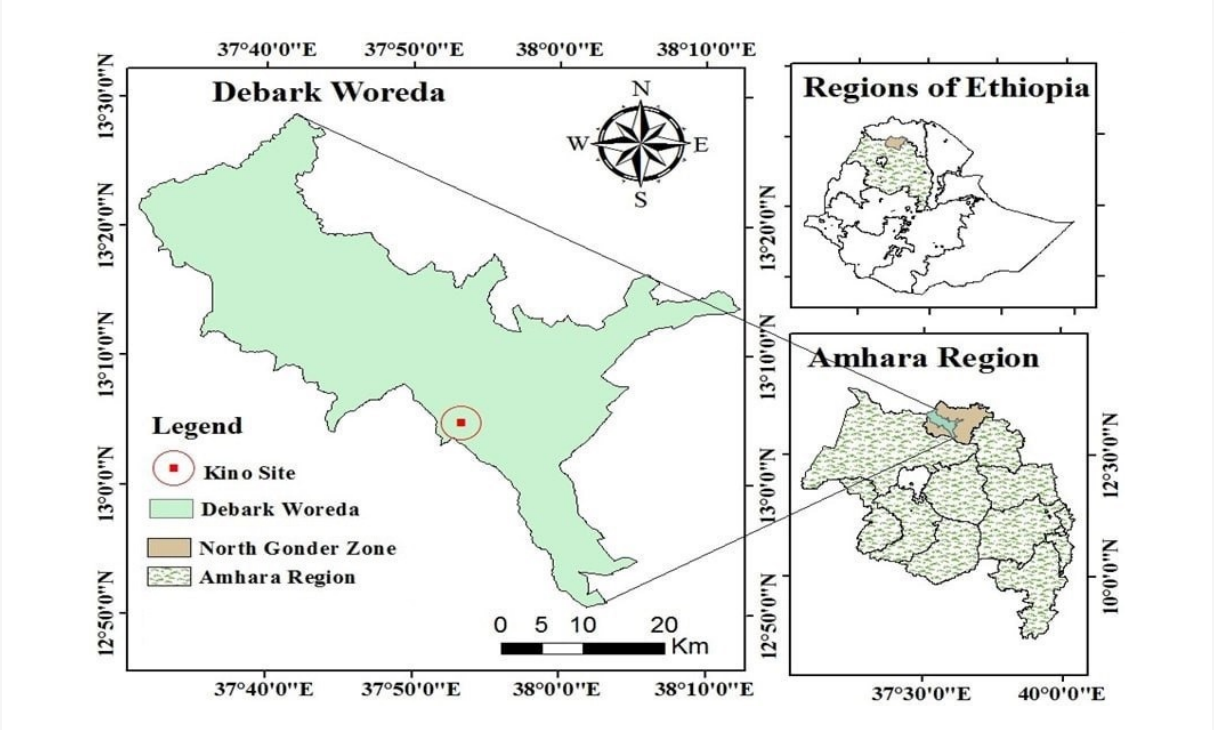

Figure 1. Map of the study area: Amhara National Regional State in Ethiopia, North Gondar in Amhara region, Debark district in North Gondar, Kino Keble in Debark district.

Slika 1. Zemljevid študijskega območja: Amhara v Etiopiji, Severni Gondar v regiji Amhara, okrožje Debark v Severnem Gondarju, Kino Keble v okrožju Debark.

Treatments and experimental design

The treatments have three levels of lime [un-limed (0kg/ha), lime recommended (2000 kg/ha) and farmer practised (1000 kg/ha)], three levels of fertilizer [control (0 kg/ha), farmer practised (100 kg/ha), recommended (165 kg/ha)] and two potato varieties [Belete and Gudenie]. There were eighteen treatment combinations laid out in a randomized complete block design, and each was replicated three times. There were a total of 18 X 3 = 54 plots with an individual plot size of 1.2m X 3m = 3.6 m^2. The spacing between rows and plants was 75 cm and 30 cm, respectively. A distance of 0.75m between plots and 1m between blocks was maintained. The gross net area of all plots (including the inter-plot and inter-block distances) was 34.35m X 11m = 377.85 m^2. Eight plants from the middle rows, leaving the plants growing in the two ridge rows, were used for data collection.

Experimental procedure

Medium-sized and well-sprouted potato tubers were planted at the bottom of a well-prepared ridge at a spacing of 75 cm between rows and 30 cm between plants. Each treatment was supplied with an equal amount of NPS fertilizer (19% N, 38% P2O5, and 7% S) at a rate of 100 kg per hectare as a phosphorus source, applied during planting. Other cultural practices, such as weeding and hoeing, were used uniformly for all experimental plots. Lime, which is locally available in the market, was powdered and sieved to pass through a 100-mesh sieve. The lime was incorporated in the 0-20 cm layer of soil 15 days prior to sowing the potato seeds according to the treatment. A preliminary survey was conducted to determine the fertilizer and lime application rate practised by farmers. Fifty potato growers were identified, and among them, 25 randomly selected farmers were interviewed on their trend of fertilizer and lime application rate per hectare. The average application rate of the 25 farmers was used as farmer-practiced (Fp) treatment for this experiment. Urea was used as a source of nitrogen fertilizer. To supply N, urea was applied three times: 2/3rd was applied during planting and mid-vegetative growth stage, and the remaining 1/3rd was applied at the initiation of tubers according to the treatment.

Crop Data collected and Measurements

After the total tubers were categorized into small (<45g), medium (46-70g) and large tubers (>70g), the total number and weight of large, medium and small tubers were measured (Lung'aho et al., 2007). The weight of marketable tubers was measured as the weight of medium, large and healthy tubers from eight plants and the central two rows. The total tuber yield (t/ha) is estimated using the gross tuber weight obtained from the plot. Data on yield and its various components were collected 120 days after planting. This period was chosen to allow sufficient time for all experimental plots to reach maturity, ensuring that the yield and associated components, such as tuber size, number, and weight, could be accurately assessed.

Soil Sampling and Chemical Analysis

Pre- and post-harvest soil samples (0-30 cm) were collected using a W-shaped pattern to ensure an accurate representation of each treatment level. The samples were processed at the Gondar Agricultural Research Centre soil laboratory, where they were mixed, homogenized, air-dried in the shade, ground to pass through a 2 mm sieve, and then analyzed for key physico-chemical properties. These included soil pH, total nitrogen (N), available phosphorus (P), organic carbon, cation exchange capacity (CEC), exchangeable calcium (Ca^{2+}), exchangeable potassium (K^{+}), and electrical conductivity (EC). Soil pH was determined using a 1:2.5 soil-to-water ratio suspension, measured with a digital pH meter attached to a glass electrode. Organic carbon and total nitrogen were analyzed using the wet digestion method of Walkley and Black (1934) and the Kjeldahl method (Jackson, 1973), respectively. Available phosphorus was quantified according to the method of Olsen et al. (1954), while CEC was determined by saturating soil samples with ammonium acetate and measuring through distillation, as per Bremner and Mulvaney (1982). Exchangeable calcium was assessed using atomic absorption spectrophotometry, and exchangeable potassium content was determined via flame photometry, following the technique outlined by Toth and Prince (1949).

Statistical Data Analysis

Prior to conducting statistical analysis, the normality of the collected data was assessed using the Shapiro-Wilk test (Shapiro and Wilk, 1965), confirming that the data met the assumptions required for ANOVA. Based on this, an analysis of variance (ANOVA) was performed using SAS software

version 9.2 to evaluate significant differences among the groups for the dependent variables. To further assess the treatment means, the Least Significant Difference (LSD) test was applied at a 0.05 significance level. Graphical representations of the data were created using R software for clear visualization.

Results

Weight and Number of tuber per hill

The number of tubers per hill was significantly ($p<0.05$) affected by the main effect of variety, lime and nitrogen. The weight of the tuber per hill was significantly affected by the main effect of variety ($p<0.001$), lime ($p<0.05$) and nitrogen ($p<0.001$). However, all the interaction effects were not significant (Table 1). The highest weight and number of tuber per hill was recorded for Belete as compared to Gudenie (Table 2). Application of lime has also improved the weight and number of tubers per hill, and no difference in the mean weight and number of tubers per hill among the rate practised by farmer (1000 kg ha^{-1}) and recommended level (2000 kg ha^{-1}) of lime application. Nitrogen application also increased both the weight and the number of tubers per hill.

Weight of large, medium and small tuber

Variety and nitrogen showed a significant ($p<0.001$) effect on the weight of large tuber. However, the main effect of lime did not significantly affect the weight of large tubers, and all two- and three-way interactions were also not significant ($p<0.05$) (Table 1). Belete has a higher weight of large tuber than Gudenie (Table 2), which might be because of the inherent genotypic capability of the variety to have a better weight of large tuber. The application of nitrogen fertilizer at the recommended level results in a large tuber with a high weight, while the lowest was recorded for the control group (Table 2).

The weight of the medium tuber was significantly affected by the main effect of variety ($p<0.01$), lime ($p<0.05$) and nitrogen ($p<0.01$) (Table 2). Not all the interaction effects were significant (Table 1). Regarding the variety, Belete gives a higher weight of medium tuber than Gudenie, which signifies that the two varieties are genotypically different. The application of lime improved the weight of medium tuber (Table 2). The highest weight of medium tuber was recorded by application of the recommended level of nitrogen (165 kg ha^{-1}) and the rate of fertilizer practised by the farmer (100 kg ha^{-1}) (Table 2).

The weight of the small tuber was significantly ($P<0.05$) affected by the application of lime. However, the main effects of variety, nitrogen, and interaction were not significant (Table 1). Even if they are statistically at par, the highest weight of small tuber was recorded for Belete (Table 2). Liming also improved the weight of small tubers; however, a similar weight of small tubers was recorded for the lime rate practised by the farmer and recommended rate (Table 2).

Marketable and total tuber yield per hectare

Marketable yield and total tuber yield per hectare were significantly ($p<0.001$) affected by the main effect of variety and nitrogen. Application of lime and all the interaction effects were not significant (Table 1). Higher marketable and total tuber yield per hectare was recorded for Belete than Gudenie (Table 2). The highest marketable yield was obtained under the recommended level of nitrogen application, while the lowest was obtained under the control group (Table 2). The application of nitrogen fertilizer also improved the yield obtained per hectare, and accordingly, the highest yield per hectare was obtained from the recommended (165 kg ha^{-1}) and farmer-practised (100 kg ha^{-1}) level of nitrogen application (Table 2).

Effect of nitrogen and variety interaction on tubers

The number of large tubers was significantly affected by the main effect of variety ($p<0.01$), nitrogen ($p<0.001$) and the interaction effect of variety*nitrogen ($p<0.01$), while lime application and the rest interaction effect were not significant (Table 1). The highest number of large tubers was obtained from variety Gudenie under the recommended rate of nitrogen fertilizer application, which is statistically at par with variety Belete, which received different levels of nitrogen level and the lowest was obtained from Gudenie under the control group (Table 3).

Number of medium tubers was significantly affected by the main effect of variety ($p<0.001$), lime ($p<0.01$), nitrogen ($p<0.001$) and the interaction effect of variety*nitrogen ($p<0.01$). Except for the variety*nitrogen, the rest of the interaction effects were not significant (Table 1). Variety Belete, with the recommended rate of fertilizer application,

Table 1. ANOVA table for the effect of lime and nitrogen rate on yield and yield components of potato varieties.

Tabela 1. Preglednica ANOVA za vpliv apna in odmerka dušika na pridelek in komponente pridelka sort krompirja

Source of variation	NL	WL	NM	WM	NS	WS	NTH	WTH	MY	YH
Replication	27.02NS	0.21NS	14.29NS	0.51*	208.04NS	0.07NS	4.12NS	0.01NS	17.28NS	7.73NS
Variety	244.91**	6.90***	580.17***	2.21**	238.56NS	0.41NS	47.56**	0.35***	522.23***	687.15***
Lime	17.91NS	0.09NS	261.46**	0.49*	846.26**	0.46*	24.59**	0.03*	21.73NS	35.56NS
Nitrogen	239.13***	3.54***	828.07***	1.13**	601.43*	0.02NS	25.98**	0.15***	267.14***	300.65***
Variety*lime	36.68NS	0.15NS	28.39NS	0.38NS	571.89NS	0.05NS	18.54NS	0.02NS	26.96NS	39.83NS
Variety*nitrogen	151.24**	0.03NS	193.55**	0.33NS	347.78*	0.13NS	3.78NS	0.01NS	7.23NS	14.86NS
Lime*nitrogen	78.51NS	0.30NS	32.68NS	0.15NS	138.74NS	0.16NS	7.52NS	0.02NS	23.78NS	46.20NS
Variety*nitrogen*lime	30.35NS	0.21NS	69.78NS	0.28NS	495.53NS	0.14NS	13.46NS	0.02NS	18.50NS	38.64NS

where Nl= number of large tubers, WL= weight of large tuber, NM= number of medium tubers, WM=weight of medium, NS= number of small, WS= weight of small, NTH=number of tuber per hill, WTH= weight of tuber per hill, MY= marketable yield , YH =yield per hectare, NS= non-significant, *= significant at 5%, **= significant at 1%, ***= significant at 0.1%

Table 2. The mean weight of large, medium, and small tubers and tubers per hill of potato varieties is affected by the application of different rates of nitrogen and lime and by location.

Tabela 2. Povprečna masa velikih, srednjih in majhnih gomoljev ter gomoljev na gomilo pri sortah krompirja, na katere vpliva uporaba različnih odmerkov dušika, apna in lokacije.

Treatment	Weight of large tuber	Weight of medium tuber	Weight of small tuber	Weight of tuber per hill	Number of tuber hill^{-1}	Marketable yield (t Ha^{-1})	Yield (t Ha^{-1})
Varieties							
Belete	1.59a	1.51a	1.10a	0.52a	14.96a	17.22a	23.49a
Gudenie	0.87b	1.11b	0.92a	0.36b	13.08b	10.99b	16.36b
LSD	0.22	0.19	0.17	0.05	1.07	1.73	2.32
Lime (kg ha^{-1})							
Control (0)	1.19a	1.12b	0.83b	0.39b	12.68b	12.84a	18.32a
Farmer-practiced (1000)	1.19a	1.44a	1.15a	0.47a	14.86a	14.62a	20.88a
Recommended (2000)	1.32a	1.36ab	1.05ab	0.46a	14.50a	14.86a	20.60a
LSD	0.26	0.24	0.21	0.06	1.31	2.12	2.84
Nitrogen (kg ha^{-1})							
Control (0)	0.76c	1.05b	0.97a	0.35c	12.63b	10.08c	15.49b
Farmer-practiced (100)	1.29b	1.32a	1.03a	0.45b	14.72a	14.49b	20.74a
Recommended (165)	1.64a	1.55a	1.03a	0.53a	14.69a	17.75a	23.55a
LSD	0.26	0.24	0.21	0.06	1.31	2.12	2.84
CV	31.81	26.71	31.27	13.84	20.19	22.22	21.01

Where CV= coefficient of variation, LSD= list significant difference at a 5% probability level

gives a higher number of medium tubers, while the lowest was obtained from variety Gudenie with zero fertilizer application (Table 3).

The application of lime ($p<0.01$) and nitrogen ($p<0.05$) significantly affected the number of small tubers. The interaction effect of variety*nitrogen was also significant ($p<0.05$). However, the main effect of variety and the rest interaction effect were not significant (Table 1). The highest number of small tubers was obtained from a variety of Belete under the rate of nitrogen application practised by farmers (100 kg ha^{-1}), which is statistically at par with the recommended (165 kg ha^{-1}) nitrogen application (Table 3). The lowest number of small tubers was obtained from the Gudenie variety under the control group (0 kg ha^{-1}) nitrogen application. Likewise, Negero (2017) also reported that increasing the rate of nitrogen increases total tuber yield per plot, yield, tuber number per plant and small tuber size.

Post-harvest soil chemical properties

A notable difference was observed in the soil's chemical properties after harvest, particularly with respect to the varieties, lime, and nitrogen application rates. The plot planted with the Belete variety exhibited lower levels of soil phosphorus, exchangeable potassium, and calcium compared to other Gudenie. Liming the soil resulted in improved pH levels, as well as enhanced calcium and potassium content. Additionally, the application of nitrogen fertilizer contributed to an increase in soil organic matter, calcium levels, and cation exchange capacity, further enhancing the overall quality of the soil (Figures 2 and 3).

Discussion

Marketable and total tuber yield per hectare

According to MoA (2018), about 36 different varieties of potato have been released in Ethiopia, which vary in yield, quality, and adaptability to different environments. The varietal difference in total and marketable yield between Belete and Gudenie is due to the inherent genetic potential of the genotypes for yield. In line with the present finding, it has been reported that the total tuber yield of potatoes was affected by the variety used (Fantaw et al., 2019). The result is also in agreement with Dash et al. (2018), who had previously reported a significant difference in marketable and total tuber yield among potato varieties. Likewise, Ebrahim et al. (2018) and Alemayehu et al. (2018) reported a significant variation in the marketable and total tuber yield among potato varieties. A high total and marketable yield of the Belete variety, compared to other varieties, was also reported (Kolech et al., 2015; Tessema et al., 2022; Seid and Tessema, 2024).

Although statistically non-significant, a slight improvement in mean total and marketable yield was observed with increasing lime rates. The result shows that Belete and Gudenie can yield well in moderately acidic soil (pH 5.6–6) by tolerating existing conditions. Nitrogen application rate is positively correlated with marketable yield, and total yield signifies the role of nitrogen for yield improvement in its indirect role of promoting vegetative growth. Previous findings also reported that the application of 100% nitrogen of the recommended dose boosted the growth and

Table 3. Interaction effect of variety and nitrogen rate on yield parameters of potato varieties.

Tabela 3. Interakcijski učinek sorte in odmerka dušika na parametre pridelka sort krompirja.

Variety	Nitrogen rate (kg ha^{-1})	Number of large tubers	Number of medium tubers	Number of small tubers
Belete	Control	17.22ab	29.33b	62.11bc
	Farmer-practised	18.44ab	30.22b	72.89a
	Recommended	18.78ab	43.33a	66.55ab
Gudenie	Control	7.78c	19.44c	66.17ab
	Farmer-practised	13.11bc	31.22b	69.67ab
	Recommended	20.78a	32.55b	53.11c
	CV	20.45	7.44	8.32
	LSD	5.959	4.197	9.455

Where CV= coefficient of variation, LSD= list significant difference at a 5% probability level

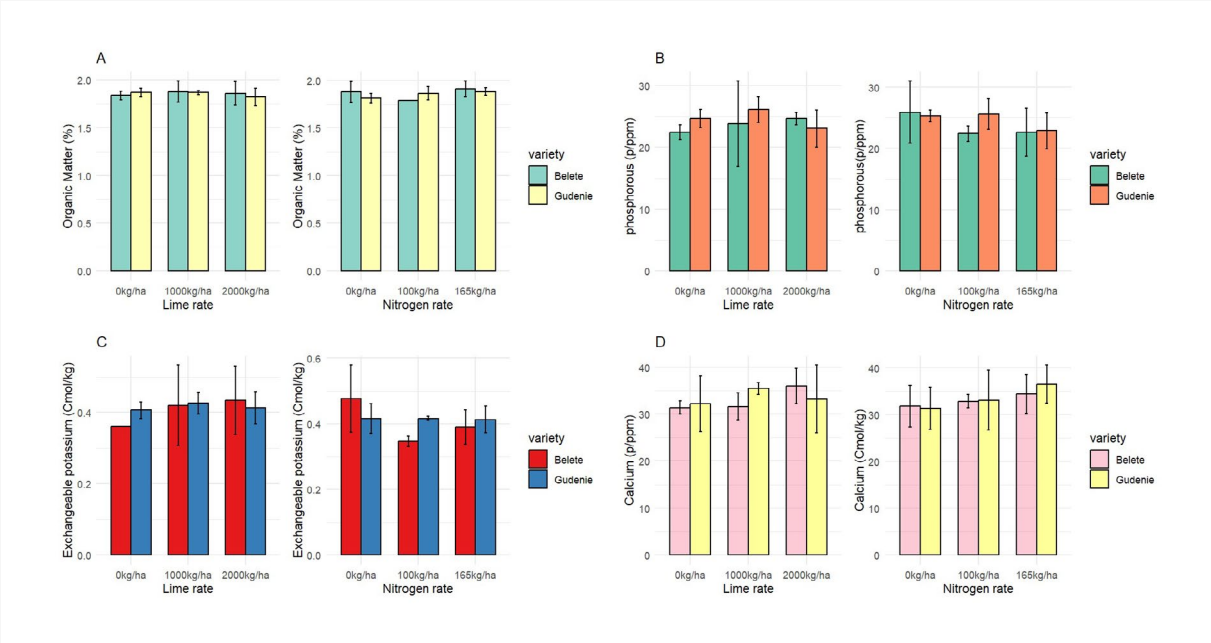

Figure 2. Effects of lime application and nitrogen rate on post-harvest soil chemical properties. A) organic matter (%), B) phosphorous in p/ppm, C) Exchangeable potassium (Cmol/kg), and D) Calcium in p/ppm.

Slika 2. Vpliv uporabe apna in odmerka dušika na kemijske lastnosti tal po spravilu pridelka. A) organska snov (%), B)fosfor v p/ppm, C) izmenljivi kalij (Cmol/kg), D) kalcij v p/ppm.

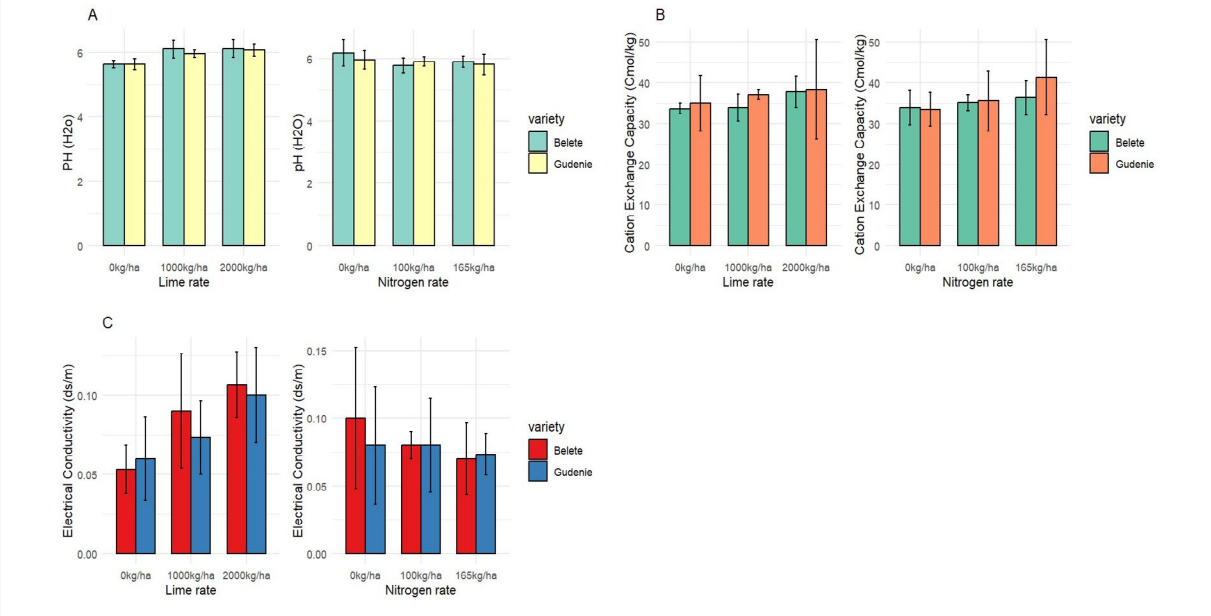

Figure 3. Effects of lime application and nitrogen rate on post-harvest soil chemical properties. A) Soil acidity pH (H2O), B) Cation Exchange capacity Cmo/kg, C) Electrical conductivity (ds/m).

Slika 3. Vpliv uporabe apna in odmerka dušika na kemijske lastnosti tal po spravilu pridelka. A) kislost tal pH (H2O), B)kationska izmenjalna kapaciteta Cmol/kg, C) električna prevodnost (ds/m).

yield of potatoes (Singh and Gupta, 2005). The absence of a significant difference between the farmer's practised nitrogen rate (100 kg/ha) and the recommended level (165 kg/ha) suggests that 100 kg/ha is sufficient to boost potato production in the study area as further increases raise production costs. Similarly, Alemaye et al. (2015) reported that total marketable and total tuber yields were increased by an application of nitrogen at a rate of 110 kg ha^{-1}, but increasing the rate of nitrogen beyond 110 kg ha^{-1} has statistically non-significant increment on the tuber yields. Belachew (2016) also reported that nitrogen application at rates of 110 kg ha^{-1} and 165 kg ha^{-1} resulted in the highest marketable yield, though the difference was statistically non-significant. A significant increase in tuber yield parallel to the increasing rate of nitrogen application was reported by Barghi et al. (2012) and Marthha et al. (2010).

Weight of large, medium and small tuber

The higher weight of large and medium tubers obtained from Belete compared to Gudenie demonstrates the greater genetic potential of the Belete variety. In the same manner, Tessema et al. (2022) and Tsegaye et al. (2018) also reported the presence of cultivar differences in weight of different-sized tuber. Increasing areas of agricultural land in high-rainfall regions of Sub-Saharan Africa, where crop production was once reliable, are affected by soil acidity, which can be mitigated through liming (Agegnehu et al., 2021). In this experiment, the application of lime increased the weight of medium and small tubers of potato due to its soil-reclaiming ability and its role in making applied fertilizers more available.

The application of nitrogen at the recommended level (165 kg/ha) increased the weight of large and medium tubers, highlighting the role of nitrogen in improving yield. This might be due to more luxurious growth, more foliage and leaf area, and a higher supply of photosynthate, which helped produce bigger tubers, resulting in higher yields (Sharma and Arora, 1987). In line with the present finding, an increase in tuber yield with the application of higher levels of nitrogen was also reported (Marthha et al., 2017). In addition to this, a significant increase in tuber yield was recorded with the increase in the nitrogen dose (Barghi et al., 2012). Other scholars like Etemad and Sarajuoghi (2012) and Negero (2017) also confirmed the presence of a positive relationship between potato tuber yield and dose of nitrogen application. All growth parameters and yield parameters, including different grade tubers, mean total tuber yield and tuber bulking, increased with increasing rates of nitrogen (Kumar et al., 2002; Negero, 2017; Barghi et al., 2012). Conversely, the application of nitrogen fertilizer at a higher rate may be associated with soil acidity and N2O emissions. Ning et al., 2023 reported that nitrogen application at a rate of 166 kg ha^{-1} was adequate for a target yield of <34 t ha^{-1}, but for target yields of 40, 50, and 60 t ha^{-1}, the recommended rates increased to 182, 211, and 254 kg N ha^{-1}, respectively, while keeping N2O emissions low at an emission factor of 0.2%.

Weight and number of tuber per hill

The superior performance of the Belete variety compared to Gudenie is attributed to the inherent genotypic differences between the two varieties. In line with the present study, genotypic variability in tuber weight and number of tubers per hill has been reported (Fantaw et al., 2019; Dash et al., 2018; Tsegaye et al., 2018). A significant difference in the level of lime application signifies that there is a considerable amount of soil acidity, which can limit the weight and number of tubers per hill. However, lime can be applied at the rate practised by farmers (1000 kg ha^{-1}) in the study area, as it is statistically similar to the recommended rate (2000 kg ha^{-1}), and since the farmer's rate is half of the recommended one, it helps reduce production costs by half. The increase in weight and number of tubers per hill with nitrogen application might be due to the pivotal role of nitrogen in the growth, development and translocation of photosynthesis products from source to sink (Lakshmi et al., 2001). In accordance with the present finding, an increase in total tuber yield with increasing rates of nitrogen application has been reported. (Kumar et al., 2002; Belachew, 2016; Marthha et al., 2010).

Effect of nitrogen and variety interaction on tubers

Even though Belete gives a high yield compared to Gudenie, a higher number of large-sized tubers was recorded when variety Gudenie was at a rate of recommended level (165kg ha^{-1}) of nitrogen application. Although Belete yields higher than Gudenie, a greater number of large-sized tubers was recorded for Gudenie when nitrogen was applied at the recommended rate of 165 kg ha^{-1}. In agreement with this finding, nitrogen is reported as the primary potato yield-lim-

iting nutrient, followed by phosphorous (Alemu et al., 2024). Significant interaction effect of nitrogen fertilization and variety was also reported (Belachew, 2016; Marthha et al., 2010). The high number of large tubers and low tuber weight of the Gudenie variety indicates that it has lower dry matter content compared to Belete, and therefore, Gudenie cannot be recommended for production in the study area. At the recommended nitrogen rate, Belete produced a higher number of medium and small-sized tubers than Gudenie, suggesting that Belete responds better to nitrogen fertilization. Likewise, Negero (2017) also reported that increasing the rate of nitrogen increases total tuber yield plot^{-1}, yield, tuber number plant^{-1} and small tuber size.

Post-harvest soil chemical properties

The low post-harvest soil nutrient composition for variety Belete compared to Gudenie might be because of the vigorous vegetative growth nature of Belete so that the variety efficiently used the available soil nutrients. The application of lime improves the soil's exchangeable potassium, calcium, pH, cation exchange capacity and electrical conductivity. In line with the present finding, Yenesew (2024) reported that lime application significantly ($P \leq 0.01$) improved the soil's chemical properties, increasing the pH to 6.26, reducing exchangeable acidity to 0.32 Cmol(+) kg^{-1}, eliminating exchangeable aluminium, enhancing cation exchange capacity to 29.43 Cmol(+) kg^{-1}, raising available phosphorus to 13.32 mg kg^{-1}, and boosting organic carbon content to 2.06%. It has also been reported that a significant decrease in exchangeable acidity (0.17 cmolc.kg^{-1}) was observed in soil treated with 6 tons of $CaCO_3$·ha^{-1} lime applied alone (93%), with an even more pronounced effect when combined with vermicompost (Bekele et al., 2018).

Deficiencies of calcium, magnesium, potassium and molybdenum have also been reported to limit crop yield in acid soils, which can be alleviated by liming (Wilkinson et al., 2000). It was also reported that Soil acidity converts available soil nutrients into unavailable forms, and soils affected by soil acidity are poor in their basic cations, such as Ca, K, Mg, and some micronutrients, which are essential to crop growth and development (Wang et al., 2006). In line with the present finding, Agegnehu et al. (2006) also indicated that soil pH consistently increased from 4.37 to 5.91 as the lime rate increased. Furthermore, it has been reported that acidity leads to the adsorption of phosphorus (P) onto colloidal fractions, as well as deficiencies in calcium, magnesium, potassium, and molybdenum (Wilkinson et al., 2000; Hocking, 2001; Brady and Weil, 2008).

Nitrogen fertilizers may acidify the soil mainly after nitrification, followed by nitrate leaching. In the present finding, nitrogen application increases soil organic matter and decreases soil pH, phosphorus, and electrical conductivity. It has been reported that the application of different types of nitrogen fertilizers decreased the soil pH linearly over time (Dal Molin et al., 2020).

Conclusions

Potato is one of the most widely cultivated vegetable crops in the highlands of Ethiopia. The yield and productivity of potatoes are far below the national average yield in the study area. Among various factors, the lack of improved varieties, improper timing and rates of fertilizer application, and the loss of soil fertility due to the leaching of basic cations from the soil surface are the most significant contributors. Given the aforementioned problem, a field experiment was conducted in the Debark district of Northern Ethiopia, with the objective of evaluating the main and interactive effects of liming and nitrogen fertilizer on soil properties and yield components of potato varieties in acidic soil.

Belete was found to perform the best in terms of weight, number of differently sized tubers, and total marketable yield compared to Gudenie. Thus, Belete can be recommended for farmers compared to Gudenie in the study area. Liming also improves the soil pH values of calcium and potassium content. The application of nitrogen fertilizer improves the soil's organic matter, calcium, and cation exchange capacity. Furthermore, liming improved the weight and number of differently sized tubers, and although it was not statistically significant, an improvement in the mean total and marketable yield of different potato varieties was observed. The application of different levels of nitrogen fertilizer improved potato yield, organic matter, and cation exchange capacity while decreasing soil pH and electrical conductivity. There was no significant difference in total tuber yield per hectare between the nitrogen fertilizer rate used by farmers (100 kg/ha) and the recommended level (165 kg/ha). However, applying nitrogen at the recommended level (165 kg/ha) did improve the marketable tuber yield. The rate of nitrogen fertilizer practised by farmers is not significantly different from the recommended level, but it could be applied to a recommended level to have a better marketable yield.

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Author Contributions

Investigation, S.H.A. and M.H.B.; Conceptualization, A.A.B. and A.W.A.; Methodology, A.A.B. and A.W.A.; Formal analysis, A.T.E.; Software, A.T.E.; Writing - review & editing, S.H.A., M.H.B., A.A.B., A.A.M., A.W.A., and A.T.E.; Writing - review & editing, A.T.E. All authors have read and agreed to the published version of the manuscript.

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Data Availability

The data that support the fndings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conficts of interest.

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ABS

Original Research

Electromyogram-based muscle stress estimation of Gastrocnemius medialis using Machine learning algorithms

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Abstract

Surface electromyography (sEMG) is the primary technique for recording muscle activity by capturing the electrical signal from the muscles. The present study has been undertaken to analyze lower limb muscle stress of gastrocnemius medialis without and with a workout (exercise) using machine learning algorithms. Ten healthy subjects (Seven male & three female) have been chosen on the basis of their age, height and mass. The experiment has been conducted without a workout and with a workout having a set of 10 kg resistance band loads for the gastrocnemius medialis muscle of the right leg. Fourth-order Butterworth filters and full wave rectifiers have been employed in data preprocessing. Many features have been extracted, including mean absolute value (MAV), average, skewness, kurtosis, zero crossing (ZC), slope sign change (SSC), waveform length (WL), mean frequency (MNF), median frequency (MDF), power spectral density (PSD), short-time Fourier transform (STFT) and wavelet transform (WT). Feature selection has been used to reduce the dimensionality of the data after feature extraction, which lowers computing costs and the risk of overfitting. This increases the accuracy of the classifiers. Principal component analysis (PCA) and independent component analysis (ICA) are two dimensionality reduction approaches used for EMG signal classification. Moreover, in the present study, decision trees (DT), random forests (RF) and support vector machines (SVM) have been used as classifiers. After the data has been preprocessed, the RMS value is obtained as the best-performing feature among all the other features. Then, the stress (RMS value) is analyzed for workout (warm-up), i.e., exercise and without workout cases. The stress value for a workout (warm-up) is found to be less than that without a workout case. Moreover, individuals taking up warming-up exercises proved to be giving more accuracy (79%) with respect to their contemporaries (70%). So, in order to improve the accuracy of the workout (exercise), dimensionality reduction techniques (PCA and ICA) have been applied. The dataset is divided into training, testing and validation at a ratio of 70:20:10 respectively. Better accuracy (99.2%) is obtained when PCA is used as a dimensionality reduction technique, and SVM is used as a classifier. The proposed technique attains 99.2% accuracy with RMS as feature extraction, PCA as a dimensionality reduction and SVM as a classifier.

Keywords

EMG signal, Gastrocnemius medialis, With workout, Without workout, PCA, SVM.

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Ocenjevanje mišične obremenitve Gastrocnemius medialis na podlagi elektromiograma z uporabo algoritmov strojnega učenja

Izvleček

Površinska elektromiografija (sEMG) je primarna tehnika za beleženje mišične aktivnosti z zajemanjem električnega signala iz mišic. Ta študija je bila izvedena za analizo mišičnega stresa spodnjih okončin gastrocnemiusa medialisa brez in z vadbo (vadbo) z uporabo algoritmov strojnega učenja. Deset zdravih oseb (sedem moških in tri ženske) je bilo izbranih na podlagi njihove starosti, višine in mase. Poskus je bil izveden brez vadbe in z vadbo z nizom 10 kg upornih obremenitev za mišico gastrocnemius medialis desne noge. Butterworthovi filtri četrtega reda in polnovalni usmerniki so bili uporabljeni pri predprocesiranju podatkov. Izločenih je bilo veliko funkcij, vključno s srednjo absolutno vrednostjo (MAV), povprečjem, asimetrijo, kurtozo, prečkanjem ničle (ZC), spremembo predznaka naklona (SSC), dolžino valovne oblike (WL), srednjo frekvenco (MNF), srednjo frekvenco (MDF), spektralno gostoto moči (PSD), kratkotrajno Fourierjevo transformacijo (STFT) in valovno transformacijo (WT). Izbira funkcij je bila uporabljena za zmanjšanje dimenzionalnosti podatkov po ekstrakciji funkcij, kar znižuje stroške računalništva in tveganje prevelikega opremljanja. To poveča natančnost klasifikatorjev. Analiza glavnih komponent (PCA) in analiza neodvisnih komponent (ICA) sta dvodimenzionalna pristopa zmanjšanja, ki se uporabljata za klasifikacijo signala EMG. Poleg tega so v tej študiji kot klasifikatorji uporabljeni odločitvena drevesa (DT), naključni gozdovi (RF) in podporni vektorski stroji (SVM). Po predhodni obdelavi podatkov se pridobi RMS vrednost kot najuspešnejša funkcija med vsemi drugimi značilnostmi. Nato se stres (RMS vrednost) analizira za vadbo (ogrevanje), torej primere vadbe in brez vadbe. Ugotovljeno je, da je vrednost stresa za vadbo (ogrevanje) manjša od tiste brez primera za vadbo. Poleg tega se je izkazalo, da so posamezniki, ki izvajajo ogrevalne vaje, bolj natančni (79 %) v primerjavi s svojimi vrstniki (70 %). Torej, da bi izboljšali natančnost vadbe (vadbe), so bile uporabljene tehnike zmanjšanja dimenzij (PCA in ICA). Nabor podatkov je razdeljen na usposabljanje, testiranje in validacijo v razmerju 70:20:10. Boljša natančnost (99,2 %) je dosežena, če se PCA uporablja kot tehnika zmanjševanja dimenzij, SVM pa se uporablja kot klasifikator. Predlagana tehnika doseže 99,2-odstotno natančnost z RMS kot ekstrakcijo značilnosti, PCA kot zmanjšanje dimenzij in SVM kot klasifikator.

Ključne besede

EMG signal, Gastrocnemius medialis, Z vadbo, Brez vadbe, PCA, SVM.

Introduction

Stress is a natural reaction that affects everyone. There are two types of stress: positive and negative. A normal workout (approximately five minutes of running) has a positive effect on our body by relaxing muscles and increasing flexibility and mobility. However, rigorous exercise instantly has a negative effect, i.e., muscle fatigue. The decline in maximal force produced by a muscular contraction is a sign of muscle stress (Hayder et al., 2019). Acute stress impairs our capacity for lifting and movement. Several research studies have been carried out to detect and investigate muscular stress. Janssen et al. (2000) estimated skeletal muscle mass based on gender, age, and height. Molinari et al. (2006) assessed fatigue during muscle contraction. Dingwell et al. (2008) and Al-Mulla et al. (2011) estimated muscle fatigue and movement kinematic changes. Wavelet analysis was used by Tscharner (2002) to evaluate light exercises. González-Izal et al. (2012) observed both non-linear and linear methods for estimating muscle fatigue. These detection techniques are insufficient for monitoring muscle–fatigue conditions.

So, currently, surface electromyography (sEMG) is the primary technique for recording muscle stress by capturing the electrical signal from the muscles (Tankishi et al., 2020; Birbeck et al., 2021). The characteristics of EMG signals, i.e. time or frequency domain, evaluate the muscle condition (Liu et al., 2019; Liu et al., 2014). Human evolution has resulted in a greater number and development of lower limb muscles. The decline of lower limb muscles is greater

than that of upper limb muscles (Kotman et al., 2013; Abe et al., 2014; Abe et al., 2011). During activity, every muscle has its unique functions and produces different EMG signals (Wang et al., 2021). Muscles of the lower limb, including the gastrocnemius medialis (GM), play an important role in flexing the feet (Ugbolue et al., 2021). It is possible to quantify gastrocnemius in the legs before and after exercises. Using EMG signals, researchers examine the gastrocnemius, a major muscle in the leg's calf. The gastrocnemius muscles are located in the back part of the lower leg and play a crucial role in walking, running, and jumping (Mohd Khairuddin et al., 2012). They are responsible for plantar flexion of the foot, lifting the heel off the ground, and pushing the body forward while walking.

The present study focuses on muscle stress estimation of gastrocnemius medialis muscle without workout (without exercise) and with workout (with exercise) using the Electromyographic technique. Therefore, the recorded EMG data is divided without and with workouts and then assessed accordingly. The gastrocnemius is an essential muscle in the body since it flexes the foot and is used constantly for the most basic activities like walking (Giulio et al., 2009). An imbalance in the body is caused by fatigue in the gastrocnemius. It has been noted that mediolateral sway increases during plantar flexion, impairing postural control. EMG signals are a complex process that involves analyzing muscle activity using surface electrodes placed on the skin. Researchers have explained different optimization techniques for biomedical signals (Chawla and Duhan, 2014). Regular physical activity has numerous health benefits; exercise can enhance mental well-being, lessen worry tension and enhance cognitive performance. Regular exercise is important for health, particularly weight-bearing exercise such as walking, which helps increase bone density and reduce the risk of osteoporosis.

There are several methods for analyzing biomedical signals (Manoj et al., 2011). While selecting a method is contingent upon the particular research and experimental design. Furthermore, Kumar et al. (2022) observed different time and frequency domain approaches. Amplitude-based techniques estimate muscle activation by measuring the EMG signal's amplitude. The peak/ RMS amplitude of the EMG signal is used for muscle stress. Frequency-based methods analyze the frequency content of the EMG signals for estimating muscle stress. The PSD of the EMG signal is used to estimate the frequency distribution of muscle activity. Moreover, Time-domain methods analyze time-varying properties of EMG signal in order to estimate muscle stress. The present study emphasizes machine learning algorithms to analyze the differences in sEMG signal characteristics for lower limb muscles with and without workouts. Section 1 includes an introduction, section 2 shows the materials and methods, Section 3 consists of results & discussion, and section 4 includes conclusion & future scope.

Materials and Methods

EMG Database

The present study is carried out at the DCRUST, Murthal, Haryana, India. Seven male & three female subjects have been chosen on the basis of their age, height, mass and workout (exercises). EMG signals have been acquired from the gastrocnemius medialis muscle using Acknowledge 4.2 software, Biopac MP150 (BIOPAC Systems, Inc., Santa Barbara, CA). The EMG signal is acquired using two electrodes, with the third electrode serving as a reference electrode, which is placed between two surfaces. The EMG activity of each muscle is measured using the Biopac System and bipolar electrodes made of Ag-AgCl of 10 mm. EMG procedures have been followed as per standards endorsed by the International Society of Electrophysiology and Kinesiology, 1999. The sampling rate of 1000 Hz, input impedance of 1015 Ω/0.2 pF, and common mode rejection ratio (CMRR> 90 dB) have been used to acquire EMG signals.

Ten healthy subjects, age group between (18 to 25 years), height (1.62 to 1.80 m), mass (48 to 84 kg) and BMI (15.9 to 25.9 kg/m^2) as shown in Table 1, have been investigated without and with workout. Right leg gastrocnemius medialis has been used to record the EMG data. Each subject has signed a written consent form certifying that they are free from any muscular diseases. Experiments are carried out using a dumbbell that weighed 10.0 Kg. The weight of the dumbbell has been chosen based on preliminary trials to prevent overstress in subjects. EMG signals have been recorded and analyzed using Acknowledge 4.2 software, Biopac MP150 (BIOPAC Systems, Inc., Santa Barbara, CA).

In this study, bipolar electrodes (silver chloride electrodes with a distance between electrodes of 20 mm) are used over the gastrocnemius medialis of the right legs. To prepare the skin area for electrode placement, it has been rubbed (with spirit and towel) and shaved in the designated area. The readings of subjects are taken without workout

Table 1. Physical characteristics of subjects (n=10)

Tabela 1. Fizične značilnosti subjektov (n=10)

Subjects	Gender	Age	Mass (kg)	Height (m)	BMI (Kg/m^2)
1	Male	18	56	1.63	21.1
2	Male	18	75	1.75	24.5
3	Male	19	55	1.62	20.9
4	Male	19	75	1.78	23.7
5	Male	20	84	1.8	25.9
6	Male	23	78	1.72	26.3
7	Male	25	76	1.8	23.5
8	Female	18	48	1.74	15.9
9	Female	18	53	1.63	19.9
10	Female	19	54	1.63	20.3

with a dumbbell of resistance band load of 10 Kg in both hands while standing on tiptoes position at 45^0 angles. The Medigauge electronic digital goniometer has been used to measure angle position. The subjects (participants) have never gone to the gym or engaged in athletic exercise. The data is recorded for 10 seconds (10000 samples) for each subject. The procedure is repeated 8 times. After 2 days' rest, the same procedure is repeated with a workout (exercise) in which all participants (subjects) run fast for 5 mins. After acquiring the dataset, it is divided into training, testing, and validation, with a ratio of 70:20:10. The dataset is split in order to check the performance of the model. A training set is used to train the model. The test set is independent of the training set; it is used to evaluate the performance of the trained model. The validation set offers a preliminary assurance that the model can produce insightful forecasts.

Signal Pre-processing

Band-pass filtered signal: Motion artifacts and high-frequency noise have been minimized by the use of band-pass filtered signals with frequency 20 – 450 Hz along with Zero lag fourth-order Butterworth filter.

Full wave rectifier signal: A full wave rectifier is used to take the absolute value of the signal. An EMG signal has been analyzed using a full-wave rectifier to retain all the energy of the signal so that the lower limb muscle can be estimated.

Feature extraction

It involves identifying relevant information from raw EMG signals that can be used for various applications, such as prosthetic control, clinical diagnosis, or human-computer interaction. Feature extraction can be done in three domains, *viz.* time, frequency and time-frequency. The relevant information for classification is obtained by extracting features from the preprocessed signals. Common characteristics consist of:

Time Domain: Time domain analysis of EMG data is a method for looking at the time components of these signals (Ekincl et al., 2023).

Mean absolute value (MAV): The mean of the EMG signal's absolute values.

Average: The average mean of EMG data is a valuable metric in understanding muscle function and provides important insights in a range of fields. It also helps in the identification and quantification of muscle fatigue, which is characterized by a reduction in muscle activity over time.

Standard Deviation (SD): The variability or dispersion of signal across time is measured by the SD of the EMG data. It offers crucial details regarding the signal's properties, including the muscle activation length, frequency, and amplitude. Additionally, it's utilized to find any possible noise or artifacts in the signal.

Root Mean Square (RMS): RMS calculates the degree of

muscular activity by measuring the amplitude of the EMG signal. An EMG signal's RMS value indicates the average power of the signal for a given duration. In EMG analysis, the RMS value is computed, which yields a more precise measurement of muscle activation than the raw EMG signal. Determining the degree of muscle activity in the raw EMG data can be challenging due to noise, artifacts, and other factors. The EMG signal's RMS value offers a more accurate indicator of the contraction of muscles. When the RMS value of the EMG signal is calculated, the level of contraction of muscles during a specific time period can be determined and compared to other time periods or to other individuals.

Skewness: Skewness analysis helps identify the type of muscle activity by determining whether the signal is symmetric or skewed to the left or right.

Kurtosis: The shape of a probability distribution can be described statistically by kurtosis. In the context of electromyography (EMG) signals, kurtosis is used to analyze the shape of the distribution of muscle activation.

Zero Crossing (ZC): How many times the signal intersects zero, indicating muscle activity frequency.

Slope Sign Change (SSC): The number of times the slope of the EMG signal's slope shifts in either direction.

Waveform Length (WL): The EMG waveform's total length over time.

Frequency Domain: Frequency domain analysis of EMG data is a method for looking at the frequency components of these signals (Tsai et al. 2015).

Mean Frequency (MNF): The average frequency of the EMG signal, providing information about muscle fatigue.

Median Frequency (MDF): The frequency at which there are two equal portions in the power spectrum.

Power Spectrum Density (PSD): This represents the distribution of power into frequency components.

Time-Frequency Domain: Short-Time Fourier Transform (STFT): Analyzes the frequency content of the signal over short time windows (Woldemariam et al. 2016).

Wavelet Transform: Decomposes the signal into different frequency bands, capturing both time and frequency information (Chowdhury et al. 2013).

Feature Selection: It's a critical step in processing electromyography (EMG) signals due to their high-dimensional and often noisy nature. Techniques like PCA and ICA are widely used for this purpose.

Principal Component Analysis (PCA): Data is divided into a collection of orthogonal (uncorrelated) components using PCA, a dimensionality reduction technique. Usually, the first few components represent the majority of the data's volatility. Compute the covariance matrix of the signals, perform eigenvalue decomposition, and project the signals onto the principal components. PCA is used for noise and dimensionality reduction (Mishra et al., 2017). PCA is beneficial due to following reasons:

Noise Reduction: PCA helps in reducing noise by focusing on the most significant components, effectively filtering out the less important, noisy parts of the signal.

Data Compression: It reduces the dimensionality of the data.

Visualization: PCA can transform the data into 2D or 3D space for visualization.

Independent Component Analysis (ICA): Another method for reducing dimensionality is called independent component analysis (ICA), which breaks down a multivariate signal into additive parts. ICA is better suited for applications requiring signal separation and artifact removal. Use algorithms like Fast ICA to decompose the signals into independent components. It is particularly useful for EMG signals for the following reasons:

Artifact Removal: ICA can separate the EMG signal into components that are likely to be artifacts (e.g., from electrical noise or motion) and those that are true muscle signals, making it easier to isolate andremove unwanted noise.

Signal Source Separation: It helps in identifying and separating the sources of EMG signals, which is useful when multiple muscles are being monitored simultaneously.

Enhanced Feature Extraction: Independent components can provide features that are more meaningful for specific tasks like gesture recognition or muscle activity analysis. Both PCA and ICA are valuable tools for feature selection in EMG signal processing. They help reduce noise, compress data, and extract meaningful features, ultimately enhancing the performance of subsequent analyses and applications.

Classification

Decision Tree: Decision tree is a supervised algorithm applied to both regression and classification problems. These are non-parametric supervised learning models that predict the value of a target variable using basic decision rules obtained from the data variables. The decision trees split at each node based on a chosen criterion. Popular decision tree algorithms include:

CART: Classification & regression trees use gini impurity by default for classification and variance reduction for regression, and binary trees are also built. There are four steps in the CART procedure. The first is building a tree by recursively splitting nodes where the splitting requirements are met. The second is to cease the tree-building process once the learning data set has been shaped and fitted using the attributes. The third is tree trimming, which involves removing significant nodes to create smaller, more straightforward trees. The final step is to choose the best tree from the list of pruned trees that, when fitted to the learning dataset, do not overfit (Lewis, 2000). Assigning a minimum of two terminal nodes and five folds for cross-validation pruning yields efficient results.

C4.5: C4.5 is the successor of ID3 and CART, which deal with both continuous and discrete attributes and missing values. The first step in the C4.5 algorithm is choosing an attribute to test the tree's root and verify the viability of each attribute; training instances are compared by statistical calculations once each possible attribute value is examined in relation to training samples.

Random Forest: RF classifier gained popularity in the field of bioinformatics and biomedical engineering as a machine learning option. Because random forests are good at handling complex data structures, multidimensional feature space, and small sample sizes, they are being used more and more in the field of computational biology (Gokgoz et al., 2015). Two stages are involved in applying randomness. In order to construct each tree, first use distinct bootstrap sample data. Subsequently, each tree node is divided using the best subset of predictors rather than all of the predictors based on a random subset selection (Yi and Pan, 2010). Bootstrapping is done to reduce generalization error and to increase classification accuracy when random features are leveraged.

Support vector machines: SVM are powerful supervised learning models used in classification and regression tasks. When there are more dimensions (features) than samples in the dataset, they perform well in high-dimensional spaces. It consists of linear SVM and non-linear SVM.

Linear SVM: For a binary classification problem, SVM determines the best hyperplane with the largest margin to divide the data points of several classes.

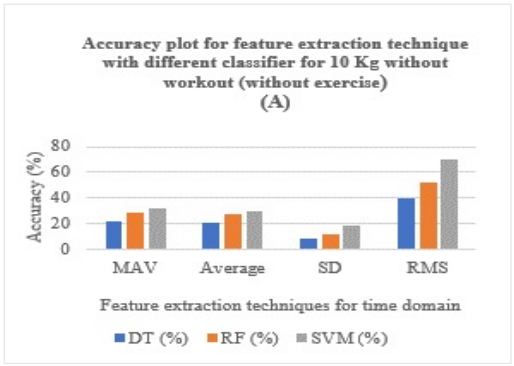
Non-linear SVM (with the kernel): SVM can handle non-linear separable data by mapping the input vectors into a higher-dimensional feature space using a kernel function $K(X_i, X_j)$.

Results and Discussion

There are 10 users in this research problem, and each user must go through 8 trials. Consequently, the total number of trials increases to 10×8, or 80, which becomes the dataset rows. The sample size is 10000 for one subject per trial, so the total sample size for this data is 10×8×10000 samples. The performance of the provided dataset has been evaluated using a variety of techniques. The optimal features are obtained from the feature set (MAV, Average, SD, RMS, MDF, PSD, STFT and WT) based on the accuracy attained after using various classification methods (DT, RF and SVM), as shown in Fig. 1 and 2 (for 10 Kg load with and without workout) from the above feature set. RMS has given maximum accuracy; hence, it has been chosen as the best feature.

Figure 3 represents the RMS and standard deviation values for without and with workouts. RMS is an essential feature which gives necessary information about a specific data set (Velliangiri et al., 2019). The stress value (as evident in terms of RMS value) has been reduced with a workout (with exercise) as compared to without a workout. It pertains from the result that the RMS value of stress is reduced from 13.64% (minimum) to 26.08% (maximum). However, the p-value that has been calculated for with and without workout is $p < 0.001$, which indicates that data is highly significant statistically. Our results are in lieu of the study of Kauranen et al. (1999) and Chen et al. (2021). The study is also corroborated with the study of Callewaert et al. (2013), who found that the stress value reduced with the exercise of trained boy groups. Similarly, Halin et al. (2002) found that the frequency-domain characteristics of gymnasts are lower than those of trained boys. The gastrocnemius muscle showed variable levels of long-term dependability, according to Gollhofer et al. (1990) and Goodwin et al. (1999). Mohr et al. (1998) reported that in gastrocnemius muscle, post warm-up EMG activity is significantly less than the pre-warm EMG activity.

The important prerequisite for the feature selection technique is to reduce the number of features, noise and irreverent data (Jia et al., 2022). Dimensionality reduction (DR) is the pre-processing technique (Velliangiri et al., 2019), which is used to improve feature accuracy, training time and computation cost reduction. In the present work, DR techniques like principal component analysis (PCA) and independent component analysis (ICA) have been applied to feature selection. PCA and ICA are statistical methods (Abdi and Williams, 2010; Comon, 1994; Hyvarinen and Oja, 2000). The use of PCA and ICA decreases the computational



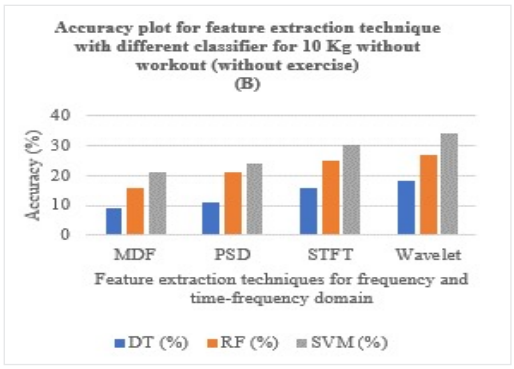
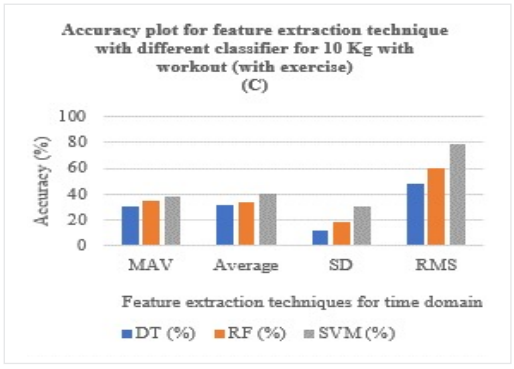


Figure 1. Accuracy attained with various classifiers for different feature extraction techniques without workout (without exercise).

Slika 1. Natančnost, dosežena z različnimi klasifikatorji za različne tehnike ekstrakcije funkcij brez vadbe.



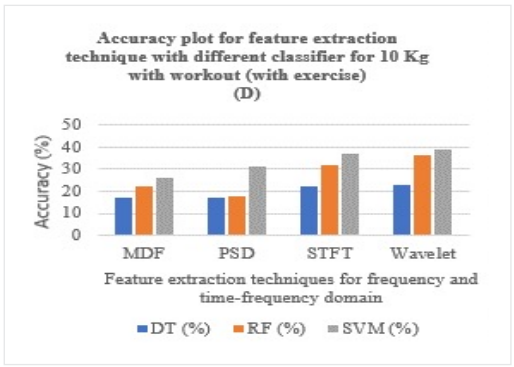


Figure 2. Accuracy attained with various classifiers for different feature extraction techniques with workout (with exercise).

Slika 2. Natančnost, dosežena z različnimi klasifikatorji za različne tehnike ekstrakcije značilnosti z vadbo.

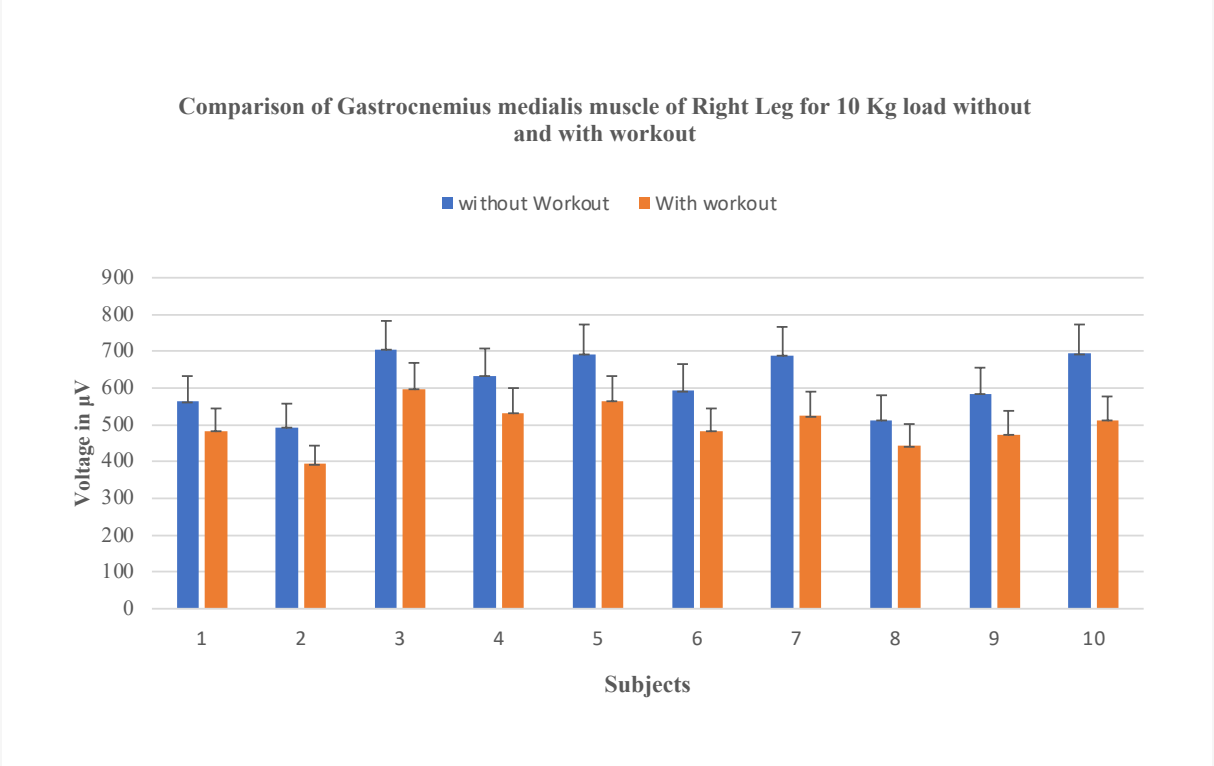


Figure 3. Comparison of RMS values of stress for gastrocnemius medialis muscle of subjects (n=10) with 10 kg resistance load without and with workout.

Slika 3. Primerjava RMS vrednosti za mišico gastrocnemius medialis preiskovancev (n=10) z uporno obremenitvijo 10 kg z in brez vadbe.

Table 2. Values of Accuracy, Precision, Recall and F1 Score have been attained after using various dimensionality reduction strategies for a 10 kg load with a workout (exercise).

Tabela 2. Vrednosti natančnosti, natančnosti, priklica in rezultat F1 so dosežene po uporabi različnih strategij za zmanjšanje dimenzionalnosti za obremenitev 10 kg z vadbo.

Classifier	Dimensionality Reduction	Accuracy (%)	Precision (%)	Recall (%)	F1 Score (%)
DT	'None'	48	46	45	49
RF	'None'	60	58	59	62
SVM	'None'	79	74	76	78
DT	PCA	93	89	91	90
RF	PCA	95	93	90	89
SVM	PCA	99.2	94	97	96
DT	ICA	89	87	86	90
RF	ICA	92	94	89	91
SVM	ICA	94	91	92	94

complexity of the model because the signal's dimensionality is reduced, and calculation time is also reduced (Jia et al., 2022; Wang and Chang, 2006). Table 2 illustrates the accuracy, Precision, Recall and F1 Score values that have been obtained after applying different dimensionality reduction techniques with workout (with exercise). 'None' in Table 2 shows that the feature selection (dimensionality reduction) technique is not being used. SVM with PCA (dimensionality reduction technique) shows a higher accuracy of 99.2 with the workout.

Conclusion and Future Scope

The experiment has been conducted to analyze stress with and without workout for the gastrocnemius medialis muscle. Fourth-order Butterworth filters and full wave rectifiers have been employed in data preprocessing. RMS value has been chosen as best performing feature among all features. It also finds that stress value is reduced in the case of a workout (exercise) as compared to without a workout. Exercise (warm-up) relaxes the muscles, reducing physical stress. Moreover, warm-up (exercise) is applied to enhance tissue flexibility. The result suggests that an accuracy 99.2% is achieved with RMS as a feature, PCA as a feature selection and SVM as a classifier for optimal performance. Table 3 illustrate the comparison between various machine learning-based methods for EMG signal. So, this study is helpful for rehabilitation treatment, sports training and movement recognition to improve their overall quality of life.

In future work, present research may integrate different datasets to evaluate the effectiveness of the proposed feature extraction and selection methodology for EMG signal classification.

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Autor Contribution

Conceptualization, A.K.; methodology, A.K.; software, A.K.; validation, A.K.; formal analysis, A.K.; investigation, A.K.; data curation, A.K.; writing—original draft preparation, A.K.; writing—review and editing, A.K., M.D. and P.S.; visualization, M.D. and P.S.; supervision, M.D. and P.S. All authors have read and agreed to the published version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

Table 3. Comparison with existing Machine learning techniques.

Tabela 3. Primerjava z obstoječimi tehnikami strojnega učenja.

Reference	Features	Classifiers	Accuracy (%)
Wang et al. (2021)		The LSTM	95.18
		CNN	92.72
		SVM	90.30
Ramos et al (2020)	RMS	SVM	82
Worassa et al (2024)	RMS	MLP	90.6
		ID CNN	93
		Bi-LSTM	95
Proposed Model	RMS+PCA	SVM	99.2

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Original Research

Phytochemical screening and in vitro assessment of antioxidant and anti-inflammatory activity of cornsilk (*Stigma maydis*) extracts

Asmaa Belgharbi [1*], Chourouk Chennoufi [2], Sara Sitayeb [1], Mohamed Ouahdi [2], Hadjer Kada [2], Nour El Houda Maghraoui [2]

Abstract

Cornsilk (*Stigma maydis*) has significant anti-inflammatory and antioxidant activities, making it a valuable component in traditional medicine. In this context, our study focuses on the phytochemical, antioxidant and anti-inflammatory analysis of methanolic and aqueous extracts of *Stigma maydis* using various methods (phytochemical screening, anti-oxidative activity (DPPH), ferric reducing power (FRAP), TAC and red blood cell membrane stabilisation method). Preliminary results showed that these extracts were rich in polyphenols (with 80.60 ±0.03* vs 45.99 ±0.01* at $p \leq 0.05$ for methanolic and aqueous extracts respectively) and flavonoids (with 27.14 ±0.07* vs 9.70 ±0.05 at $p \leq 0.05$. Furthermore, the results of the antioxidant activity showed that the methanolic fraction had the highest antioxidantcapacity (195 mg EAA/1 g EXS) compared to the aqueous extracts (105.2 mg EAA/1 g EXS). However, the anti-inflammatory evaluation showed that the highest percentage of haemolysis inhibition was observed with the aqueous extract of *Stigma maydis*, which can be explained by the fact that the haemolytic activity of each extract is related to its chemical composition. These results confirmthat *Stigma maydis* extracts have excellent antioxidant and anti-inflammatory properties, which could recommend them as a real alternative to chemical drugs.

Keywords

Aqueous extract, Anti-inflammatory, Antioxidant activity, Methanolic extract, phytochimical screening, *Stigma maydis*

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Fitokemični pregled in in vitro ocena antioksidativne ter protivnetne aktivnosti izvlečkov koruzne svile (*Stigma maydis*)

Izvleček

Koruzno mleko (*Stigma maydis*) ima pomembne protivnetne in antioksidativne lastnosti, zato je dragocena sestavina tradicionalne medicine. V tem kontekstu se naša študija osredotoča na fitokemično, antioksidativno in protivnetno analizo metanolnih in vodnih izvlečkov *Stigma maydis* z uporabo različnih metod (fitokemični pregled, antioksidativna aktivnost (DPPH), železova redukcijska moč (FRAP), TAC in metoda stabilizacije membrane rdečih krvnih celic). Preliminarni rezultati so pokazali, da so ti ekstrakti bogati s polifenoli (80,60 ±0,03* proti 45,99 ±0,01* pri $p \leq 0,05$ za metanolni oziroma vodni ekstrakt) in flavonoidi (27,14 ±0,07* proti 9,70 ±0,05 pri $p \leq 0,05$. Poleg tega so rezultati antioksidativne aktivnosti pokazali, da je imela metanolna frakcija največjo antioksidativno zmogljivost (195 mg EAA/1 g EXS) v primerjavi z vodnimi izvlečki (105,2 mg EAA/1 g EXS). Vendar pa je protivnetna ocena pokazala, da je bil najvišji odstotek inhibicije hemolize ugotovljen pri vodnem izvlečku *Stigma maydis*, kar je mogoče pojasniti z dejstvom, da je hemolitična aktivnost vsakega izvlečka povezana z njegovo kemično sestavo. Ti rezultati potrjujejo, da imajo izvlečki *Stigma maydis* odlične antioksidativne in protivnetne lastnosti, zaradi česar bi jih lahko priporočili kot pravo alternativo kemičnim zdravilom.

Ključne besede

Vodni izvleček, protivnetno, antioksidativno delovanje, metanolni izvleček, fitokemični pregled, *Stigma maydis*

Introduction

Interest in medicinal plants and their traditional uses has increased significantly in recent decades, reflecting a global movement to promote traditional medicine. Today, nearly 80% of theworld's population relies on traditional medicine for healthcare (WHO, 2022). Medicinal plants, in particular, remainan important source of novel compounds and continue to play an essential role in the discovery of new therapeutic agents, contributing to the development of future medicines (Chaughule and Barve, 2024).

Algeria, with its diverse flora and wealth of aromatic and medicinal plants, offers a promising resource for pharmacological research. However, despite the wide range of biological and therapeutic activities associated with many local plants, scientific studies on their medicinal potential remain under explored (Becheneb and Bouchetti, 2022).

One such under utilised plant is corn silk (*Stigma maydis*), the fine, thread-like stigmas of the female corn (*Zea mays*) flower, which is native to Mesoamerica and was domesticated in Mexico some 9000 years ago. Today, maize silk is cultivated world wide (Buckler et Stevens, 2006). Historically, it has been used in traditional medicine to treat a variety of deseases, including bladderand prostate inflammation and urinary tract irritation (Apampa and Adedapo, 2024).

Rich in flavonoids and phenolic compounds, corn silk has long been recognised for its antioxidant properties, making it a natural alternative to synthetic antioxidants commonly used in modern medicine (Ebrahimzadeh et al., 2008).

In addition, it has a number of therapeutic applications in folk medicine, including use as an insecticide, disinfectant, antioxidant, antibiotic, immune stimulant, as well as an oral hypoglycaemic and anti-inflammatory agent (Kim et al., 2004; Hasanudin et al., 2012; Wang et al.,2017).

Notwith standing its extensive utilisation across numerous regions globally, the potential of corn silk remains under utilised in Algeria and the broader North African region, despite the prevalence of corn cultivation in these areas. The pharmacological potential of corn silk in these regions has not been extensively explored. The present study aims to address this knowledge gap by investigating the antioxidant and anti-inflammatory properties of corn silk from the Mascara region of Algeria. The objective is to promote its more wide spread use and to explore its potential as a valuable resource in local and global health applications.

Materials and Methods

Collection of plant material

The biological material used in this study is cornsilk (*Stigma maydis*), which refers to the yellowish filiform strands of the female corn flower (Figure 1).

The plant material was harvested in November 2022 from theregion of Mascara (Algeria) and was identified by the Laboratory of Plant Biology in the Faculty of Natural and Life Sciences at the University of MustaphaS tambouli of Mascara. The samples were separated from the mother plant, sorted, and cleaned. Then, they were dried in a ventilated place and in the dark to obtain a better extraction and to standardize the residual moisture content. Once dried, the samples were ground to a fine powder (≤ 250 µm) using an electric laboratory micromill (model MF 10 Basicfrom IKA 0002836001). The powder obtained is stored in there frigerator at 4°C in an opaque glass box coveredvwith tin foil, protected from light to prevent photo-oxidation of the active substances (Figure 2) (Chuo et al., 2020).

Figure 1. Samples of *Stigma maydis*.

Slika 1. Vzorci *Stigma maydis*.

Figure 2. Steps of sample preparation (A: Fresh plant; B: Drying; C: Grinding and Sieving; D: conservation).

Slika 2. Koraki priprave vzorca (A: sveža rastlina; B: sušenje; C: mletje in presejanje; D: konzerviranje).

Preparation of *Stigma maydis* methanolic and Aqueous Extracts

The objective of this extraction process is to recover the bioactive molecules present in corn silk, with a particular focus on phenolic compounds and flavonoids. Solid/liquide xtraction is carried out by maceration of the powder from our samples in two appropriate solvents, aqueous and methanolic, to extract the biologically activec ompounds (Figure 3). This extraction is carried out at room temperature using magnetic stirring for 24 hours to prepare methanolic extracts (Me.E) (Harborne, 1998) and in distilled water for the aqueous extracts (Aq.E) (Sasidharan et al., 2011).

The extracts were stored in small, shadedvials at 4°C until use. The extraction yield (Y%) is determined by the ratio between the mass of the dry extract obtained after the evaporation of the solvent (m') and the mass of the plant powder (m), according to the following formula (Harborne, 1998):

$$Y\% = \frac{m'}{m} \times 100$$

Qualitative screening

The free quinones: were determined by mixing 2 ml of extract with a few drops of sodium hydroxide (NaOH) (0.1N). The mixture was then left at room temperature for between five and ten minutes. The presence of free quinones was confirmed by the observation of a yellow to orange colour in the mixture (Katoch, 2011).

Steroids: In order to detect the presence of steroids in the sample, 0.1 ml of chloroform ($CHCl_3$) and 0.1 ml of concentrated sulphuric acid (H_2SO_4) were added to 0.1 g of extract. The observation of a red layer during this reaction indicates the presence of steroids (Siddiqui et al., 2009)

Flavonoids: 0.5 g of magnesium (Mg) powder was weighed and added to 2 ml of plant extract. Then, 2 dropsof 2N HCl solution were added to the mixture and gently stirred. The appearance of a yellow, pink or red colouration, after a period of 3 to 5 minutes at room temperature, indicated the presence of flavonoids in the extract (Alqethami and Aldhebiani, 2020).

Tanins: The Braymer Test is a method employed to detect the presence of tannins in a solution. To this end, drops of a 10% FeCl3 solution were added to 2 ml of extract. The presence of tannins is revealed by the appearance of a greenish grey or dark blue colour(Siddiqui et al., 2009).

Alkaloids: The Mayer Test was employed to detect the presence of alkaloids in the extracts under investigation.1 ml of methanolic or aqueous extract was mixed with 3 drops of Mayer's reagent, prepared fresh in a test tube. The appearance of a white colour or the formation of an insoluble precipitate indicates the presence of alkaloids (Kumar et al., 2020).

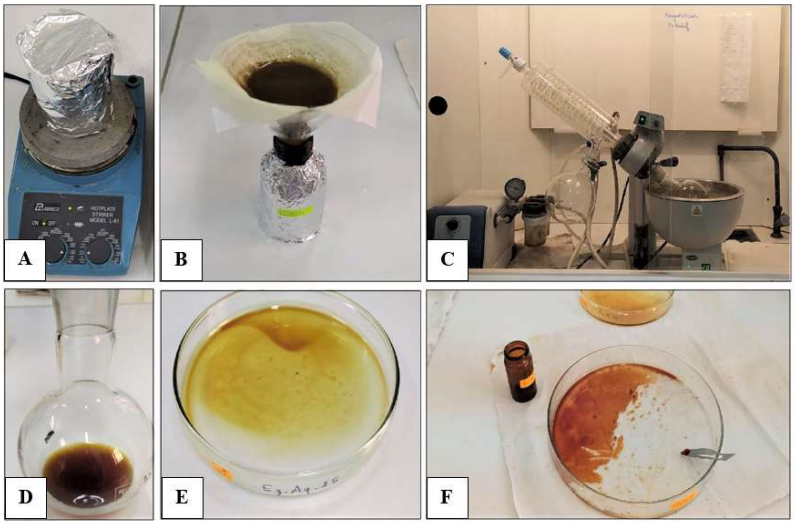

Figure 2. Techniques for preparing extracts of *Stigma maydis* (A: maceration; B: filtration; C: evaporation by rotavapour; D: liquidextract; E: drying; F: powdered extract).

Slika 2. Tehnike priprave izvlečkov *Stigma maydis* (A: maceracija; B: filtracija; C: izhlapevanje z rotacijo; D: tekoči izvleček; E: sušenje; F: izvleček v prahu).

Saponins: The Foam Test technique is uses for the detection of saponins in a given solution. The procedure involves the mixing of 2ml of distilled water with 2ml of extract. The mixture is then subjected to vigorous shaking.The presence of a foam greater than 1 cm in height, which persists for a minimum of 1 hour, is indicative of the abundant presence of saponins in the extract (Alqethami and Aldhebiani, 2020).

Anthocyanins: In this experiment, 2 ml of anthocyanin extract was mixed with 2ml of hydrochloric acid (2N). When the mixture exhibited a pink-red hue, ammoniac was introduced. The colourc hange to a violet-blue hue indicated the presence o fanthocyanins (Ukoha et al., 2011).

Glycosides: The detection of glycosides is based on the use of the Kellar-Kiliani Test, in which 1 ml glacial acetic acid, 1 ml $FeCl_3$ and 1 ml H_2SO_4 are added to 2 ml extract. The appearance of a green-blue colour is indicative of the presence of glycosides (Ukoha et al., 2011).

Resins: A precipitate test was performed by mixing 1.5 ml of extract with 2 ml of distilled water. The formation of a precipitate is an index of the presence of resins (Harborne, 1998).

Coumarins: 1 g of powder was placed in a test tube with a few drops of distilled water. The tube was then covered with paper soaked in dilute NaOH and brought to the boil. The presence of coumarin is indicated by yellow fluorescence observed under ultraviolet light (Shaik and Patil, 2020).

Mucilages: In a separate test tube, 1 ml of extract was mixed with 5 ml of absolute alcohol. The formation of a flaky precipitate, after shaking, indicates the presence of mucilages (Benzidia et al., 2004).

Quantitative detection of Total Phenolic and Flavonoid content

The total phenolic content of our samples was determined using the Folin-Ciocalteu reagent (Chang et al., 2002). However, the flavonoid content was determined according to the protocol using aluminium trichloride (Brand-Williams et al., 1995). All substances were then measured at 765 nm for total phenolics and at 430 nm for flavonoid susing a UV-visible spectrophotometer (Shimadzu UV-1280, Japan).

Antioxydant activity assessment

In order to highlight the antioxidant activity of ourextract, we have chosen the following techniques:

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging: The DPPH free radical reducing power of our extracts was determined using the method reported by Brand-Williams et al. (1995) and Bozin et al. (2008) with minor modifications. In this technique, 2 ml of methanolic DPPH solution (Sigma Chemical Co., USA) (0.1 mM) was mixed with 2 ml of each extract at different concentrations. After incubation for 30 min in the dark at room temperature, the absorbances were measured at 517 nm. The IC50 was calculated by linear regression with the concentration of the tested compounds on the abscissa and theantioxidant activity in percentage (%) on the ordinate (Mishra et al., 2012).

Reducing power of iron (FRAP): The reducing powerwas determined according to the method of Benzie and Strain (1996). 0.5 ml of the extract at different concentrations (200, 100, 50, 25, 0) was mixed with 1.25 ml of 0.2 mM phosphate buffer solution (pH=6.6) and 1.25 ml of 1% potassium ferricyanide $K_3Fe(CN)_6$ solution (1 mg/100 ml distilled water). The combination was incubated in a water bath at 50°C for 20 min. Then 1.25 ml of 10% trichloroacetic acid ($C_2HCl_3O_2$) was added to stop the reaction. The tubes were centrifuged at 3000 rpm for 10 min; 1.25 ml of the supernatant was combined with 1.25 ml of distilled water and 0.25 ml of a freshly prepared 0.1% aqueous solution of $FeCl_3$ (ferric chloride). The absorbance of the reaction medium is read at 700 nm against a similarly prepared blank. The positive control is an antioxidant standard.

Determination of total antioxidant capacity (TAC): It is determined by the phosphomolybdate reduction method (Prieto et al., 1999).In this method, 200 µl of each extract at different concentrations are added to a tube containing 2000 µl of a reagent consisting of H_2SO_4 (0.6 M), Na_2PO_4 (28 mM) and ammonium molybdate (4 mM). The tube was then sealed and incubated at 95°C for 90 minutes. After cooling, the absorbance was measured at 695 nm. The control consisted of 200 µl of methanol mixed with 2000 µl of the above reagent. Samples and controls are incubated under the same conditions. The results obtained are expressed as mg ascorbic acide quivalents per gram of extract dry matter (mg EAA/g EXS).

In vitro-anti-inflammatory activity assessment

The in vitro anti-inflammatory activity of plant extracts is determined using the protein denaturation inhibition method (Esho et al., 20-21). The techniques used in ourstudy are described below:

Red bloodcell membrane stability test: This involves

mixing 2 ml of blood with 2 ml of Alsevers solution (2% glucose + 0.8% sodium citrate + 0.5% citric acid + 0.42% NaCl + distilled water).The various blood samples obtained were centrifuged at 3,000 rpm for 10 min to remove plasma and polynuclear cells. The erythrocyte pellet was then washed three times with a suitable volume of iso-saline solution. After this step, the volume wa smeasured and reconstituted as a 5% (v/v) suspension (GRH) with iso-saline solution and used immediately (Shinde et al., 1999).

Haemolysisby heat: In haemolysistubes, 0.5 mLof extracts (methanolic/aqueous of *Stigma maydis*), 1 mL of phosphate buffer (0.15 M, pH 7.4) and 2 mL of hyposaline solution (0.42% NaCl) were mixed and incubated at 37°C for 20 min. A volume of 0.5 mL of erythrocyte suspension (5%) was then added to each tube and incubated at 56°C for 30 minutes. The tubes were in cold water for 20 min to stop the reaction and then centrifuged at 2500 rpmfor 10 min. The absorbance of the supernatant was read at 560 nm using a spectrophotometer (Shimadzu UV-1280, Japan) (Gadamsetty et al., 2013).

Statistical analysis

All experiments were performed in triplicate. Data are presented as mean ± standard deviation (SD). Analysis of variance (ANOVA) was used to assess significant differences between treatment groups for each parameter. A p≤ 0.05 was considered statistically significant.

Results and Discussion

Extraction yield and phytochimical screenning: Qualitative and Quantitative Analysis

The extraction yields, along with the qualitative and quantitative assessment of total phenols and flavonoids in methanolique and aqueous extracts of *Stigma maydis*, are presented in Table 1.

The results of our study revealed differences in yield between aqueous and methanolic extracts of *Stigma maydis*. The aqueous extract showed a significantly higher yield of 19.31 ±0.015* compared to the yield of 14.6 % and 9.55 % reported in a the study of Nurhanan and WanRosli (2012) and Ammor et al. (2021) respectively .for the methanolic extract, our study showed a yield of 16.7 %,which is lower than the 33.10 % and 20.7 % reported in the same studies.

In other hand; According to the tests performed in Table 1, the phytochemical analysis of the aqueous and methanolic extracts of corn silk revealed the presence of polyphenols, flavonoids, tannins, glucosides and saponosides in ourextracts, which are in perfect agreement with thework of Ammor et al. (2019). Thus, according to the study of Nurhanan and WanRosli (2012), methanolic extracts are richer in polyphenols and flavonoids than aqueous extracts, which is in agreement withour results.

These variations can be attributed to several factors

Table 1. Extraction yield, qualitative and quantitative analysis of the bioactive components of *Stigma maydis* extract.

Tabela 1. Ekstrakcijski izkoristek, kvalitativna in kvantitativna analiza bioaktivnih sestavin izvlečka *Stigma maydis*.

Plant Extract	Gender	Me.E	Aq.E.
Yield (%)		16.7 ±0.02	19.31 ±0.015
Qualitative Screenning	Free quinon	+++	++
	Steroids	+++	+++
	Flavonoids	+++	+++
	Tannin	+++	+++
	Alkaloid	-	-
	Saponins	-	-
	Anthocya-nins	-	-
	Glycosids	+++	+++
	Resins	-	-
	Coumarins	++	++
	Mucilage	-	-
Quantitative Screening	Total phenol (mg EAG/g)	80,60 ±0.03*	45,99 ±0.01*
	Flavonoid (mgEqQ/g)	27,14 ±0.07*	9,70 ±0.05*

Absent (-) ,Weaklypresent (+) , Moderatelypresent (++) , Stronglypresent (+++)/ (*) significant (p ≤ 0.05)

such as the conditions of sample collection, extraction techniques and analytical methods used, which may lead to differences in the results obtained.

In addition, the results of the quantitative analysis of the active compounds showed different concentrations of total phenolic compounds in the *Stigma maydis* extracts. The methanolic extract showed the highest concentration of phenolics measured at 80.60 ±0.03 vs 45.99 ±0.01 mg EAG/g for the aqueous extract. These results are in perfect agreement with those obtained by Ammoret al. (2021) with total polyphenol concentration of 75.20 ±1.83 vs 41.54 ±7.90 for hydro-alcolic and aqueous extract respectivetly. In contrast, the results obtained by Dong et al. (2014) showed much lower values, ranging from 4.3 to 7.6 mg EAG/g extract.

Overall, these differences in the concentrationof phenolic compounds and flavonoids can be attributed to various factors, such as genetic and environmental factors and the extraction techniques used. In addition, the stage of maturity of the plant or its parts can also lead to variations in concentration; in some cases, concentrations may be higher in the early stages of plant development, while in other cases they may be higher in later stages (Bibi et al., 2022).

Results of antioxydant activity assessment

The results of this test on our two *Stigma maydis* extracts and on the ascorbic acid (Vitamin C) control are shown in Figure 3.

From these data it can be seen that the percentage inhibition of the free radical for both extracts was lower than that of the standard at all the concentrations used. These percentages correspond to a total inhibition of DPPH, which is reflected by the complete discolouration of DPPH from purple to paleyellow. The antioxidant capacity of the different extracts was then determined from the IC50 (Table 2).

These results show that the methanolic extract has an IC50 (in the order of 36.89 µg/ml) much lower than that of the aqueous extract (64.347 µg/ml). The anti-free radical activity of the methanolic extract is therefore higher than that of the aqueous extract. These results are in perfect agreement with the work of Emmanuel et al. (2016), who demonstrated that the methanolic extracts of *Stigma maydis* have a much higher antioxidant activity.In fact, it was shown that the antioxidant activity depends not only on the concentration of polyphenols, but also on the nature and structure of the antioxidants present in the extract. In general, polyphenols with a high number of hydroxyl groups show high antioxidant activity (Maksimović et al., 2005).

Nurhanan and wanRosli (2012) reported that methanol-extracted corn silk exhibited a higher level of DPPH scavenging activity (81.7 % at 1,000 µg/ml) than the aqueous extract (63.5 %) at the same concentration.Inaddition, the result of Aourabi et al. (2021) shows that the IC50 of DPPH scavenging activity of water extracts were 95.2 µg/ml which is more important than thats showed in our result.

Ferric reducing power (FRAP) results.

The assessment of the reducing power of iron at different concentrations of *Stigma maydis* is shown in Figure 4.

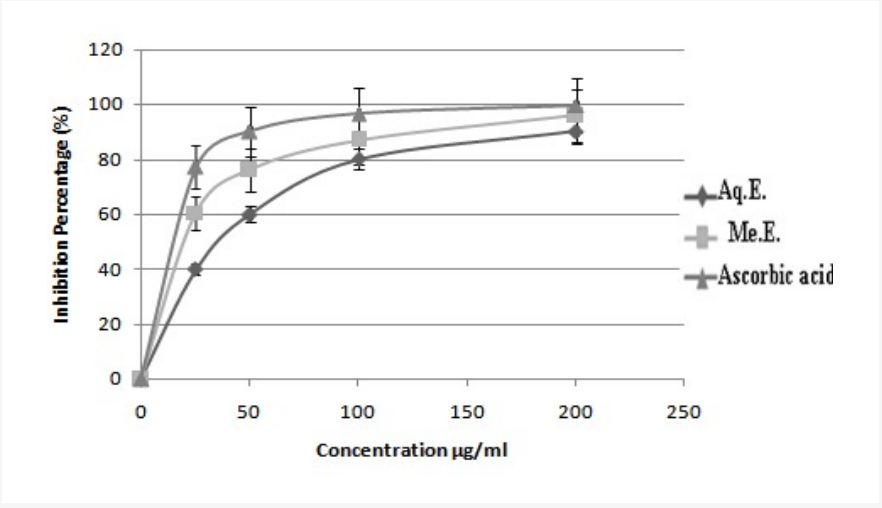

Figure 3. Percentage of DPPH radical inhibition in *Stigma maydis* extracts.

Slika 3. Odstotek inhibicije radikala DPPH v izvlečkih *Stigma maydis.*

Table 2. Antioxidant power (expressed as IC50 in µg /ml) of reference antioxidants and *Stigma maydis* extracts.

Tabela 2. Antioksidativna moč (izražena kot IC50 v µg /ml) referenčnih antioksidantov in izvlečkov *Stigma maydis*.

Sampletested	IC 50 value (µg/ml)
Ascorbic Acid	9.77
Aqueous extract	64.347
Methanolic extract	36.89

These results show a proportionality between the reducing capacity and the increase in concentration of the extracts. We note that ascorbic acid has a greateriron-reducing capacity than the methanolic extract (0.4) and the aqueous extract (0.33) ($p<0.05$). This activity could be mainly attributed to the polyphenols present in the plant (Bekara et al., 2007).

Indeed, our results are in perfect agreement with those of Nurhanan and WanRosli (2012), who showed that the reducing powerof corn silk extract was significantly lower than that of ascorbic acid. This can be justified by the action of polyphenols and flavonoids, which play a very important role in chelating transition metals involved in the Fenton reaction (formation of hydroxyl radicals resulting from the reaction of iron with hydrogen peroxide) (Bougandoura and Bebdimerad, 2012).

Total Antioxidant Capacity (TAC) assay

The results obtained are expressed as mg ascorbic acid equivalent per g of dry extract (mg EAA/1g EXS). They are shown in the figure 5.

The results clearly indicate that the methanolic and aqueous extracts have an antioxidant capacity to reduce Mo6+ ions to Mo5+ ions. According to these results, the estimation ofthe total antioxidant capacity of the extracts showed variability depending on the type of solvent used for extraction. Indeed, at a concentration of 600µg/ml, the methanolic fraction had the highest antioxidant capacity (195 mg EAA/1g EXS) compared to the aqueous extracts (105.2 mg EAA/1g EXS). This antioxidant capacity observed in the two extracts may be essentially due to the richness of the extracts in polyphenols, especially flavonoids, and also to the chemical structures of the bioactive molecules. This difference in activity between the two extracts is related to the difference in chemical composition of each extract and the content of phenolic elements.

The antioxidant activity depends on the content of phenolic compounds and the position of the hydroxyl groups (Heim et al, 2002).In general, polyphenols with a high number of hydroxyl groups have the highest antioxidant activity due to their ability to donate more atoms to stabilise free radicals.

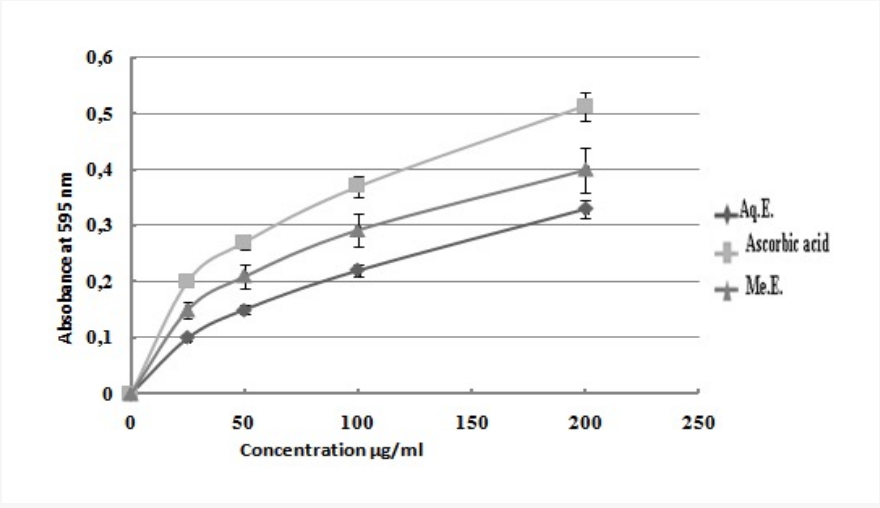

Figure 4. Assessment of the antioxidant activity of *Stigma maydis* extracts using the FRAP method.

Slika 4. Ocena antioksidativne aktivnosti izvlečkov *Stigma maydis* z metodo FRAP.

Assessment of the in vitro anti-inflammatory activity of *Stigma maydis* extracts

The variations in anti-inflammatory activity, expressed as a percentage of albumin protection, as a function of the concentrations of extracts used are shown in Figure 6.

From these results it can be seen that the highest percentage of haemolysis inhibition was observed with the aqueous extract of *Stigma maydis*, which can be explained by the fact that the haemolytic activity of each extract is linked to its chemical composition (Saheed et al., 2020).

These phytochemicals composition include flavonoids, which are involved in the oxidation of haemoglobin, disruption of membrane structure and increased conductivity, and therefore haemolysis of erythrocytes, due to pro-oxidant effects that can be exerted at high concentrations (Galati et al., 2002).

Furthermore, *Stigma maydis* extracts play an important role in anti-inflammatory activity; indeed, they significantly reduced iNOS and COX-2 levels without inducing cytotoxicity and suppressed NO secretion in LPS-stimulated macrophages (Ansari and Sahoo, 2022).

All in all; these observations demonstrate that *Stigma maydis* extracts have an anti-inflammatory and anti-nociceptive effects, which may provide further scientific evidence for it suse as a potential therapeutic agent for the treatment of inflammation.

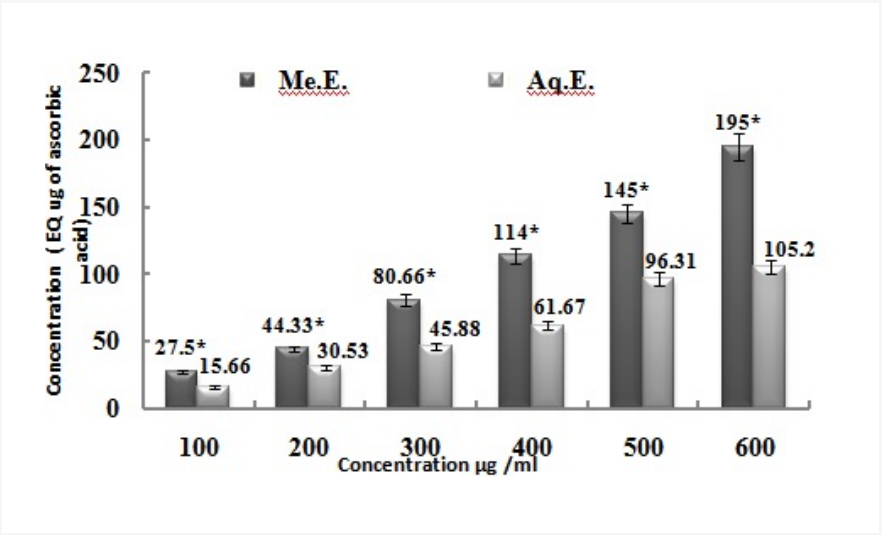

Figure 5. Total antioxidant capacity (TAC) of *Stigma maydis* extracts.

Slika 5. Skupna antioksidativna zmogljivost (TAC) ekstraktov *Stigma maydis*.

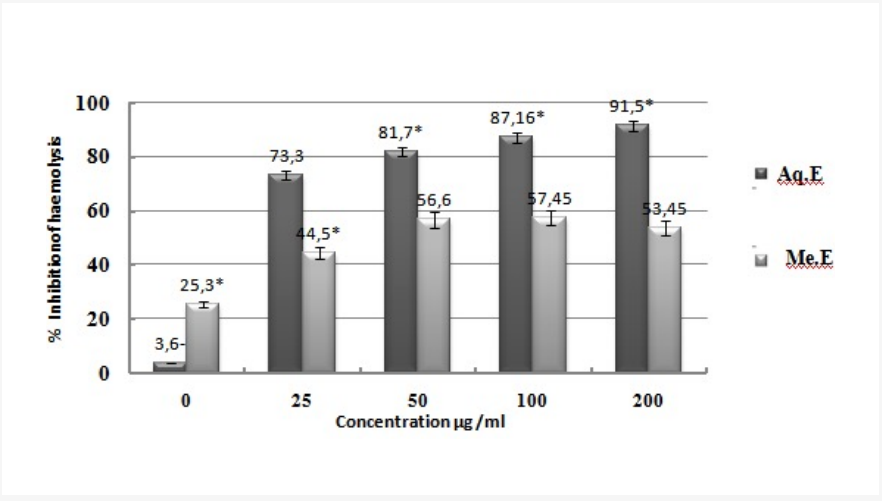

Figure 6. Inhibition Percentage (%) of haemolysis of *Stigma maydis* extracts.

Slika 6. Odstotek inhibicije (%) hemolize *Stigma maydis*.

Conclusions

The present study highlights the significant in vitro antioxidant and anti-inflammatory potential of aqueous and methanolic extracts of corn silk (*Stigma maydis*), emphasising its pharmacological relevance. Phytochemical screening confirmed the presence of bioactive compounds such as steroids, flavonoids, tannins and alkaloids, known for their biological properties.

Quantitative analysis revealed a high concentration of polyphenols and flavonoids, the main contributors to the antioxidant potential of the extracts. The evaluation of antioxidant capacityusing DPPH, TAC and FRAP assays showed a remarkable free radical scavenging activity, suggesting that the observed effects are strongly related to the chemical composition of the extracts. These finding shigh light the potential use of cornsilk as a natural antioxidant, which could serve as an alternative to synthetic antioxidants widely used in the pharmaceutical and food industries.

Furthermore, theanti-inflammatory activity assessed by the thermal denaturation inhibition method confirmed that both extracts exhibited significant inhibitory effects at a concentration of 200 µg/ml, supporting their potential therapeutic application in inflammation-related diseases. The presence of bioactive compounds with anti-inflammatory properties further strengthens the medicinal importance of *Stigma maydis*.

All in all; our findings suggest that corn silk could be a promising natural source of antioxidant and anti-inflammatory agents with potential applications in pharmacology, nutraceuticals and functional food development. However, to fully exploit its therapeutic potential, further studies, including in vivo investigations and molecular mechanism analyses, are required to validate its efficacity and safety for clinical applications.

Author Contributions

Conceptualization, A.B.andC.C.; methodology: A.B.; software: M.A.A.; validation, A.B.and S.S.; formal analysis: N.K., N.E.M.and C.C.; data curation, writing—original draft preparation: A.B.; writing—review and editing, visualization and supervision: A.B. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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ABS

Original Research

Botanical remedies for livestock: A quantitative ethnoveterinary study in the Northeastern coastal region of Odisha, India

Keshari Prasad Mohanty [1,2], Suman Patra [3], Ramya Ranjan Paul [4], Kshan Prabha Sahoo [5*]

Abstract

Compared to other coastal regions of India, research on the quantitative approach of ethnoveterinary useful plants is still scarce in the northeastern coastal region of Odisha. To fill this knowledge gap, we investigated the use value (UV), informant consensus factor (ICF), and fidelity level (FL %) of medicinal plants used for veterinary purposes. We conducted semi-structured interviews with 63 individuals to collect essential ethnoveterinary information. A total of 52 plant species belonging to 51 genera of 36 families were recorded to cure 12 different livestock aliment categories. Among all the plant parts, the indigenous community primarily uses leaves to prepare remedies instead of underground parts of plants to minimize the disturbance of the natural habitat and population of plants. In our study region, oral consumption (50%) and paste (40.38%) are the common modes of consumption and formulation, respectively. *Pongamia pinnata* (L.) Pierre was the most common medicinal plant used by the majority of indigenous communities, having a UV of 0.86. *Alocasia macrorrhizos* (L.) G. Don. and *Justicia adhatoda* L. species are frequently used by the community for treating respiratory system disorders, with an ICF of 0.96. We identify *Curcuma longa* L. as a highly medicinal value for maximum treatments (skin/surface traumatic lesion, reproductive system disorder, and respiratory system disorder) having higher FL% (83.87%). Our documentation provides precious information on ethnoveterinary medicine that helps in-situ conservation of the highly medicinal plant and conserves traditional knowledge at both the local and national levels.

Keywords

Ethnoveterinary, northeastern coastal region, India, ailments category, use value, informant consensus factor, fidelity level

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Botanična zdravila za živino: Kvantitativna etnoveterinarska študija v severovzhodni obalni regiji Odisha, Indija

Izvleček

V primerjavi z drugimi obalnimi regijami Indije so raziskave o kvantitativnem pristopu k etnoveterinarskim uporabnim rastlinam v severovzhodni obalni regiji Odisha še vedno redke. Da bi zapolnili to vrzel v znanju, smo tu raziskali uporabno vrednost (UV), faktor soglasja informatorjev (ICF) in stopnjo zvestobe (FL %) zdravilnih rastlin, ki se uporabljajo v veterinarske namene. Opravili smo polstrukturirane intervjuje s 63 posamezniki, da bi zbrali bistvene etnoveterinarske informacije. Skupaj je bilo zabeleženih 52 rastlinskih vrst, ki pripadajo 51 rodovom iz 36 družin, s katerimi se zdravi 12 različnih kategorij prehrane za živino. Med vsemi rastlinskimi deli avtohtona skupnost za pripravo zdravil uporablja predvsem liste namesto podzemnih delov rastlin, da bi čim manj posegala v naravni habitat in populacijo rastlin. V naši študijski regiji sta najpogostejša načina uživanja (50 %) in pripravljanja (40,38 %) oralno uživanje oziroma pripravljanje paste. *Pongamia pinnata* je bila najpogostejša zdravilna rastlina, ki jo je uporabljala večina avtohtonih skupnosti, z UV 0,86. Vrsti *Alocasia macrorrhizos* in *Justicia adhatoda* skupnost pogosto uporablja za zdravljenje motenj dihalnega sistema, pri čemer je ICF 0,96. *Curcuma longa* smo opredelili kot visoko zdravilno vrednost za največje število zdravljenj (zdravljenje ran, reproduktivne motnje in motnje dihalnega sistema), saj ima višji FL % (83,87 %). Naša dokumentacija zagotavlja dragocene informacije o etnoveterinarni medicini, ki pomagajo pri ohranjanju visoko zdravilnih rastlin in situ ter ohranjanju tradicionalnega znanja na lokalni in nacionalni ravni.

Ključne besede

Etnoveterina, severovzhodna obalna regija, Indija, kategorija bolezni, uporabna vrednost, faktor soglasja informatorjev, stopnja zvestobe.

Introduction

Ethnobotany, a subfield of ethnobiology, explores the intricate relationship between humans and plants, focusing on their use, management, and cultural significance (Jan et al., 2017). In recent years, there has been increasing emphasis on the sustainable utilization and governance of economically significant plant species due to their role in fulfilling human needs, supporting household economies, ensuring food security, and providing medicinal treatments (Akhtar et al. 2018). Indigenous communities possess a wealth of knowledge about natural resources, which has significantly contributed to the discovery of modern allopathic medicines (Sajem and Gosai 2006; Uniyal et al. 2006; Kirtikar and Basu 1918; Pradhan and Badola 2008). This traditional knowledge encompasses cognitive skills, social interactions, cultural values, and resource management practices (Gosal et al. 2019; Hassan et al. 2023).

In developing countries, approximately 80% of the population depends on plants for medicinal purposes (Ekor 2014), highlighting their role in enhancing healthcare access, addressing food insecurity, and alleviating unemployment (Olofsson 2021). India, with its rich biodiversity and cultural heritage, has a deep-rooted tradition of plant-based medicine. The widespread use of herbal remedies is attributed to their affordability, accessibility, safety, and cultural acceptance (Jan et al., 2022; Sureshkumar et al., 2017). With an estimated 2,500 medicinal plant species utilized in traditional healing systems, India has a long history of herbal therapy and indigenous medicine (Sheng-Ji 2001).

Closely linked to ethnobotany is the field of ethnoveterinary medicine, which focuses on traditional veterinary practices involving the use of plants and other natural substances for treating animal ailments. Communities worldwide have long relied on indigenous plant-based remedies to manage livestock health, addressing issues such as infections and injuries. This knowledge remains particularly relevant in regions where access to modern veterinary care is limited.

India, with a livestock population of approximately 536.76 million in 2019, plays a vital role in global milk production and animal husbandry. The country has witnessed significant growth in cattle, buffalo, sheep, mithun, and goat

populations, with increases of 1.34%, 1.06%, 14.12%, 30.00%, and 10.14%, respectively, since 2012 (MFAHD 2021). About 65% of India's population depends on agriculture and livestock (Meen et al., 2020). Traditional ethnoveterinary practices offer cost-effective and accessible healthcare solutions, particularly for resource-limited farmers. Despite their significance, these practices are often overlooked in favour of modern veterinary methods (Meen et al. 2020).

In rural India, plant-based therapies remain a viable alternative for treating livestock ailments. Community elders often share their knowledge of herbal medicine either freely or for a nominal fee. However, the transmission of this knowledge faces challenges, including limited interaction between generations and a declining interest among younger individuals. The lack of adequate investment in preserving traditional expertise raises concerns about its potential loss (Heera et al., 2023).

In Odisha, an agro-dependent state, most of the population relies heavily on livestock, particularly cattle, for economic and social well-being. Despite the availability of veterinary facilities, many pastoralists and farmers continue to prefer traditional remedies due to factors such as high costs, adverse effects of modern medicine, social norms, and limited access to essential pharmaceuticals.

Although some ethnoveterinary research has been conducted in Odisha (Mohapatra et al. 2020; Lenka et al. 2018; Panda et al. 2017; Sahu and Sahu 2017; Sen and Behera 2016; Panda and Mishra 2016; Rautrey et al. 2015; Panda and Dhal 2014; Mishra 2013; Mallik et al. 2012; Mishra 2011; Satpathy 2010), specific studies on the Balasore district remain scarce. While previous ethnoveterinary studies in Odisha provide well-documented accounts of traditional knowledge, they primarily focus on qualitative descriptions. These studies lack a robust quantitative approach to assess the frequency, reliability, and selection criteria of medicinal plants used in veterinary healthcare. This study addresses that gap by employing quantitative analytical methods to evaluate ethnoveterinary practices in the Balasore district.

Materials and Methods

Study area

The study was carried out in the Balasore district, which is the northeastern coastal region of Odisha and spans 3634 square kilometres. The region is situated within a Latitude of 20.48° to 21.59° N and a Longitude of 86.16° to 87.29° E (Figure 1). According to the 2011 census, the district has a total population of 2,320,529 and a literacy rate of 79.79% (Census 2011). This region is characterized by diverse and dense flora. The district's forested areas include the Kuldiha Wildlife Sanctuary, Nilgiri Hills, Panchalingeswar Hills, and Dhobisila Hills, among others. Our study area consisted of 4 blocks and 19 villages i.e. Nilgiri (Garadihi, Bhagasahi, Tihudiabada, Sapabania, Naranpur, Jamudiha, Kanthiakatikar, Guhalia, Sindhua), Jaleswar (Raibania, Bahargarh, Pinchabani, Dudhiakhali), Basta (Patharjhara, Pegarpada, Baharda) and Baliapal (Jamunasul, Dagara, Jagei) (Figure 1). The population in these areas possess a deep understanding of forest flora and its sustainable utilization, especially its therapeutic properties. Animal husbandry in this region is a crucial component of the local economy and the livelihoods of its population. The region engages in various kinds of animal husbandry, comprising cattle, buffalo, goats, sheep, pigs and poultry. These operations provide essential nutritional resources such as milk, meat, and eggs but also contribute to the economic stability of several rural families.

Demographic features of informants

A total of 63 individuals were questioned, consisting of 39 males and 24 females. The informants' ages ranged from 26 to 73 years (Table 1). The primary informants were chosen based on their expertise in traditional plant-based medicine for treating various ailments. Following this, snowball methods applied, i.e. a referral-based technique where initial participants recruit others from their network, who may not be easily accessible through random sampling, were employed to identify the potential informant (Jan et al. 2022). To verify the authenticity of ethnic knowledge, a consistent connection was kept with the local community during the study. The majority of chosen informants were unable to read or write, while only a few of them had received education up to the graduation level. Barely any of them were employed. All of the informants chosen had different religions, i.e., Hindu, Christian, and Islamic. Hinduism is the predominant religion in the region. Odia and Santali are the primary languages used for interaction.

Data Collection

The study utilized informants to discuss indigenous medicinal herbs used for treating several veterinary

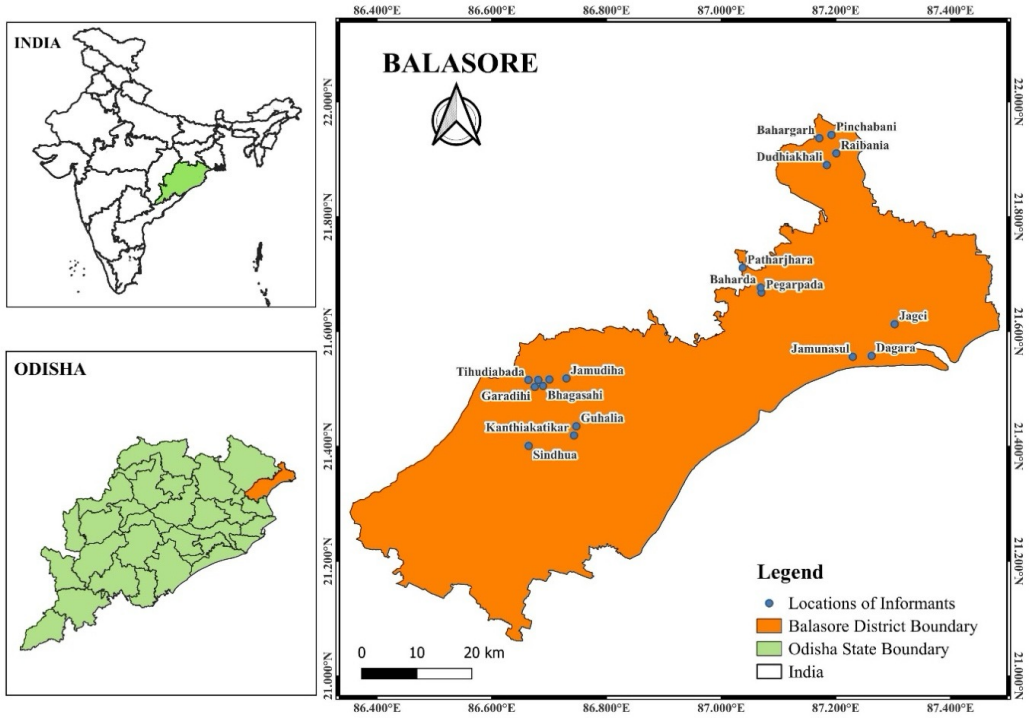

Figure 1. Study map in the northeastern coastal region of Odisha.

Slika 1. Zemljevid študije v severovzhodni obalni regiji Odisha.

Table 1. Demographic details of respondents.

Tabela 1. Demografski podatki anketirancev.

Variable	Category	Total	Percentage
Gender	Male	39	61.90
	Female	24	38.10
Age Group	26-35	13	20.63
	36-45	17	26.98
	46-55	12	19.05
	56-65	14	22.22
	66-73	7	11.11
Qualification	Illiterate	9	14.29
	Primary	7	11.11
	Secondary	29	46.03
	Higher Secondary	12	19.05
	Graduation	6	9.52
Occupation	Farmer	41	65.08
	Business	11	17.46
	Employed	3	4.76
	Housewives	8	12.70

diseases, using semi-structured interviews and standard questionnaires with group discussion (Martin 1995). The study was conducted from April 2022 to March 2023. The study ensured that all participants were fully briefed on its objectives. They granted permission to disclose their expertise, including the aetiology and manifestations of the illness, local name, techniques for preparing herbal remedies and methods of treatment. The data was recorded in the local language and later transcribed into the English language. The survey included two methodologies: In the first method, informants were presented with plant materials to confirm their medicinal properties or the name of the plant they had used as medicine previously, while the second method involved organized excursions to the forest region to locate and use significant medicinal plant species (Martin 1995; Maundu 1995) (Figure 2). Multiple interviews were undertaken to ensure and maintain uniformity. Medicinal plants were collected under the guidance of local experts and tagged with common names. Collected specimens were identified with the help of available literature and flora (Saxena and Brahmam 1994; Saxena and Brahmam 1995; Saxena and Brahmam 1996) and deposited in the Department of Botany, North Orissa University, Baripada herbarium. The revised classification of the plant was updated using the online database Plants of The World Online (https://powo.science.kew.org).

Ailment categories

Based on the information obtained from the Indigenous community of the study area, 38 ailments were grouped into 12 categories as represented (Table 2). The classification into 12 ailment categories was based on traditional ethnoveterinary practices, clinical manifestations, affected organ systems, etiological factors (infectious or non-infectious), and observable clinical symptoms commonly recognized by local veterinary healers and livestock owners during ethnoveterinary surveys.

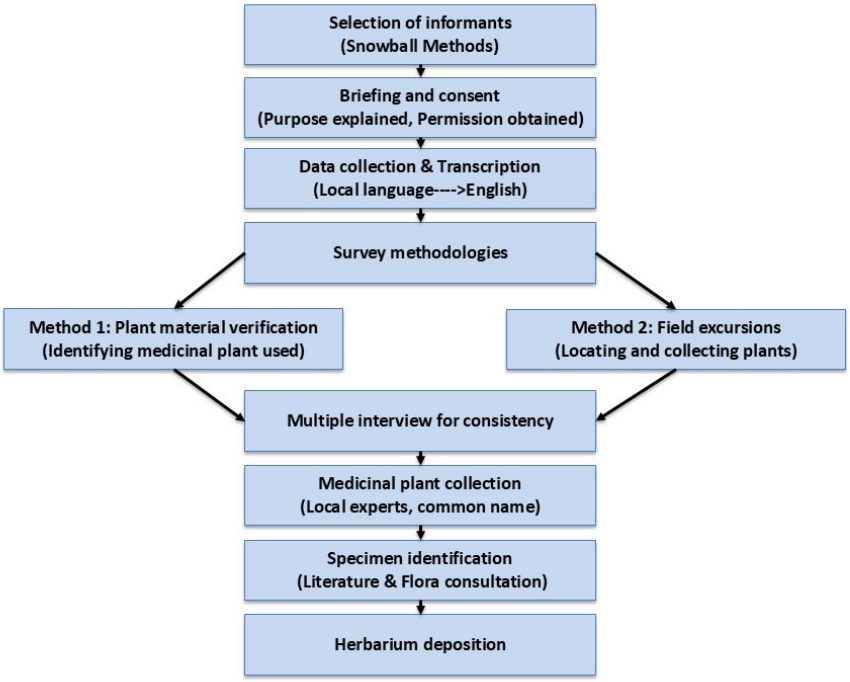

Figure 2. Flowchart showing the data collection process.

Slika 2. Diagram poteka, ki prikazuje postopek zbiranja podatkov.

Table 2. The category of ailments was found in livestock from the study area.

Tabela 2. Kategorije bolezni, ugotovljene pri živini na območju študije.

Ailments categories	Diseases/Clinical Challenges
Respiratory system disorder	URTD- cough, cold LRTD- bronchitis (non-infectious), pneumonia (infectious)
Digestive system disorder	dysentery, diarrhoea, indigestion, gas formation in the stomach
Worm infestation	roundworm, tapeworm
Reproductive system disorder	retention of placenta, vaginal ostium, uterus cleaning, vaginal bleeding, labour pain
Urinary tract disorder	bacterial infection, blood discharge during urination
Infectious disease (Systemic and contagious conditions)	hemorrhagic septicemia, fever, septicemia
Lactation insufficiency	increase lactation period, milk production
Skin/surface traumatic lesions	damage horn, wound healing, injury, crack
Dermatological disorder	Fungal infection- scaly lesions, discolouration, persistent skin damage Non-infectious conditions- skin inflammation, allergic reactions, environmental dermatitis. Infectious condition- foot rot affecting hooves Cooling Therapies: treatments for heat stress and inflammation.
Ectoparasitic condition	eradication of mosquitoes, repellent of ticks, mites, lice, filariasis
Bone fracture	joining of a fractured bone
Oral disorder	foot and mouth disease (local name fatua)

*URTD- Upper Respiratory Tract disorder, LRTD- Lower Respiratory Tract disorder

Data analysis

Use value (UV): This index aims to evaluate the significance of each medicinal plant species used by the indigenous community. The UV of a medicinal plant species increases when there are multiple of its uses. In the present investigation, UV has been determined by using the following by Phillips and Gentry (1993).

Informant consensus factor (ICF): It is a metric employed to assess the degree of consensus or similarity among participants in a study area about the utilization of plants for certain diseases. If the ICF value for a specific disease group is maximum, it indicates that the population is very selective in choosing plants for treatment or that there is a strong interchange of knowledge regarding the usage of plants within the informants. Conversely, a low value signifies a lack of consensus among the informants about the use of a specific plant species. Additionally, it highlights the broadly approachable feature of alternative allopathic medicines. The permissible range of ICF values is between 0 and 1 (Canales et al. 2005; Heinrich et al. 1998). In the present investigation, ICF was determined using Heinrich et al. (1998).

Fidelity level (FL): The Fidelity level is a measure of respondent's acceptance of certain plant species for treating specific medical conditions, which is crucial in selecting the most effective plant species (Musa et al. 2011). In the present investigation, FL was determined using the Friedman et al. (1986) formula.

Results

Diversity of ethnoveterinary plants

A total of 52 species from 51 genera across 36 families in Odisha's northeastern coastal region, including 19 trees, 16 herbs, nine shrubs, five climbers, two parasites, and one aquatic plant, are useful for treating 12 ailments (Figure 3, Table 3). Fabaceae and Poaceae exhibited co-dominance with 5 and 3 species, respectively, while Acanthaceae, Amaryllidaceae, Apocynaceae, Asteraceae, Convolvulaceae, Cucurbitaceae, Euphorbiaceae, Moraceae, Myrtaceae and Vitaceae were represented by two species.

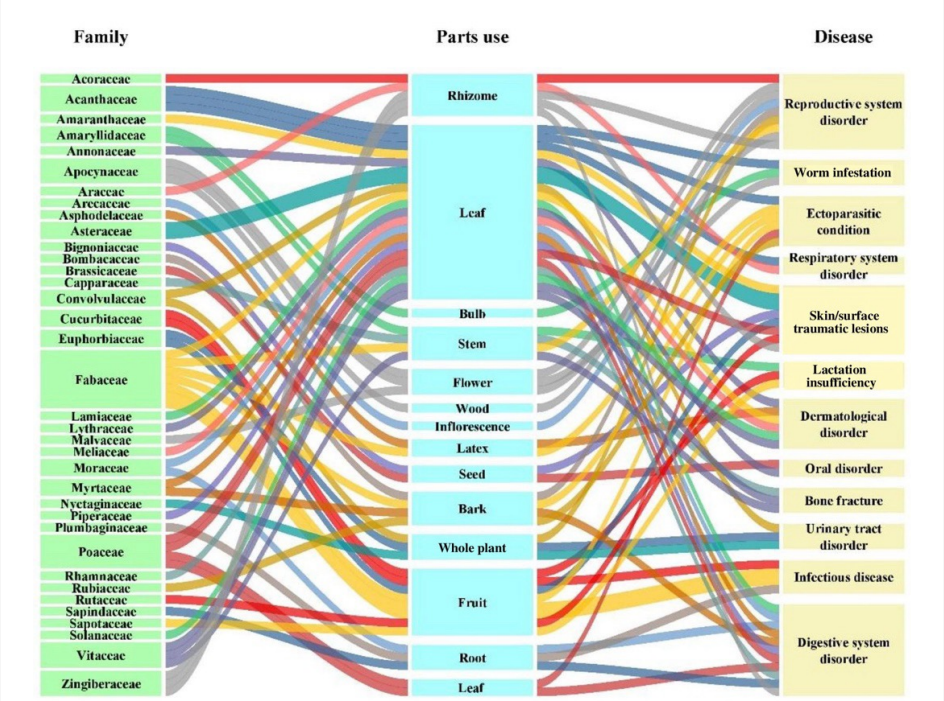

Figure 3. The alluvial diagram illustrates the relationship between different plant families and parts of the plants used to treat the ailments by the indigenous community in the northeastern coastal region of Odisha.

Slika 3. Aluvialni diagram prikazuje povezavo med različnimi rastlinskimi družinami in deli rastlin, ki jih avtohtona skupnost v severovzhodni obalni regiji Odisha uporablja za zdravljenje bolezni.

Method of preparation

The current study found that leaves (38.5%) were the most used plant parts for ethnoveterinary purposes, either alone or in combination with other parts, followed by fruit (13.46%) (Figure 4). Multiple medicinal remedies were utilized for treating various ailments (Figure 5). The predominant mode of preparation was paste, mostly made up of leaves and root, making up 40.38%, followed by direct feeding (15.38%), decoction (15.38%), and raw as well as boiled juice (13.46%) (Figure 6). Oral consumption (50%) was the common mode of consumption, followed by dermal consumption (46.15%), both oral and dermal (3.85%).

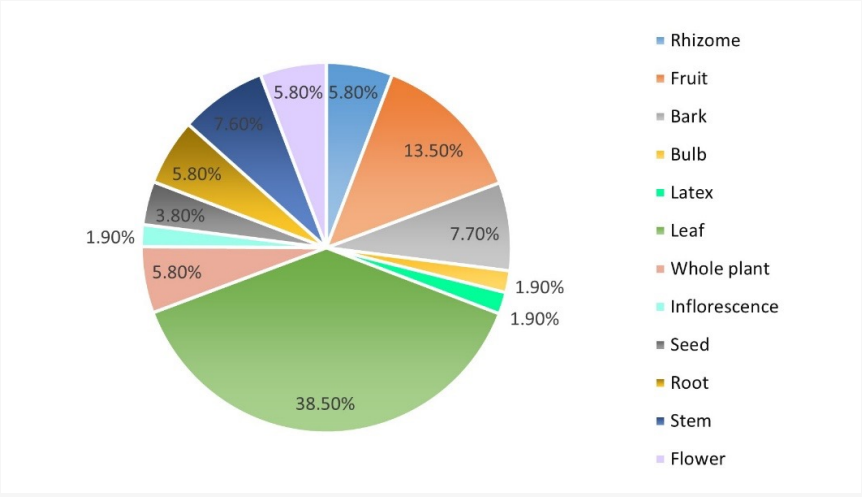

Figure 4. Pie-chart representing percentages of plant parts used for ethnoveterinary purposes in north eastern coastal region of Odisha.

Slika 4. Krožni diagram, ki prikazuje odstotke delov rastlin, ki se uporabljajo v etnoveterinarske namene v severovzhodni obalni regiji Odisha.

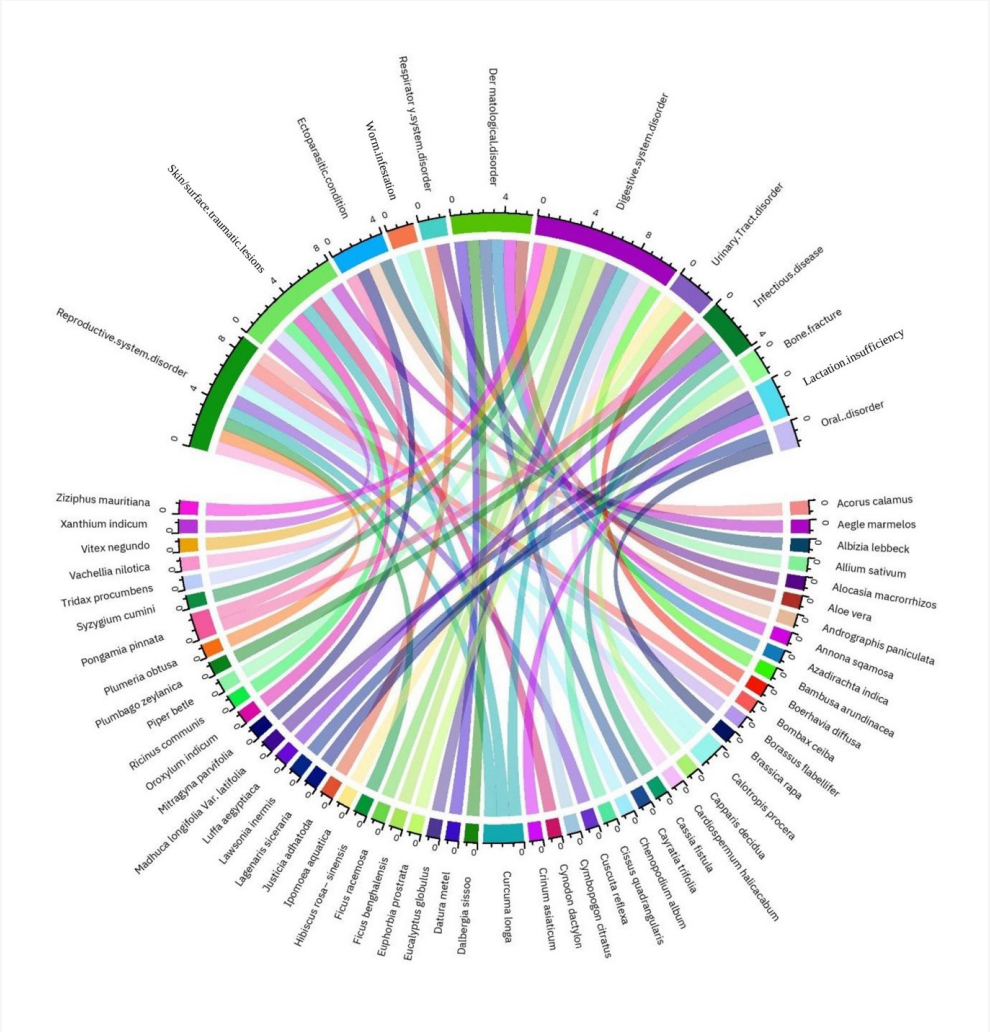

Figure 5. The chord diagram represents the indigenous community that used different plant species to treat various ailments in the northeastern coastal region of Odisha.

Slika 5. Akordni diagram predstavlja avtohtono skupnost, ki uporablja različne rastlinske vrste za zdravljenje različnih bolezni v severovzhodni obalni regiji Odisha.

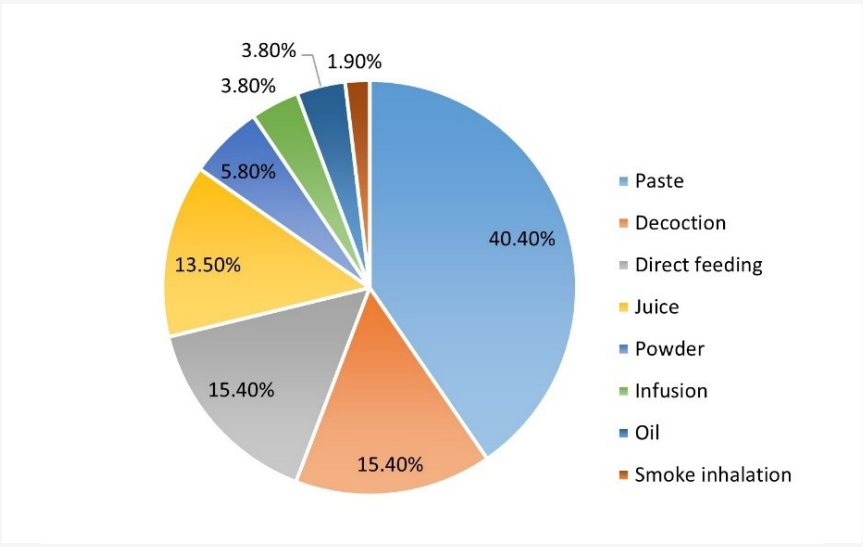

Figure 6. Pie-chart showing percentages mode of formulation in the northeastern coastal region of Odisha.

Slika 6. Krožni diagram, ki prikazuje odstotke, način formulacije v severovzhodni obalni regiji Odisha.

Use Value (UV)

The UV index was estimated through citation analysis, enabling the assessment of the significance of medicinal plant species in designated areas. The value varied from 0.08 to 0.86 (Table 3). The result highlights the ethnobotanical significance of various plant species, with notable trends observed in both the number of uses reported (UR) and the corresponding use value (UV). Plants with the highest use values, such as *Pongamia pinnata* (0.86), *Ricinus communis* (0.83), and *Tridax procumbens* (0.81), are of considerable importance, suggesting their widespread use and versatility in traditional practices. Additionally, plants like *Curcuma longa* (0.81), *Azadirachta indica* (0.75), and *Syzygium cumini* (UV = 0.75) exhibit both highly reported uses and high UV, reinforcing their multifaceted roles in local medicinal and cultural contexts. Moreover, several species, including *Chenopodium album* (0.73), *Hibiscus rosa-sinensis* (0.73), and *Ficus benghalensis* (0.73), show a balanced representation of moderate use values and number of uses, indicating their consistent reliance on traditional knowledge systems. On the other hand, some plants with lower UVs, such as *Acorus calamus* (0.10), *Cymbopogon citratus* (0.14), and *Capparis decidua* (0.13), although reported in various uses, display niche or specialized applications rather than widespread utility. Notably, plants with high medicinal value, such as *Andrographis paniculata* (0.67) and *Azadirachta indica* (0.75), further emphasize the importance of these plants for modern pharmaceutical research.

Table 3. Inventory of plant species utilized by indigenous inhabitants of Balasore district, Odisha, India.

Tabela 3. Seznam rastlinskih vrst, ki jih uporabljajo avtohtoni prebivalci okrožja Balasore, Odisha, Indija.

Botanical Name		Habit	Vernacular Name (Odia)	Part used	Mode of Consumption	Disease Category	Mode of Application	No. of use reported (UR)	Use Value (UV)
Andrographis paniculata (Burm.f.) Wall. ex Nees	Acanthaceae	Herb	Bhuinimbo, Chireita	Leaf	D	DW, EC	Decoction	42	0.67
Acorus calamus L.	Acoraceae	Herb	Bacha	Rhizome	O	RD	Paste	6	0.10
Justicia adhatoda L.		Shrub	Basang	Leaf	O	RSD	Paste	38	0.60
Chenopodium album L.	Amaranthaceae	Herb	Bathua	Leaf	D	SL	Decoction	46	0.73
Allium sativum L.	Amaryllidaceae	Herb	Rasuna	Bulb	O	DW	Paste	37	0.59
Crinum asiaticum L.		Herb	Panikenduli, Arisa	Stem	O	LE	Decoction	18	0.29
Annona sqamosa L.	Annonaceae	Tree	Ata, Neua	Leaf	D	DD	Paste	15	0.24
Calotropis procera (Aiton) W.T. Aiton	Apocynaceae	Shrub	Arakha	Flower, Wood	D	DW, RD	Powder	17	0.27
Plumeria obtusa L.		Shrub	Katha champa	Flower	O	RD	Direct feeding	13	0.21
Alocasia macrorrhizos (L.) G. Don.	Araceae	Herb	Mana saru, Mantara saru	Rhizome	O	RSD	Decoction	19	0.30
Borassus flabellifer L.	Arecaceae	Tree	Tala, Talo	Inflorescence	O	RD	Powder	17	0.27
Aloe vera (L.) Burm.f.	Asphodelaceae	Herb	Ghi kuanari	Latex	D	DD	Decoction	23	0.37
Tridax procumbens L.	Asteraceae	Herb	Bisalya Karani	Leaf	D	SL	Juice	51	0.81
Xanthium indicum Koenig		Herb	Mendha godia	Leaf	D	SL	Paste	17	0.27
Oroxylum indicum (L.) Vent.	Bignoniaceae	Tree	Phanphania, Pependi	Seed	D	SL	Paste	5	0.08
Bombax ceiba L.	Bombacaceae	Tree	Simili, Semulo	Bark	D	RD	Paste	28	0.44
Brassica rapa L.	Brassicaceae	Herb	Sorisa	Seed	O	OD	Paste	33	0.52

Capparis decidua (Forssk.) Edgew.	Capparaceae	Tree	Karira	Stem	D	BF	Paste	8	0.13
Cuscuta reflexa Roxb.	Convolvulaceae	Parasitic	Nirmuli	Whole Plant	D	RD	Powder	14	0.22
Ipomoea aquatica Forssk.		Aquatic	Kalam saga, Titi saga	Leaf	O	UTD	Direct feeding	37	0.59
Lagenaris siceraria (Molina) Standl.	Cucurbitaceae	Climber	Lau	Fruit	O	LE	Decoction	17	0.27
Luffa aegyptiaca Mill.		Climber	Pita Taradi, Tarada	Fruit	D	ID	Smoke Inhalation	36	0.57
Euphorbia prostrata Aiton	Euphorbiaceae	Parasitic	Rangotoli	Whole plant	O	UTD	Decoction	32	0.51
Ricinus communis L.		Shrub	Jada, Gaba	Fruit	D	SL	Oil	52	0.83
Albizia lebbeck (L.) Benth.	Fabaceae	Tree	Sirisi, Tinia	Bark, Latex	D	EC	Juice	16	0.25
Cassia fistula L.		Tree	Sunari, Argavada	Fruit	D	ID	Infusion	33	0.52
Dalbergia sissoo Roxb. Ex DC.		Tree	Sisu, Simsapa	Leaf	D	DD	Paste	27	0.43
Pongamia pinnata (L.) Pierre		Tree	Karanja	Fruit	D	ID, EC	Oil	54	0.86
Vachellia nilotica (L.) P.J.H. Hurter & Mabb.		Tree	Baburi, Bambur	Stem	O	RD	Infusion	29	0.46
Vitex negundo L.	Lamiaceae	Shrub	Begunia, Nirgundi	Leaf	D	DSD	Juice	36	0.57
Lawsonia inermis L.	Lythraceae	Shrub	Menjuati, Mahendi, Manghati	Leaf	D	OD	Paste	36	0.57
Hibiscus rosa-sinensis L.	Malvaceae	Shrub	Mandara	Flower	O	RD	Direct feeding	46	0.73
Azadirachta indica A. Juss.	Meliaceae	Tree	Nima, Limba	Leaf	D	DD	Paste	47	0.75
Ficus benghalensis L.	Moraceae	Tree	Bara, Bata	Prop root	O	DSD	Paste	46	0.73
Ficus racemosa L.		Tree	Dimiri	Leaf	O	DSD	Direct feeding	41	0.65
Eucalyptus globulus Labill.	Myrtaceae	Tree	Eucalipatas	Leaf	O	DSD	Direct feeding	18	0.29
Syzygium cumini (L.) Skeels		Tree	Jamu, Jamkoli	Bark	O	DSD	Juice	47	0.75
Boerhavia diffusa L.	Nyctaginaceae	Herb	Puruni, Kechna, Kharkharia	Whole Plant	O	UTD	Decoction	16	0.25
Piper betle L.	Piperaceae	Climber	Pana	Leaf	O	DSD	Paste	31	0.49
Plumbago zeylanica L.	Plumbaginaceae	Shrub	Sweta chitapa-ru, Chintamani, Ogni	Root	O	ID	Paste	36	0.57
Bambusa arundinacea Willd.	Poaceae	Herb	Baunsa	Leaf	O	DSD	Direct feeding	24	0.38
Cymbopogon citratus (DC.) Stapf		Herb	Dhanwantari	Leaf	O, D	DSD, EC	Juice	9	0.14
Cynodon dactylon (L.) Pers.		Herb	Duba	Leaf	D	SL	Paste	45	0.71
Ziziphus mauritiana Lam.	Rhamnaceae	Tree	Barakoli, Badori, Boyer	Leaf	O	DSD	Paste	38	0.60

Mitragyna parvifolia (Roxb.) Korth.	Rubiaceae	Tree	Mundikama, Kalakadamba, Mitikunia	Bark	O	EC	Juice	18	0.29
Aegle marmelos (L.) Corr.	Rutaceae	Tree	Bela	Fruit	O	SL	Direct feeding	31	0.49
Cardiospermum halicacabum L.	Sapindaceae	Herb	Phut Phutika, Kanphuta	Root	O	DSD	Paste	29	0.46
Madhuca longifolia var. *latifolia* (Roxb.) A. Chev.	Sapotaceae	Tree	Mahula, Madgi, Mahua	Fruit	O	LE	Direct feeding	44	0.70
Datura metel L.	Solanaceae	Shrub	Durdura	Leaf	D	DD	Juice	37	0.59
Cayratia trifolia L.	Vitaceae	Climber	Nata nirimisi, Ambiliti	Leaf	D	DD	Paste	12	0.19
Cissus quadrangularis L.		Climber	Hadavanga, Hadasinkusa	Stem, Leaf	D	BF	Paste	32	0.51
Curcuma longa L.	Zingiberaceae	Herb	Haladi	Rhizome	O, D	SL, RD, RSD, DSD	Paste	51	0.81

*O-oral, D-dermal, RSD- respiratory system disorder, DSD- digestive system disorder, DW- worm infestation, RD- reproductive system disorder, UTD-urinary tract disorder, ID- infectious disease, LE- lactation insufficiency, SL - skin/surface traumatic lesions, DD- dermatological disorder, EC- ectoparasitic condition, BF-bone fracture, OD- oral disorder, UR (use report) and UV (use value). The highlighted text refers to species reported with fewer than five citations from the study area.

Informant consensus factor (ICF)

The ethnoveterinary analysis identified 12 disease categories, with ICF values between 0.71 and 0.96, reflecting differing levels of consensus among informants (Table 4). The highest level of consensus was found for respiratory system disorders (0.96), subsequently followed by oral disorders (0.94), infectious diseases, and lactation insufficiency (both 0.93). The categories exhibited a lower number of taxa; however, they suggested a high rate of use reports, indicating reliable traditional knowledge. Dermatological disorders exhibited the highest number of usage reports (46), though the ICF was slightly lower at 0.89. Bone fractures (0.88) and skin/surface traumatic lesions (0.83) showed significant agreement. Lower ICF values were observed in ectoparasitic conditions (0.77), reproductive disorders (0.74), digestive disorders (0.71), and worm infestations (0.71), suggesting a requirement for more diverse treatment approaches.

Table 4. Informant consensus factor (ICF) of various medicinal plants.

Tabela 4. Faktor soglasja informatorjev (ICF) za različne zdravilne rastline.

Disease Category	Nur	Nt	ICF
Respiratory system disorder	25	2	0.96
Oral disorder	19	2	0.94
Infectious disease	41	4	0.93
Lactation insufficiency	28	3	0.93
Urinary tract disorder	27	3	0.92
Dermatological disorder	46	6	0.89
Bone fracture	17	3	0.88
Skin/surface traumatic lesions	36	7	0.83
Ectoparasitic condition	23	6	0.77
Reproductive disorder	32	9	0.74
Digestive system disorder	36	11	0.71
Worm infestation	8	3	0.71

* Nur- total number of mentions for a disease category, Nt- total species used by each participant, ICF- Informant consensus factor.

Fidelity Level (FL)

This study measures the significance of 23 plant species' FL values in treating specific ailments by analyzing their preference in that area (Table 5). The FL values of these medicinal plants ranged from 31.58% to 83.87%. The analysis documented 12 disease categories, each associated with distinct Fidelity Level (FL) values, indicating the efficacy and preference of particular plants. *Curcuma longa* (83.87%) was the most preferred for digestive disorders, whereas *Madhuca longifolia* Var. *latifolia* (79.17%) and *Cissus quadrangularis* (75.00%) were significantly preferred for lactation insufficiency and bone fractures, respectively. Moderate FL values were recorded for *Tridax procumbens* (63.33%) and *Aegle marmelos* (61.11%) concerning skin injuries, *Pongamia pinnata* (68.97%) related to infectious diseases, *Piper betle* (69.23%) associated with digestive issues, and *Justicia adhatoda* (60.00%) pertaining to respiratory disorders. *Andrographis paniculata* (60.87%) was commonly utilized for ectoparasitic conditions, showing significant agreement among informants. Lower FL values for *Azadirachta indica* (48.84%) and *Aloe vera* (42.42%) in the context of dermatological disorders indicate a range of treatment options. *Lawsonia inermis* (44.44%) and *Brassica rapa* (43.48%) were noted for oral disorders with lower consensus, while *Borassus flabellifer* (33.33%) and *Hibiscus rosa-sinensis* (31.82%) were identified for reproductive disorders, indicating varied treatment strategies.

Table 5. The Fidelity level (FL %) of commonly utilized medicinal plants by local indigenous communities.

Tabela 5. Stopnja zvestobe (FL %) zdravilnih rastlin, ki jih pogosto uporabljajo lokalne avtohtone skupnosti.

Disease Category	Plant	Np	N	FL %
Bone fracture	Cissus quadrangularis L.	9	12	75.00
Worm infestation	Allium sativum L.	14	21	66.67
Digestive system disorder	Bambusa arundinacea Willd.	10	18	55.56
	Cardiospermum halicacabum L.	8	13	61.54
	Ficus benghalensis L.	11	21	52.38
	Curcuma longa L.	26	31	83.87
	Piper betle L.	9	13	69.23
	Ziziphus mauritiana Lam.	7	12	58.33
Infectious disease	Pongamia pinnata (L.) Pierre	20	29	68.97
Lactation insufficiency	Madhuca longifolia Var. latifolia (Roxb.) A.Chev.	19	24	79.17
Oral disorder	Lawsonia inermis L.	8	18	44.44
	Brassica rapa L.	10	23	43.48
Ectoparasitic condition	Andrographis paniculata (Burm.f.) Wall. ex Nees	14	23	60.87
Reproductive disorder	Borassus flabellifer L.	4	12	33.33
	Hibiscus rosa- sinensis L.	7	22	31.82
Respiratory system disorder	Justicia adhatoda L.	18	30	60.00
Dermatological disorder	Aloe vera (L.) Burm.f.	14	33	42.42
	Azadirachta indica A. Juss.	21	43	48.84
Urinary tract disorder	Ipomoea aquatic Forssk.	6	19	31.58
Skin/surface traumatic lesions	Aegle marmelos (L.) Corr.	11	18	61.11
	Chenopodium album L.	13	22	59.09
	Ricinus communis L.	16	28	57.14
	Tridax procumbens L.	19	30	63.33

* Np- number of respondents who reported using a particular plant for a specific category of disease, N- Overall number of those who reported using the plant of any other disease category, FL%- percentages of Fidelity level.

Discussion

Utilizing native plant species for veterinary purposes is a significant aspect of the Eastern Ghats mountain communities in the Odisha region. Animal husbandry has a crucial position in supporting the local economy and people's livelihoods. Local farmers, tribes and nomadic communities rely on plants for use as therapeutic herbs to treat various animal ailments. Ethnomedicines are frequently used over conventional therapies due to their easy accessibility and affordability (Tariq et al. 2014).

The present study recorded a total of 52 medicinal plant species from the Balasore district, Odisha, that are being used by native practitioners to cure livestock diseases. Similarly, different number of medicinal plants reported from different districts of Odisha by other researchers are 44 from Kendrapara (Panda et al., 2017), 56 from Koraput (Lenka et al., 2018), 46 from Ganjam and Kandhamal (Garnayak et al., 2023) and 187 from Dhenkanal (Satpathy et al., 2024). The observed differences in the number and type of plant species reported in our study compared to others can be attributed to several factors. Firstly, the characteristics of the study area play a significant role. Balasore district, with its distinct climatic, edaphic, and ecological conditions, differs considerably from other areas, influencing the composition of vegetation and the availability of medicinal plants. Additionally, methodological differences, including variations in sampling techniques, interview approaches, and informant selection, can affect the species documented in different studies. The extent of urbanization and anthropogenic influence also contributes to variations in plant diversity. For instance, Balasore, with its significant coastal influence and urban expansion, may limit access to certain medicinal plants.

Fabaceae was found to be the most dominant family in the study area and was also reported to be a dominant family in another district of Odisha, such as the Kendrapara district (Panda et al. 2017). This family was recognized as one of the most abundant families not only in the different regions of India, like the Junagarh district of Gujarat (Bhatt et al. 2019), Bhandara district of Maharastra (Gadpayale et al. 2014), etc. but also in other countries like Nepal (Uprety et al. 2022) and Ethiopia (Tilahun and Shewage 2019). The abundance refers to the high species richness of Fabaceae rather than the sheer number of individual plants, as it is one of the most species-rich families across different regions.

Besides the utility of different habits (herb, shrub and tree) for ethnoveterinary purposes, herbaceous species were reported as frequently used (Mandal and Rahaman, 2022; Uprety et al., 2022), but contradictory result was observed in the present study that showed the utility of tree species is comparatively more than shrub or herbs in the northeastern coastal region of Odisha. Cultural and ethnobotanical preferences further explain the dominance of tree species in our study, reflecting local healing traditions. This contrasts with studies such as Mandal & Rahaman (2022) and Uprety et al. (2022), which may have documented a higher number of herbaceous plants due to differences in ethnomedicinal practices. Finally, regional plant diversity and conservation status play a critical role in shaping medicinal plant utilization. Balasore's coastal and inland ecosystems support a unique flora, contributing to the regional specificity of ethnoveterinary knowledge. These factors collectively provide an explanation for the variations observed in plant species documentation, highlighting the importance of considering the regional context in ethnoveterinary studies.

The results of the present study coincide with the previous studies (Heera et al. 2023; Jan et al. 2017) that the Indigenous community primarily uses leaves for preparing remedies due to various advantages like ease of gathering in larger amounts and minimising the disturbance of natural habitat and population of plants. However, contradictory results for the frequent utilization of roots and underground components of plants (Mandal and Rahaman, 2022) are also available.

Among the different modes of consumption by livestock, oral consumption (50%) and paste (40.38%) are the common modes of consumption and formulation, respectively, which aligns with previous studies (Aruna and Shrinitha 2023; Bhat et al. 2023; Mandal and Rahaman 2022; Abebe 2022; Chakale et al. 2022).

The highest UV (0.86) was found in *Pongamia pinnata* (L.) Pierre, the major utilized species in the present study, which could be attributed to the frequent distribution of the plant (Figure 7). For economic purposes, the locals utilize this plant product in various ways, such as the crude oil used for constipation or as a moisturizer. Thus, Internationally, multiple studies have been conducted on the effective exploitation of this plant (Kebede and Shibeshi 2022; Lans et al. 2006; Verma 2014). The therapeutic properties of *Pongamia pinnata* are attributed to several important phytochemical constituents. The flavonoids, including karangin, pongamol, and glabrin, exhibit strong antimicrobial, anti-inflammatory, and antioxidant activities. The presence of

Figure 7. *Pongamia pinnata* (L.) Pierre plant and crude oil extracted from seed.

Slika 7. Rastlina *Pongamia pinnata* (L.) Pierre in surovo olje, pridobljeno iz semena.

fatty acids such as oleic acid, stearic acid, and palmitic acid contributes to skin healing and lipid metabolism regulation. Sterols like beta-sitosterol and stigma sterol are known for their immunomodulatory and cholesterol-lowering effects. Additionally, furanoflavones and pyranoflavonoids enhance antifungal and hepatoprotective activities. These bioactive compounds collectively support the plant's pharmacological mechanisms, which include antimicrobial, anti-inflammatory, antioxidant, hepatoprotective, and wound-healing properties, making it a valuable resource in traditional and modern medicine (Al Muqarrabun et al. 2013; Preethima et al. 2019) by following *P. Pinnata*, two species *T. procumbens* and *C. longa*, having a UV of 0.81, plays a crucial role in ethnoveterinary practices. *T. procumbens* contains active compounds like flavonoids, alkaloids, and tannins, which provide antimicrobial, hemostatic, and anti-inflammatory effects (Patel et al., 2011). In traditional practices, the crushed leaves are applied directly to wounds to speed up healing and prevent infection.

This study's findings provide significant insights into the use of medicinal plants by indigenous communities, emphasizing both agreement and variation in plant choices across different disease categories. There was a notable consensus in the categories of Respiratory system disorder, Oral disorder, and Infectious disease, reflected by high Informant Consensus Factor (ICF) values of 0.96, 0.94, and 0.93, respectively. This suggests a collective and good understanding of the plants utilized for these ailments. This indicates that these conditions are widely recognized within the community, with specific plants being regularly utilized for their therapeutic properties. Align with this study, a similar result was also reported by Madisha and McGaw (2023) in the respiratory system disorder category from South Africa. *Justicia adhatoda* L. and *Alocasia mycorrhizas* (L.) G. Don. were commonly used against respiratory system disorders in the present study area, which also aligns with the report of Satpathy (2010). The second highest ICF value was Oral disorder (0.94), which was cured by *B. rapa* and *L. inermis* in our study area. This ailment category aligns with the work of Traore et al. 2020, which reported Foot and mouth disease was the most reported disease in West Africa. On the other hand, reproductive disorders and urinary tract disorders exhibited lower ICF values (0.74 and 0.92), suggesting increased variability

in plant utilization, which may indicate a less uniform approach or increased individual preference in managing these ailments. Fidelity Levels (FL%) supported the findings of significant utilization and cultural importance of various plant species within the community. *Curcuma longa* L., with an FL% of 83.87, stands out for its prominent use in treating digestive system disorders, indicating its reliability as a therapeutic option passed down through generations. This high FL value aligns with its comparable Use Value (UV) and suggests its significance in ethnoveterinary practices, particularly in regions where modern veterinary care may be less accessible. Moreover, the high FL% for *C. longa* was also reported in neighbouring countries like Pakistan (Mussarat et al., 2014). Curcumin, the primary bioactive component of *C. longa*, is well-documented for its diverse pharmacological properties (Iweala et al., 2023; Srivastava et al., 2022), while other components like sesquiterpenes and polysaccharides, such as arabinogalactans, contribute to its antimicrobial and anti-inflammatory effects (Murtadlo et al., 2024; Chandrasekaran et al., 2023). However, a more detailed phytochemical study is required to pinpoint the precise bioactive components responsible for these therapeutic benefits (Tariq et al., 2014). Alongside *C. longa*, *Madhuca longifolia* (L.) demonstrated an FL% of 79.17 for lactation enhancement, making it the second most prominent species in ethnoveterinary practices. Its flowers are commonly prepared as decoctions or mixed with feed to stimulate milk production in dairy animals, a practice confirmed by Gupta et al. (2012) and Sinha et al. (2017), who also reported its frequent use to improve lactation in cows and buffaloes. In contrast, other species exhibited lower FL%, indicating either less prevalent or more variable application. For example, *Borassus flabellifer* L., with an FL% of 33.33, is used for reproductive disorders, while *Ipomoea aquatica* Forssk., with an FL% of 31.58, is used for urinary tract disorders. These species highlight the diversity in plant utilization, showing the community's adaptation to ecological availability and individual preferences.

This study demonstrates that the medicinal plants of Balasore, Odisha, possess substantial potential for utilization due to their rich diversity and adaptability to the region's tropical monsoon climate. The area's climatic conditions, characterized by high humidity, moderate to heavy rainfall (especially during the monsoon season), and mild winters, support the growth of a wide range of medicinal plant species. Additionally, the presence of both coastal and inland ecosystems enhances plant adaptability to varying moisture levels, salinity, and seasonal temperature fluctuations, further contributing to their resilience and pharmacological potential. Nevertheless, there is a lack of studies, and only a small number of plants are now being utilized for their pharmacological qualities in the veterinary sector. The sustainable utilization of biodiversity has the potential to promote the advancement in pharmaceutics, leading to economic and social benefits for the country.

Conclusions

The study aims to investigate regional biodiversity and traditional knowledge pertaining to animal health and productivity in rural communities and practitioners. A total of 52 medicinal plants belonging to 36 different families were identified for their efficacy in treating 12 specific ailments in livestock. The predominant therapies consisted of utilizing leaves as plant parts, paste as the preparation method, and oral consumption. The Fabaceae family showed the highest diversity in ethnoveterinary plants. This research is crucial for preserving medicinal plants and traditional knowledge. In future, research should encompass toxicological investigations, determination of the most favourable periods for harvesting, and assessments of therapeutic efficacy. It is essential to provide education to rural people about traditional ethnoveterinary methods in order to protect and maintain natural plant life.

Author Contributions

Conceptualization, K.P.M. and S.P.; methodology, K.P.M. and S.P. ; software, S.P and R.R.P.; formal analysis, S.P and R.R.P.; investigation, K.P.M. and S.P.; writing—original draft preparation, K.P.M. and S.P.; writing—review and editing, K.P.S; supervision, K.P.S. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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ABS

Review

A Review on Uses of Ethno-Medicinal Plants for Treatment of Whooping Cough: A Highly Contagious Respiratory Tract Infection

Tanujaya Taran [1,2], Alok Bharadwaj [2*], Tapan Majumdar [3]

Abstract

Phytotherapeutic medicinal plants are an important part of the world's widely distributed vegetation. One well-known technique for identifying the main constituents that can result in the development of trustworthy therapeutic agents is the evaluation of pharmacokinetics from the plant's resources. Since ancient times, ethnomedicine has been documented as being used in India's traditional medical systems. Therefore, this thorough review aims to look into the worldwide documentation of ethnomedical uses of various plant part extracts against whooping coughs, as well as the taxonomic distribution of medicinal plant species, methods for preparing remedies, and documented reports from various Indian states. Throughout this review, a total of 89 plant species are recorded from 41 families and 70 genera. This review also documented that *Saussurea sp., Solanum sp.,* and *Cassia sp.* are the superior plant species that are applied to treat whooping cough disease in different states of India. Asteraceae is also the superior family, under which most ethnomedicinal plants are applied to treat whooping cough. This document outlines the traditional treatments for colds and whooping cough in various Indian provinces. According to the review, India has the greatest amount of ethnomedical evidence supporting using plant extracts as a therapy for whooping cough. This is why it is important to assess and document these valuable plant parts as well as the processing techniques of ethnomedicine plants so that the focus can be on their preservation and sustainability.

Keywords

Therapeutic agents; Plant resources; Plant extracts; Medicinal plants; Whooping cough

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Pregled uporabe etnomedicinskih rastlin za zdravljenje oslovskega kašlja: Visoko nalezljiva okužba dihalnih poti

Izvleček

Fitoterapevtske zdravilne rastline so pomemben del svetovno razširjene vegetacije. Ena od znanih tehnik za prepoznavanje glavnih sestavin, ki lahko privedejo do razvoja zaupanja vrednih terapevtskih sredstev, je vrednotenje farmakokinetike iz rastlinskih virov. Že dolgo je znano, da se etnomedicina uporablja v indijskih tradicionalnih medicinskih sistemih, zaradi česar je cilj tega pregleda preučiti: svetovno dokumentacijo o etnomedicinski uporabi izvlečkov različnih rastlinskih delov proti oslovskemu kašlju; taksonomsko razporeditev zdravilnih rastlinskih vrst; metod za pripravo zdravil in dokumentiranih poročil iz različnih indijskih držav. V tem pregledu je zabeleženih 89 rastlinskih vrst iz 41 družin in 70 rodov. V tem preglednem delu je tudi dokumentirano, da so vrste *Saussurea sp., Solanum sp.* In *Cassia sp.* najprimernejše rastlinske vrste, ki se uporabljajo za zdravljenje bolezni oslovskega kašlja v različnih državah Indije. Asteraceae je tudi najpomembnejša družina, v okviru katere najdemo večino etnomedicinskih rastlin uporabljanih za zdravljenje oslovskega kašlja. V tem pregledu so opisani tradicionalni načini zdravljenja prehlada in oslovskega kašlja v različnih indijskih provincah. Glede na pregled ima Indija največ etnomedicinskih dokazov, ki podpirajo uporabo rastlinskih izvlečkov kot terapijo za oslovski kašelj. Zato je pomembno oceniti in dokumentirati te dragocene rastlinske dele ter tehnike predelave etnomedicinskih rastlin, da bi se lahko osredotočili na njihovo ohranjanje in trajnost.

Ključne besede

terapevtske snovi, rastlinski viri, rastlinski izvlečki, zdravilne rastline, oslovski kašelj

Introduction

Around the world, pulmonary disease is common and considered to be a serious cause of death and illness. The common cold, cough, and whooping cough are the most familiar problems of the respiratory system (Pal & Bareth, 2023). By how long it lasts, coughing is one of the most common signs of a cold and is classified as acute, subacute, or chronic. When coughing constantly lasts for more than three weeks, this type of respiratory infection belongs to post-infectious cough (Zhao et al., 2018). Acute respiratory infections caused by *Bordetella pertussis*, which can cause the illness of whooping cough, are caused by a Gram-negative *Coccobacillus*, aerobic, motile phylum Pseudomonadota and the genus *Bordetella*. The genus *Bordetella* includes *B. pertussis, B. parapertussis*, and *B. bronchiseptica*, and by releasing various toxins like pertussis toxin, adenylate cyclase toxin, filamentous hemagglutinin, pertactin, fimbria, and tracheal cytotoxin, *Bordetella* can cause serious illness in the host (Smith et al., 2001). By ciliary adherence, the bacteria attach to the upper airway epithelium and release a multi-subunit (AB5) protein toxin to do host-pathogen interaction (Madsen, 1925). The contagious disease pertussis includes symptoms like a runny nose, a low-grade fever, nasal congestion, red, watery eyes, cough, and sometimes being familiar with the common cold. Infected people with severe coughs breathe sounds similar to "whoop". In early childhood, various difficulties are found, like life-threatening breathing difficulties, cyanosis, pneumonia, seizures, dehydration, and in severe cases, sometimes they become life-threatening also (Murthy, 2018). Harshberger first used the term ethnobotany in the1890s. This branch of ethnobiology, under the umbrella of ethnobotany, is concerned with providing all the information needed about the different plants, their medicinal uses and how they can be used to make sure that people can fulfil their basic needs, such as those for food, shelter, and clothing, people utilize wild plants in diverse ways (Rahman et al., 2019).

Herbs and shrubs are key components of human life. They contain vegetative prosperity that is needed for metabolic activities because they have a divergent range of biochemical compounds. This large network of traditional Indian medicine is supported by an enormous storehouse of herbs that are used for the treatment of diseases. Studies on therapeutic plants conducted by ethnobotanists are of utmost relevance, especially in severe climates like cold

desert regions where the contemporary medical system has not yet fully evolved. Together with community healthcare and drug development, this indigenous system of traditional knowledge preserves ecological diversity as well as cultural diversity. The ever-expanding pharmaceutical sector is also anticipating new knowledge from ethnobotanical studies (Gupta et al., 2013). The direct interactions between people and plants are the subject of ethnobotany. A vast resource such as plants can generate a wide range of goods and chemicals that can be used by all other forms of life to sustain them. The local aboriginal communities are familiar with the traditional use of herbs as medicines. Villagers in that region use numerous plant products. The ancient population was also believed to have lived in peace with nature, which is why it is thought that they were healthier than modern populations. This native information is transmitted from generation to generation (Bhushan & Kumar, 2013). The use of traditional medicines for primary healthcare depends on about 70% of the world's population. India has been described as the botanical paradise of the world because of its abundance and diversity of medicinal plants. The value of herbal medications has increased recently, and over 95% of the plants used in these treatments are gathered from forested areas. More potent and systematic plant remedies might be discovered through the study of ethnomedicine (Bhosle, 2009). The comprehensive knowledge of plants and their medicinal applications is studied in ethnobotany. More than 85,000 of the plant species that are known to exist on earth are therapeutic. India is a mega-diversity country, making it rich in ethnomedicinal plants. For thousands of years, humans have utilized these therapeutic plants in Ayurvedic remedies (Rahul, 2013). An established method for the identification of bioactive substances that can lead to the development of novel and secure therapeutic medicines is the pharmacological examination of chemicals from plants (Ozturk and Hakeem, 2018). According to ethnopharmacological literature, India has used a variety of medicinal plant species. A growing number of people are becoming aware of the value of medicinal plants and conventional medical practices in treating the world's health care issues. All medications used to be made from plants in the past, whether they were in their purest form, as unprocessed plant matter, or they were in their refined form, as leaf extracts. According to recent estimates, thousands of plants have been used as medicines, and many of these plants have been subjected to the separation and subsequent alteration of their active chemical components (Pal & Bareth, 2023). With the use of ethnobotanical knowledge of their traditional applications, a significant part of such therapeutic substances has been discovered (Zhao et al.,2018). Traditional medicine has used various parts of the plant to treat bronchitis and whooping cough, as well as other chronic diseases (Ghumare, 2014). Plant-based medicines are thought to be safer for treating a variety of diseases. Over 85% of conventional diseases are supported by herbal medications in primary healthcare systems worldwide. Almost 65% of Indians, according to estimates from the World Health Organization, rely on traditional remedies to treat their health problems (Ekor, 2014).

Around the world, numerous diseases are treated with a variety of plant parts, including aerial parts, bark, buds, bulbs, cloves, corms, fruits, leaves, rhizomes, roots, stem bark, tubers, the entire plant, and combinations or mixtures. A wide variety of plants has significantly contributed to human wellness over long periods of time, with plants being used for a range of different medicinal purposes over an extended period of time. With countless struggles against illness throughout history, humanity has developed a deep knowledge of the effectiveness of medicinal plants, which has eventually led to the discovery of the healing gems hidden within fruit bodies, barks, seeds, and other botanical marvels, a collection of empirical regional practices based on indigenous and local knowledge systems, is a subset of ethnobotany (Kalita, 2024).

The two largest nations in Asia, China and India, contain the most diverse collections of officially recognized medicinal plants. A vast number of medicinal plants are available in India. In addition to the fact that all sections of the population use these medicinal plants, they are also used as folk remedies, indirectly, through many traditional medical systems, or directly in pharmaceutical preparations in medicine. Ayurveda, Unani, and Siddha are a few of the various Indian medical systems that have contributed to the accumulation of knowledge about medicinal plants over many centuries. There are several historical sources of information about medicinal plants, including the Rigveda and Atharveda, which are dated from 2000–1000 B.C., as well as post-Vedic literature, including the Charakasamhita of 100 A.D., the Sushruta Samhita of 100–800 A.D., and the Dhanwanthari Nighantu of 1200 A.D. (Lone, 2013). Plant-based pharmaceutical products play a very important role in the healthcare of numerous indigenous populations around the world, as they are not only safer than synthetic drugs but also less expensive and more effective than

those products. People around the world use plant-based pharmaceutical products for a variety of purposes. There is a greater reliance on ethnomedicine in rural communities because of these significant effects on their health (Jenipher and Ayyanar, 2024). Therefore, the goal of this thorough review is to look into the worldwide documentation of ethnomedical uses of various plant part extracts against whooping coughs, as well as the taxonomic distribution of medicinal plant species, methods for preparing remedies, and documented reports from various Indian states.

Ethnomedicinal Plants Used for Whooping Cough

Records of Ethnomedicinal Plants Used for Whooping Cough from India

Present review work revealed that a total of 89 plant parts (aerial parts, bark, buds, bulb, cloves, corms, fruits, leaves, rhizome, roots, stem bark, tuber, whole plant, and combination/mixture, etc.) of different plants were applied for the treatment of Whooping coughs by using different extraction methods in throughout the world. Out of these 89 plant parts, the most applied plant parts are leaves (21.57%), whole plant (20.59%), and roots (16.67%) for the traditional treatment of Whooping coughs (Table 1 and Figure 1). This documentation contains 65 research reports, of which a total of 89 plant species belonging to 41 families (Figure 2) and 70 genera were studied, as seen in Table 1. All these reports are documented from different states of India, as shown in Figure 2 and Table 1. This review also revealed that 88 documents were recorded from India regarding the ethnomedicinal application of plant extract for treating whooping coughs, as shown in Table 1. This observation also revealed that these countries are rich in biodiversity, which is why most of the plants are applied for different medical treatment by the local tribes. From these, 41 families and 89 species were cited from India (Table 1). The most effective family of ethnomedicinal plants used for the treatment of whooping cough is Asteraceae, which is similar to our present documentation.

The medicinal plants used are from the eighth-largest district in the Indian state of Andhra Pradesh, the Chittoor district, which has undergone rigorous surveying and documentation. Adhatoda vasica antepartum therapy, menorrhagia, psoriasis, asthma with cough, cold, and asthma Andrographis paniculata stomach ulcers, fever, jaundice, cough, cold, and asthma (Vedavathy, 1997). Common home remedies are being used by various community members in Uttar Pradesh in order to accurately document and share knowledge among diverse communities for the benefit of humanity. The waste product spike of maize, which is used to cure ordinary cough and whooping cough, is a therapy for whooping cough (Prakash & Mehrothra, 1988). Paddar-Valley, formerly known as Sapphire Valley, is a prominent landmark in the Jammu area of the Indian state of J&K, India. The area is located in the Kishtwar district. Due to its variety of habitats, including rivers, streams, meadows, and steep mountainsides, the Paddar Valley is well known for its vast cultural and plant diversity. Traditional herbal remedies in Paddar Valley locals' observational use of traditional therapies for illnesses like malaria, cancer, digestive problems, etc. The ethnobotanical knowledge of medicinal plants would be helpful for the development of new drugs as well as the preservation of traditional cultures and biodiversity. The wide network of the traditional Indian System of Medicine is said to be supported by the Western Himalayas, which are known as an abundant source of herbal resources (Kumar,2009). In Purandhar, Maharashtra, many medicinal herbs were discovered, and a total of 77 species from 30 families and 56 genera were identified as ethnomedicinal plants in Purandhar that were found to be useful for medicinal purposes. The use of these plants is aimed at treating various diseases, such as whooping cough, asthma, diabetes, diphtheria, conjunctivitis, snake bites, scorpion bites, and more. Plants like *Pongamia pinnata* and *Cassia pumila*, etc. (Bhosle et al., 2009). The use of medicinal herbs by the residents of Taindol village in the Uttar Pradesh district of Jhansi, India, was conducted by an ethnobotanical investigation. These medicinal plants have been used for centuries to treat various diseases in rural communities, such as anaemia, aphrodisiac, jaundice, smallpox, leprosy, antiseptic cough, sores, skin problems, cancer, piles, diarrhoea, diuretics, low blood pressure, dysentery, headaches, diabetes, asthma, toothache, purifying blood, sedatives, gonorrhoea, fever, madness, disorders, and other diseases. Plants like *Abutilon indicum* and *Acacia nilotica* are found in Taindol village (Rahul, 2013).

A study of traditional remedies used by the tribal population of Buddhists in the Indian Leh-Ladakh region for colds, coughs, and fever. Ladakh is one of our country's least populous regions, with the majority of its residents living in remote communities at higher altitudes. The Amchi system

of medicine, a traditional medical practice, is the primary source of health care for the indigenous community. To cure colds, coughs, and fever in the Leh-Ladakh region, there are various medicinal plants that can be utilized, including *Arnebia euchroma, Aster diplostephioides, Aster tibeticus*, etc (Ballabh & Chaurasia, 2007). An ethnomedicinal plant like *Solanum surattense, Solanum trilobatum*, etc., was found in Theni District, Tamil Nadu, India, which was applied for different medicinal treatments (Jeyaprakash, 2011). Different medicinal plants are found in the Bangus Valley of Kashmir Himalaya, India. According to the study, 44 families and 75 plant species were discovered that were utilized as traditional medicines to treat a variety of illnesses. The *Mentha arvensis, Saussurea costus, Viola odorata*, etc., belong to the following families: Lamiaceae, Asteraceae, and Violaceae, which showed high ethnomedicinal properties (Ishtiyak and Hussain, 2017). All these research findings showed similarity with our findings review work. Bandipora district is one of the 22 districts in Jammu and Kashmir where various herbal medicinal plants were reported like *Euryale ferox, Conyza Canadensis, Adiantum venustum*, etc (Ishtiyak and Hussain, 2017). A survey on ethnobotanics was conducted in Mundakunnu village, TamilNadu, documented that *Allium sativum, Anisochilus carnosus, Bryophyllum pinnatum, Oxalis corniculata, Annona squamosa, Areca catechu*, and *Basellarubra*, etc plants are used to treat various diseases (Manikandan, 2005).

This review also revealed that some plant species are used in India to treat whooping cough disease. *Saussurea sp.* is the dominant species, and *Solanum sp.* and *Cassia sp.* are the second and third largest genera. This documentation also revealed that the ethnomedicinal application of Whooping cough and these three plant species are mostly reported from different states of India (Table 1 and Figure 1).

Records of ethnomedicinal plants used for whooping cough from other countries

A study on ethnomedicine was conducted in the southern part of Bangladesh, the districts of Barisal, Paschim Shawra, Palordi villages, and Gaurnadi Upazila, and it was discovered that the Paschim Shawra 33 families and Palordi villages 51 plant families used to treat a variety of illnesses. Different plants like *Justicia adhatoda, Tamarindus indica, Ocimum gratissimum*, etc, were used to treat coughs, chest pain, bronchitis, asthma and whooping cough, sexual disorders, leucorrhea, sexual weakness, menstrual problems, gonorrhoea, respiratory tract disorders, which showed similarity with our findings (Anup et al., 2011). A total of fifteen herbal medicinal aqueous plant extracts were tested in Palestine for antibacterial activity against eight distinct bacterial species. Medicinal plants like *Nigella sativa, Pimpinella anisum, Thymus vulgaris, Foeniculum vulgare, Majorana syriaca, Thymus origanum*, etc., were used against various diseases (Essawi & Srour, 2000).

A study on medicinal plants was done by Rajshahi's inhabitants from Bangladesh, of which 32 distinct were selected as a study area and identified the various types of medicinal plants for various treatments like asthma, cough and cold, dysentery, different skin diseases, ulcers, and leprosy. Medicinal plants like *Acacia nilotica, Butea monosperma, Caesalpinia pulcherrima, Desmodium gangeticum, Pongamia pinnata*, etc (Rahman, & Parvin, 2014). A total of 50 wild medicinal plants were identified, and their parts, like leaves, fruit, roots, seeds, whole plant, aerial parts, flower, rhizome, bark, stems, bulbs, and pods, used to treat many diseases like cold/cough, hepatitis, diuretic, sedative/narcotic, tonic, asthma, cardiac problems, jaundice, etc. The names of applied plants for the treatment of various diseases, namely, *Adiantum capillus-veneris, Allium carolinianum, Mentha longifolia*, etc, were identified (Ullah et al., 2013). Surrounding areas are diverse in both plants and cultural activities. According to, a total of 3000 plant species were identified in the region of northern Pakistan and Gilgit district, out of which 124 species showed medicinal importance. The medicinally important plant names are *Elaeagnus angustifolia, Trifolium pretence, and Thymus serpyllum* etc, which were used to treat a variety of diseases (Qureshi et al., 2007). The following plant species from India have been listed alphabetically in chronological order (Ballabh and Chaurasia, 2007) in Table 1, along with their botanical name, family local name, parts used, and utilization based on ethno-medico-botanical knowledge.

Traditional medicinal plants applied for Whooping cough

Traditional drugs may additionally function as a supply for future chemotherapy pills. Using natural substances in natural drugs is an exquisitely large price. For the improvement of medication from traditional drugs, new medical disciplines like ethnomedicine and ethnopharmacology are introduced (Mekonnen et al., 2022). Public health

worries about a noticeably contagious respiratory tract infection and whooping cough are extensive all over the globe and can inflict severe illness in all ages (Johnston et al., 1998). Due to whooping cough, toddlers and older humans are most at risk for hospitalization, morbidity, and fatality. Whooping cough can be a predominant fitness danger for both kids and adults and can even end up being threatening (Asadbeigi et al., 2014). *Bordetella pertussis* causes pertussis, which is a communicable respiratory illness. Over 300 million children worldwide pass away from it each year, making it a high global morbidity and mortality rate. The prevalence of pertussis is high in people of all ages. (Hua et al., 2024)

The ethnobotanical know-how of medicinal vegetation might be beneficial for the creation of new pills as well as the preservation of conventional cultures and biodiversity (Bussmann and Glenn, 2010). Research on therapeutic vegetation performed by using ethnobotanists is of the utmost significance, particularly in harsh climates like cold, dry locations in which the current clinical gadget is not yet absolutely advanced. Due to its style of use, flower variety performs an important role in conventional remedies. Floras are hired to remedy both internal and external diseases (Policepatel and Manikrao, 2013).

Phytochemicals are found in many plant groups, and they have been used for a variety of medical purposes over the years. A wide variety of medicinal plants contain secondary metabolites that are associated with antimicrobial and antibacterial properties. As a result of their effectiveness in treating respiratory conditions as well as a number of other illnesses around the world, these compounds are beneficial to humans as well. The greatest value of plant extracts is that they are unique and non-replaceable. They significantly increase the possibilities for therapy and the success of clinical trials. Additionally, the WHO has suggested that national health systems incorporate traditional herbal treatment. The benefits of medicinal plants also include a broad therapeutic index and significant biological activity, as herbal medicine has a much longer half-life than conventional drugs. It has become extremely popular among patients and physicians in the 21st century due to a renewed focus on this area and its potential for growth and development (Matushchak et al., 2024).

A number of illnesses can be treated with these medicinal flowers, such as whooping cough, anaemia, aphrodisiac, jaundice, smallpox, leprosy, cough, sores, skin diseases, cancer, piles, diarrhoea, diuretics, low blood pressure, dysentery, headaches, diabetes, asthma, toothache, purifying the blood, sedative, gonorrhoea, fever, issues, ulcers, and urinary discharges. Information on medicinal plants and their usage is covered in the discussions (Raj et al., 2018; Horváth and Ács, 2015)

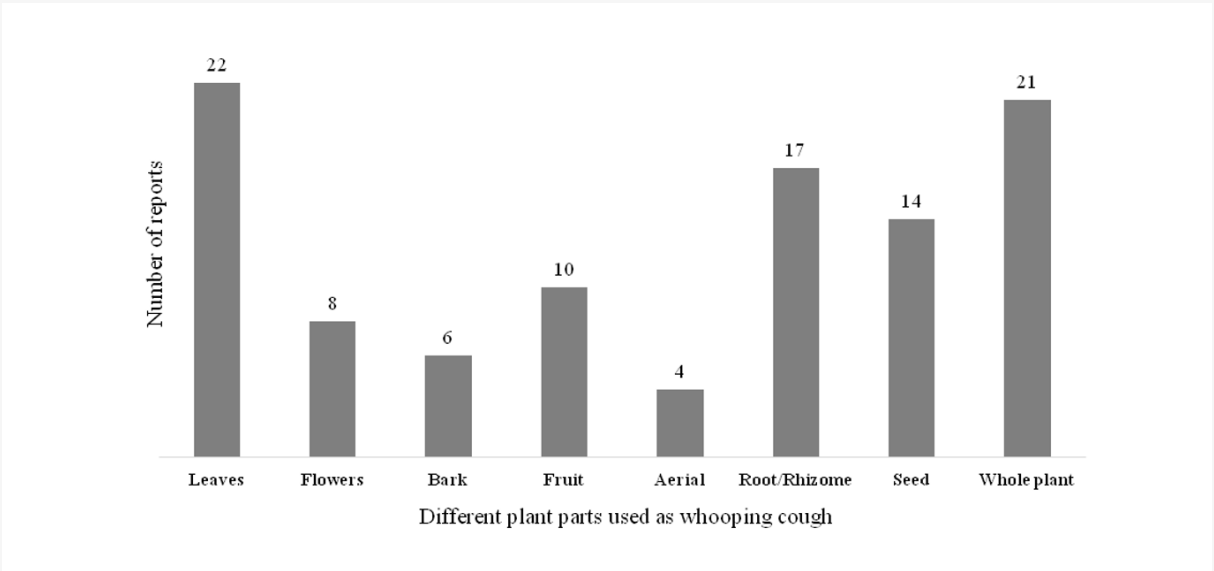

Figure 1. Different plant parts were applied for the ethnomedicinal use against the Whooping cough.

Slika 1. Različni deli rastlin, ki se uporabljajo za etnomedicinsko uporabo proti oslovskemu kašlju.

Table 1. Different ethnomedicinal plants along with their botanical name, family, local name, parts used, and medicinal properties against whooping cough.

Tabela 1. Različne etnomedicinske rastline z njihovim botaničnim imenom, družino, lokalnim imenom, uporabljenimi deli in zdravilnimi lastnostmi proti oslovskemu kašlju

Name of the plant	Scientific name	Family of the plant	Part of the plant used	Medicinal properties	States/UT of India	Ref.
Cloves	*Syzygium aromaticum*	Myrtaceae	Its dried flower buds are used.	Cloves offer very fast relief from continuous coughing	Ahmedabad	(Abdullah and Andrabi, 2021)
Kanghi	*Abutilon indicum*	Malvaceae	Seeds, Root, Leaves, Bark	Anti-inflammatory, Alexeteric, Bronchitis, Colds, Cough	Uttar Pradesh	(Rahul, 2013)
Khair	*Acacia catechu*	Mimosaceae	Stem	Useful in ailments of throat, mouth, gums, cough and diarrhea.	Tehsil Billawar, J&K	(Bhushan and Kumar, 2013)
Babul	*Acacia nilotica*	Mimosaceae	Bark, leaves, Seed	Antibacteria, Antifungal, Antiviral, Abscess, Burn, Cough, Dental care, Diarrhea, Gonorrhoea, Leucoderma, Malaria, Mouth sores, Pneumonia	Bundelkhand, Uttar Pradesh	(Rahul, 2013)
Yarrow	*Achillea millefolium*	Asteraceae	Whole plant	Used in cold and cough	Ladakh	(Ballabh and Chaurasia, 2007)
Parkanda, Aghada	*Achyranthes aspera*	Amaranthaceae	Leaves and Seeds	The roasted seed powder mixed with honey is given during cough & throat irritations.	Tehsil Billawar, J&K	(Bhushan, & Kumar, 2013)
Patrees	*Aconitum heterophyllum*	Ranunculaccae	Root	Dried roots are used to treat cough	Bangus Valley, Kashmir Himalaya	(Suroowan and Mahomoodally, 2016)
Malabar nut, adulsa, adhatoda, vasa, Vasaka	*Adhatoda vasica*	Acanthacea	From root to leaves are used	Used for cough, Cold	Chittoor district, Andhra Pradesh	(Vedavathy et al., 1997)
Malabar nut, Adhatoda, Vasaka	*Adhatoda zeylanica*	Acanthaceae	leaves	Fresh leaves was taken orally to get relief from cold, cough, breathing problems and throat pain	Western Ghats, Southern India	(Jeyaprakash, et al., 2011)
Kakbai	*Adiantum venustum*	Pteridaceae	Whole plant	Cough, jaundice, stomach ailments, headache, fever, body muscular pains and hair fall.	Jammu and Kashmir	(Lone, et al., 2013)
Bilwa, Bael	*Aegle marmelos*	Rutaceae	leaves	Fresh leaves were taken orally twice a day for a week to treat cough, breast inflammation, eye problems and to keep the body in cool	Western Ghats, Southern India	(Jenipher and Ayyanar, 2024)
Sarih, Siris	*Albizia lebbeck*	Mimosaceae	Leaves, Bark and Flowers	The leaves were also effective against cough. Bark strengthens gums. Flowers used in chronic cough and asthma.	Tehsil Billawar, J&K	(Bhushan, and Kumar, 2013)
Mulluli	*Allium sativum*	Liliaceae	Rhizome, Bulbous, flowers	For arresting whooping cough	Nilgiri district, Tamil Nadu	(Manikandan, 2005)

Aloe Vera	*Aloe barbadensis*	Asphodelaceae; Liliaceae	The plant has green, gel filled leaves is used.	Aloe vera juice mixed with honey is considered to be a cure for whooping cough in ayurveda.	Ahmedabad	(Rinchen, & Pant, 2014)
Green chiretta	*Andrographis paniculata*	Acanthaceae; Nelavemu	Whole plant	Used for Cough, Cold	Chittoor district, Andhra Pradesh	(Vedavathy, et al., 1997)
Pink arnebia	*Arnebia euchroma*	Boraginaceae	Roots	Used in cold and cough	Leh-Ladakh	(Khan, et al., 2018)
Russian wormwood, Gmelin's wormwood	*Artemisia gmelinii*	Asteraceae	Flowers	Used in cold and cough	Leh-Ladakh,	(Angmo, et al., 2021)
Creeping aster	*Aster diplostephioides*	Asteraceae	Flowers	Used in cold and cough	Leh-Ladakh	(Angmo, et al., 2019)
Aster	*Aster tibeticus*	Asteraceae	Flowers	Used in cold and cough	Leh-Ladakh	(Gupta and Singh, 2010)
Neem	*Azadirachta indica*	Meliaceae	Leaves, bark	Anthelmintic, Antifungal, Antibacterial, Antiviral, Antiseptic, Asthma, Chicken pox, Contraceptive, Cosmetics uses, Cough,	Buldenkhand, Uttar Pradesh	(Rahul, 2013)
Karal	*Bauhinia variegate*	Caesalpiniaceae	Stem bark, Root bark, flower and buds	Bark of the roots is useful in flatulence. Flowers are laxative. Flower buds are used against piles and cough	Tehsil Billawar, J&K	(Bhushan, and Kumar, 2013)
Common baggarticks	*Bidens pilosa*	Asteraceae	Leaves	Used in cold and cough	Leh-Ladakh	(Cavero and Calvo, 2014)
Makarani	*Bryophyllum pinnatum*	Crassulaceae	Leaf juice	Used for cure cough	Nilgiri district, Tamil Nadu	(Yadav, et al., 2010)
Daryai aak, Madar	*Calotropis procera*	Asclepiadaceae	Leaves and Roots	The smoke from the burning leaves is inhaled for the cure of asthma and cough	Tehsil Billawar, J&K	(Bhushan, and Kumar, 2013)
Amaltas	*Cassia fistula*	Caesalpiniaceae	Fruit, leaves, root-bark, stem-bark	Antioxidant, Blood sugar, Blood purification, Cold, Cough	Buldenkhand, Uttar Pradesh	(Rahul, 2013)
Coffee enna, Negro coffee, Coffee weed	*Cassia occidentalis*	Caesalpiniaceae	Seed	Treating the whooping cough	Andhra Pradesh	(Khisha, et al., 2012)
Harankhuri	*Cassia pumila*	Asteraceae	Leaves	Whooping cough	Pune, Maharashtra	(Haq, et al., 2020)
Iranian knaweed	*Centaurea depressa*	Asteraceae	Whole plant	Used in cold and cough	Leh-Ladakh	(Mehrnia, et al., 2021)
Karpooravalli	*Centaurea depressa*	Asteraceae	The leaf juice was used by the Paniyas.	cure whooping cough	Nilgiri district, Tamil Nadu	(Ali, et al., 2023)
Shallut	*Conyza Canadensis*	Asteraceae	Aerial portion	Indigestion, dysentery, stomach gases, internal injuries, fever and cough	Jammu and Kashmir	(Alam, 2024)
Sangi-harb	*Corydalis govaniana*	Papaveraceae	Aerial portion of plant	Used to treat cough	Bangus Valley, Kashmir Himalaya	(Krishnaraju, et al., 2005)
Haldi	*Curcuma longa*	Zingiberaceae	Whole plant	Abdominal pains, Anemia, Anti-flammatory, Antimicrobial, Antioxidant, Antispasmodic, Blood purifying, Cancerous, Cold, Cough	Buldenkhand, Uttar Pradesh	(Rahul, 2013)

Datura	*Datura metel*	Solanaceae	Leaf, twigs and fruits	The dried leaves and twigs are smoked for cure of asthma and whooping cough	Tehsil Billawar, J&K,	(Bhushan, and Kumar, 2013)
Salparni	*Desmodium gangeticum*	Fabaceae	Root	Used in fever, cough	Andhra Pradesh	(Chaurasia, et al., 1998)
White dragonhead	*Dracocephalum heterophyllum*	Lamiaceae	Whole plant	Used in cold and cough	Leh-Ladakh	(Hadi, etal., 2022)
Amla	*Emblica officinalis*	Euphorbiaceae	Fruit, Seed, bark	Antimicrobial, Anti-oxidant, Anti-ulcer, Asthma, Boils, Chicken pox, Chronic fever, Chronic head-ache, Cooling, Cough & Cold	Buldenkhand, Uttar Pradesh	(Rahul, 2013)
Broadleaf helleborine	*Epipactis helleborine*	Orchidaceae	Whole plant	Used in cold and cough	Leh-Ladakh	(Mali and Panchal, 2017)
Dudhi	*Euphorbia hirta*	Euphorbiaceae	Whole Plant	Antibacterial, Anti-viral, Asthma, Boils, Bronchitis, Cough, Diarrhea	Buldenkhand, Uttar Pradesh	(Shukla, et al., 2022)
Juwar/Kena bub	*Euryale ferox*	Nymphaeaceae	Seeds	Stomach problems, whooping cough, semen deficiency and weak libido	Bandipora, Jammu and Kashmir	(Rao, et al., 2015)
Rumbal	*Ficus racemosa*	Moraceae	Fruits and Latex	Fruits are used in treatment of dry cough and loss of voice	Tehsil Billawar, J&K	(Bhushan, and Kumar, 2013)
Bodiyaan	*Foeniculum vulgare*	Apiaceae	Whole plant	Dyspepsia, acidity, constipation, abdominal pain, Jaundice, cough, cold, chronic constipation, fever, blood purifier and joint pains	Bandipora, Jammu and Kashmir	(Thakur, et al., 2020)
Moorcroft's gentian	*Gentianella moorcroftiana*	Gentianaceae	Whole plant	Used in cold and cough	Leh-Ladakh	(Sarver, et al., 2016)
Gulaftab	*Helianthus annuus*	Asteraceae	Seeds	Whooping cough and joint pains.	Bandipora, Jammu and Kashmir	(Jan, et al., 2022)
Bazarbang	*Hyoscyamus niger*	Solanaceae	Seed, leaves, flowers	Used to treat cough	Bangus Valley, Kashmir Himalaya	(Shah, et al., 2023)
Henbane	*Hyoscymous niger*	Solanaceae	Leaves, flowers	Nervousness, asthma, and whooping cough	Paddar Valley, Kishtwar, Jammu	(Shabir, et al., 2023)
Pushkaramula	*Inula racemosa*	Asteraceae	Roots	Used in cold and cough	Leh-Ladakh	(Rawat, and Upadhaya, 2020)
Duon	*Juglans regia*	Juglandaceae	Bark, fruits and leaves	Tooth infection and toothache, tongue cleaning, mouth ulcers, dry cough	Bandipora, Jammu and Kashmir	(Beldar, and Sidat, 2020)
Adulsa	*Justicia adhatoda*	Acanthaceae	Leaves	Cough	Tamil nadu, Uttarpradesh, India	(Jishtu, et al., 2023)
Kashir Aull	*Lagenaria siceraria*	Cucurbitaceae	Fruits	Cough, cold, fever, chest pain, stomach ulcers, stomach heat up, kidney stones,	Bandipora district, Jammu and Kashmir, India	(Rather, 2023)
Kinnaur lagotis	*Lagotis kunawurensis*	Scrophulariaceae	Whole plant	Used in cold and cough	Leh-Ladakh	(Verma, et al., 2019)
Depgul	*Lancea tibetica*	Scrophulariaceae	Whole plant	Used in cold and cough	Leh-Ladakh	(Rinchen, et al., 2021)

Blue feltwort	*Lomatogonium carinthiacum*	Gentianaceae	Whole plant	Used in cold and cough	Leh-Ladakh	(Jawaid, et al., 2017)
Mash felwort	*Lomatogonium rotatum*	Gentianaceae	Whole plant	Used in cold and cough	Leh-Ladakh	(Manzoor, et al., 2023)
Maharaji Treil	*Malus domestica*	Rosaceae	Fruits	Used for cough and other chest ailments.	Jammu and Kashmir	(Gairola, et al., 2014)
Sochal	*Malva neglecta*	Malvaceae	Seed, leaves	Used to treat cough	Central Asia and W. Himalaya	(Angmo, et al., 2012)
Chinese mallow, cluster mallow	*Malva verticillata*	Malvaceae	Seeds, leaves	Used in cold and cough	Leh-Ladakh	(Smanla and Millard 2013)
Pudna	*Mentha arvensis*	Lamiaceae	Aerial portion of the plant	Used to treat cough	Bangus Valley, Kashmir Himalaya	(Lamo, et al., 2019)
Two color catmints	*Nepeta discolor*	Lamiaceae	Leaves	Used in cold and cough	Leh-Ladakh	(Bhadra, and Sethi, 2020)
Woolly catmint	*Nepeta floccose*	Lamiaceae	Leaves	Used in cold and cough	Leh-Ladakh	(Arijit, & Arpita, 2013)
Tulsi	*Ocimum sanctum*	Lamiaceae	Tulsi leaves are used.	Tulsi leaves were considered to be a very effective cure for whooping cough according to ayurveda.	Ahmedabad	(Biswas, et al., 2022)
Thulasi	*Ocimum tenuiflorum*	Lamiaceae	leaves	fresh leaves were taken orally twice a day to get relief from cold, cough and fever	Theni, Western Ghats	(Bashir, et al., 2018)
Tantu	*Oroxylum indicum*	Begnoniaceae	Stem bark, Leaf and Fruit	Mature fruits are used in treating cough, piles and cardiac disorders	Tehsil Billawar, J&K	(Bhushan, and Kumar, 2013)
Creeping woodsorrel, procumbent yellow sorrel	*Oxalis corniculata*	Oxalidaceae	The whole plant (leaves) juice	The leaves are chewed to arrest chronic cough	Nilgiri, Tamil Nadu	(Dorjey, et al., 2022)
Picrorhiza, katuka, kutki	*Picrorhiza kurrooa*	Scrophulariaceae	Roots	Used in cold and cough	Leh-Ladakh	(Lamo, et al., 2019)
Plantains, Fleaworts	*Plantago depressa*	Plantaginaceae	Whole plant	Used in cold and cough	Leh-Ladakh	(Batool, et al., 2022)
Karanj	*Pongamia pinnata*	Fabaceae	Seed oil	Whooping cough	Pune, Maharashtra	(Bhosle, et al., 2009)
Karanja, Pongam	*Pongamia pinnata*	Fabaceae	All part of plant (Seeds, leaves, etc)	Used for treatment of various ailment including bronchitis, whooping cough, rheumatism, diar-rhoea, gonorrhoea and leprosy	Tamil Nadu	(Ghumare, et al., 2014)
Large leaf primrose	*Primula macrophylla*	Primulaceae	Whole plant	Used in cold and cough	Leh-Ladakh	(Nirmala, et al., 2022)
Daduni	*Punica granatum*	Punicaceae	Bark, Roots, Seeds and Leaves	The fruit was useful against the cough and jaundice. Leaves, seeds, roots and bark are effective in anthelmintic activity	Tehsil Billawar, J&K	(Bhushan, and Kumar, 2013)
Stonecrops	*Rhodiola imbricate*	Crassulaceae	Roots	Used in cold and cough	Leh-Ladakh	(Hussain, et al.,2023)
Stonecrops	*Rhodiola heterodonta*	Crassulaceae	Roots	Used in cold and cough	Leh-Ladakh	(Haq, et al., 2023)

Common madder, Indian madder	*Rubia cordifolia*	Rubiaceae	Roots	Used in cold and cough	Leh-Ladakh	(Tali, et al., 2019)
Narrow leaved saw-wort	*Saussurea bracteata*	Asteraceae	Flowers	Used in cold and cough	Leh-Ladakh	(Dawa, et al., 2021)
Kuth	*Saussurea costus*	Asteraceae	Rhizome	Used to treat cough	Kashmir Himalaya	(Rennie, 2016)
Kuth root	*Saussurea lappa*	Asteraceae	Roots	Used in cold and cough	Leh-Ladakh	(Bhatia, et al., 2014)
King of Himalayan flowers	*Saussurea obvallata*	Asteraceae	Bracts	Cough and respiratory problems	Paddar Valley, Jammu	(Sharma, 2023)
Makoi	*Solanum nigrum*	Solanaceae	Leaves, seed, berry	Anti-dysentric, Antiseptic, Asthma, Cold, Cough, Diarrhea, Ear pain, Fever, Gout, Mouth ulcers, Ringworm, Skin diseases, Testicular Swelling, Ulcers, Whooping cough	Buldenkhand, Uttar Pradesh	(Rahul, 2013)
Kandankatthari	*Solanum surattense*	Solanaceae	leaves	Fresh leaves are heated in fire and the smoke is inhaled through nose or mouth to treat cough and asthma	Theni, Western Ghats	(Jamieson, 1973)
Thudhuvalai	*Solanum trilobatum*	Solanaceae	leaves	Fresh leaves were boiled with black pepper and tender coconut and the paste thus obtained is taken orally thrice a day for two days to get relief from cold and cough	Theni, Western Ghats	(Navchoo, and Buth, 1989)
Imli	*Tamarindus indica*	Caesalpiniaceae	Fruit, Bank	Bilious, Burns, Cough, Diabetes, Digestive disorders, Dysentery, Fever, Jaundice, Malaria, Piles, Scurvy, Sore, Throats	Buldenkhand, Uttar Pradesh	(Rahul, 2013)
Breckland thyme, Wild thyme, Creeping thyme	*Thymus serphyllum*	Lamiaceae	Whole plant	Whooping cough, epilepsy, suppression of urine and menstrual catarrh, tea substitute, roots used in havan	Paddar Valley, Jammu	(Asif, et al., 2021)
Gokshur, Puncture vine, Gokharu	*Tribulus terrestris*	Zygophyllaceae	Fruits	Used in cold and cough	Leh-Ladakh	(Afzal, et al., 2009)
Kulmanch	*Viburnum grandiflorum*	Caprifoliaceae	seed	Used to treat cough	Bangus Valley, Kashmir Himalaya	(Chauhan, et al., 2020)
Bunafsha	*Viola odorata*	Violaceae	flower	Used to treat cough	Bangus Valley, Kashmir Himalaya	(Ishtiyak and Hussain, 2017)
Banafsha	*Viola serpens*	Violaceae	Whole plant	Cough and cold	Meghalaya, Nagaland and Manipur	(Akbar and Akbar, 2020)
Bana	*Vitex negundo*	Verbenaceae	Flowers and Leaves	Leaves are chewed in cough and associated colds	Tehsil Billawar, J&K	(Bhushan and Kumar, 2013)
Mayweed	*Waldheimia stoliczkai*	Asteraceae	Whole plant	Used in cold and cough	Leh-Ladakh	(Akbar and Akbar, 2020)

Maize	*Zea mays*	Poaceae	The spike of maize without grains	Used for the treatment of ordinary cough and whooping-cough. The ear of maize (without grains) was kept inside the freshly prepared earthern roll and put in fire till it burnt to ashes. Now turned to ash was taken out from the earthen roll crushed to fine powder. That was applied as doses of 5-8 g with honey	Lucknow	(Prakash and Mehrothra, 1988)
Ginger	*Zingiber officinale*	Zingiberaceae	The root of the plant was used	Ginger juice was a very effective remedy for curing whooping cough and may mix the juice with honey or herbal tea.	Ahmedabad	(Akbar and Akbar, 2020)

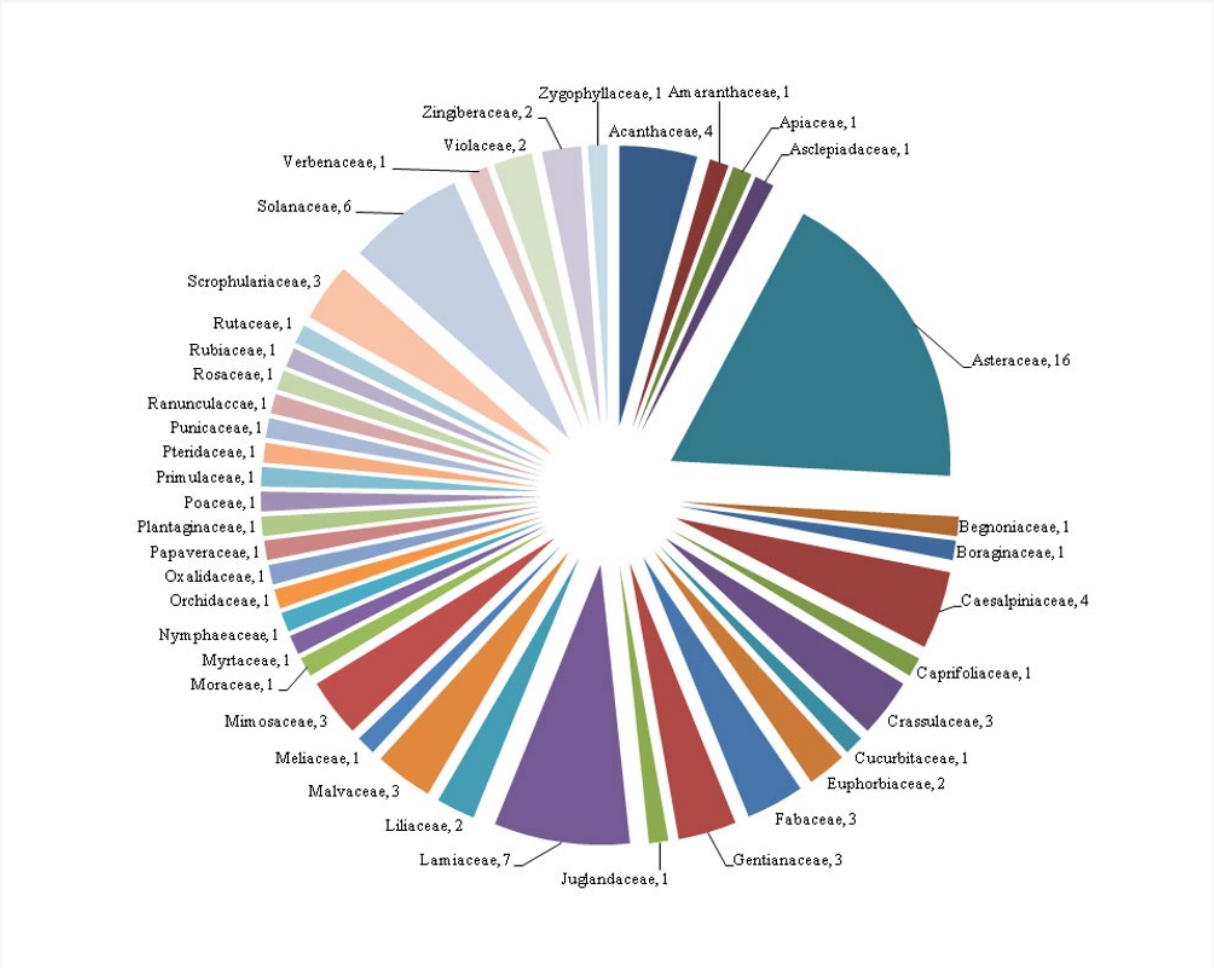

Figure 2. Number of plant species under various families of plants for the ethnomedicinal use against the Whooping cough.

Slika 2. Število rastlinskih vrst iz različnih družin rastlin za etnomedicinsko uporabo proti oslovskemu kašlju.

Conclusions

The correct phytonutrient assessment of chosen plant species for the discovery of biologically active novel chemical compounds for the development of novel medications and the conservation of endangered plant species through *ex situ* and *in situ* conservation by involving research organizations, forest departments, and local populations, which in turn will be beneficial for the overall development of traditional medical systems for the improvement of the health care system. The traditional application of plant remedies offers potential biological activity. According to the WHO, nearly one-third of the world's population uses traditional herbal remedies for their healthcare. Therefore, there is a tremendous potential for medicinal plants in healthcare, not just in remote areas of developing nations but also in the industrialized world, and there is a good chance that in the future, more people will accept the use of phytochemicals in modern medicine. A significant component of many rural communities' cultural legacy is the use of wild plants. Since the dawn of civilizations, people have utilized many plant species as a traditional source of medicine. In industrialized nations, the interaction between human populations and the usage of plant resources has been regarded as an iconic element of ecosystems and an ecological balance system. Because traditional knowledge is so dynamic and subject to generational and cultural shifts, it is imperative that it be properly and promptly documented. Humans are particularly vulnerable to hospitalization, illness, and death. Many plant parts, including leaves, fruit, roots, seeds, the entire plant, aerial parts, flowers, rhizomes, bark, stems, bulbs, and pods, are used to cure a variety of illnesses. Numerous significant medicinal plants are used to cure a variety of illnesses, including Tamarindus indica, Ocimum gratissimum, Mentha longifolia, Justicia adhatoda, Acacia nilotica, and Allium carolinianum. Ethnomedicine is both cost-effective and free of adverse effects. For most of our people living in rural and tribal areas, medicinal plants are the only easily accessible source of healthcare. 64% of people on the planet still receive treatment using traditional methods. The plant species that have been collected are renowned for their ability to treat ailments like tonsillitis, elephantiasis, tonsillitis, Alzheimer's, Parkinson's, colds, arthritis, gastritis, gonorrhoea, diarrhoea, dysentery, jaundice, and leprosy. That's why it's important to evaluate and document the valuable plant parts and processing methods of ethnomedicine plants in order to focus on their conservation and sustainable use. More experimental and clinical validation of these traditional medicinal plant parts is needed.

Author Contributions

data collection, T.T.; writing-original draft, T.T.; writing correction, T.T.; formal analysis and editing, T.T.; supervision, A.B.; project administration, A.B.; investigation, A.B.; validation and review, A.B.; concept making and methodology, T.M.; investigation, T.M.; editing and submission, T.M. All authors have read and approved the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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ABS

Review

Linking microbiome and hyperaccumulation in plants

Valentina Bočaj [1], Marjana Regvar [2], Paula Pongrac [1,2*]

Abstract

Hyperaccumulating plants can take up extraordinarily large concentrations of one or more metal(loid)s from the soil and accumulate it/them in the aboveground tissues without exhibiting any visible toxicity symptoms. Among more than 700 plant taxa reported to have evolved this unique phenotype, the most common is the hyperaccumulation of nickel (Ni), and less common is the hyperaccumulation of arsenic (As), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), manganese (Mn), lead (Pb), antimony (Sb), selenium (Se), thallium (Tl) or zinc (Zn). Metal(loid) hyperaccumulation is a result of several independent evolutionary events and despite considerable efforts, none of the proposed hypotheses on the environmental constraints driving these events has been supported fully to date. Among several tolerance strategies enabling hyperaccumulation is the allocation of metal(loid)s to competent cell types, typically away from photosynthetic apparatus, to limit damage to plant metabolism. Recently, the involvement of microorganisms colonizing roots in hyperaccumulation phenomenon has achieved increased attention due to the role of microorganisms in the mobilization of metal(loid)s in the soil. The complex interactions between hyperaccumulation and belowground microbiome are of primary interest for phytoremediation, a promising green technology for removing or immobilisation of metal(loid)s in the soil with the help of plants. In this review, we discuss and complement current reports on the contribution of microorganisms to metal(loid) hyperaccumulation.

Keywords

hyperaccumulating plants; arbuscular mycorrhizal fungi; dark septate endophytes

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Povezava med rastlinskim mikrobiomom in hiperakumulacijo

Izvleček

Hiperakumulacijske vrste lahko iz tal v svoja nadzemna tkiva privzamejo zelo velike koncentracije ene ali več (pol)kovin brez opaznih škodljivih učinkov pri rastlini. Pri več kot 700 taksonih, za katere poročajo, da so razvili ta edinstven fenotip, je najpogostejša hiperakumulacija niklja (Ni), manj pogosta pa je hiperakumulacija arzena (As), kadmija (Cd), kobalta (Co), kroma (Cr), bakra (Cu), mangana (Mn), svinca (Pb), antimona (Sb), selena (Se), talija (Tl) in cinka (Zn). Hiperakumulacija (pol)kovin je posledica več neodvisnih evolucijskih dogodkov. Kljub precejšnjim prizadevanjem pa nobena od predlaganih hipotez, ki bi razložila evolucijsko prednost hiperakumulacije, do danes ni bila v celoti podprta. Med številnimi tolerančnimi mehanizmi, ki omogočajo hiperakumulacijo, je tudi kopičenje presežnih koncentracij (pol)kovin v določenih tipih rastlinskih celic, običajno stran od fotosinteznega aparata. V zadnjem desetletju v ospredje raziskav hiperakumulacije stopa vloga mikroorganizmov, ki kolonizirajo korenine, pri mobilizaciji (pol)kovin v tleh. Zapletene interakcije med hiperakumulacijo in podzemnim rasltinskim mikrobiomom so zanimive predvsem z vidika fitoremediacije, t.j. zelene tehnologije, ki s pomočjo rastlin na naraven način odstrani (pol)kovine iz tal ali pa jih stabilizira. V tem preglednem članku razpravljamo in dopolnjujemo dosedanje študije o prispevku talnih in/ ali simbionstkih mikroorganizmov k hiperakumulaciji.

Ključne besede

hiperakumulacijske vrste; arbuskularne mikorizne glive; temni septirani endofiti

Introduction

The term hyperaccumulator was introduced by Brooks et al. (1977) to describe plants with exceptional concentrations of one or more metal(loid)s in the aboveground biomass without showing any visible toxicity symptoms (Brooks et al., 1977; Rascio, 1977). Initially, these unique plants were studied in relation to geobotany, while today, they have been at the centre of discussions concerning environmental pollution and phytoremediation. Hyperaccumulation is more than tolerance because a typical metal(loid)-tolerant plant aims to limit translocation of metal(loid) from roots to shoots, whereas in aboveground tissue of a hyperaccumulating plant, up to 1000-fold larger concentrations of metal(loid)s can be found compared to non-hyperaccumulating plants (Rascio, 1977; Reeves, 2006). Among hyperaccumulators, the largest number of species hyperaccumulate Ni, followed by other metal(loid)s like As, Cd, Co, Cr, Cu, Mn, Pb, Sb, Se, Tl and Zn (Table 1) (Baker et al., 2000; Baker & Brooks, 1989; Reeves et al., 2018; White & Pongrac, 2017). To warrant the designation of a plant as a hyperaccumulating species, the following conditions must be met: (i) the plant has to accumulate metal(loid) when grown in native soil, (ii) the shoot-to-root concentration ratio for a metal(loid) must be higher than unity, and (iii) foliar concentration (in mg g^{-1} dry weight) of a metal(loid) must exceed the thresholds 0.1 for Cd, 1 for As, Co, Cr, Cu, Ni, Pb, Sb, Se, Tl, and 10 for Mn and Zn (Baker et al., 2000; Baker & Brooks, 1989; Krämer, 2010; McGrath & Zhao, 2003; Rascio & Navari-Izzo, 2011). To date, more than 700 plant taxa are listed as hyperaccumulating, with several belonging to the Brassicaceae family (Baker et al., 2000; Krämer, 2010; Reeves et al., 2018; White & Pongrac, 2017).

Many authors argue that hyperaccumulation has independently evolved multiple times (Krämer, 2010; Reeves, 2006) as a response against herbivory and pathogens to which plants are constantly exposed (Boyd, 2007; Plaza et al., 2015). It is an extreme evolutionary trait, and although several hypotheses have been proposed, none of them is fully scientifically supported. However, the most accepted hypothesis among researchers remains the “defence against natural enemies” hypothesis, which predicts that plants (hyper)accumulate metal(loid)s in their aerial tissues to protect themselves from pathogens and herbivores. Furthermore, it also predicts the variety of defence organic compounds (Pollard, 2022; Rascio & Navari-Izzo, 2011) to complement large metal(loid) concentrations.

Although there are many studies on hyperaccumulating plants, many knowledge gaps still need to be filled to fully

Table 1. List of metal(loid)s, number of species that hyperaccumulate corresponding metal(loid) and selected hyperaccumulating species with corresponding references. Note that one species can hyperaccumulate more than one metal(loid) and that not all combinations are presented in the current table. The number of species was summarized by Reeves et al. (2018).

Tabela 1. Seznam kovin(loidov), število vrst, ki hiperakumulirajo ustrezne kovine(loide), in izbrane hiperakumulirajoče vrste z ustreznimi referencami. Upoštevajte, da lahko ena vrsta hiperakumulira več kot eno kovino(loid) in da v tej tabeli niso predstavljene vse kombinacije. Število vrst je bilo povzeto po Reeves et al. (2018).

Metal(loid)	Number of species	Selected plant species (family)	Reference(s)
As	5	*Pteris vittata* (Pteridaceae)	Xiao et al., 2021
Cd	7	*Arabidopsis halleri, Noccaea caerulescens, N. praecox* (Brassicaceae)	Bert et al., 2003; Vogel-Mikuš et al. 2005; Wójcik et al., 2005
Co	42	*Haumaniastrum robertii* (Lamiaceae)	Van Der Ent et al., 2019
Cr	1	*Spartina argentinensis* (Poaceae)	Redondo-Gómez et al., 2011
Cu	53	*Aeolanthus biformifolius* (Lamiaceae)	Van Der Ent et al., 2019
Mn	42	*Grevillea meisneri* (Proteaceae)	Bihanic et al., 2021
Ni	532	*Alyssum heldreichii, N. pindicum* (Brassicaceae)	Psaras et al., 2000
Sb	At least 1	*P. vittata* (Pteridaceae)	Wan et al., 2016
Se	41	*Stanleya pinnata* (Brassicaceae), *Symphyotrichum ericoides* (Asteraceae)	Cochran et al., 2018
Tl	2	*Biscutella laevigata* (Brassicaceae)	Pošćić et al., 2013
Zn	20	*A. halleri, N. caerulescens, N. praecox* (Brassicaceae)	Kozhevnikova et al., 2017; Mijovilovich et al., 2020; Vogel-Mikuš et al., 2008

understand the physiological mechanisms behind hyperaccumulation and furthermore, its complex interactions with the surrounding environment. One of the most prominent knowledge gaps is the understanding of the role microorganisms have in hyperaccumulation, tolerance, and/or the overall fitness of hyperaccumulators. Therefore, the main aim of this review is to appraise existing knowledge and complement current reports on the contribution of microorganisms to metal(loid) hyperaccumulation.

Plants are not alone

Seemingly, plants function as individuals. However, recent investigations revealed that they host numerous microorganisms (MO), referred to as phytomicrobiome. Phytomicrobiome consists of archaea, bacteria, fungi, nematodes, protists, and viruses (Bordenstein and Theis, 2015; Trivedi et al., 2020). Together with their plant host, they form the-so-called holobiont (Fig. 1). The interactions of MO with plant hosts can be (anthropocentrically) labelled either beneficial, harmful or neutral (Arnold et al., 2000; Brundrett, 2006; Inácio et al., 2002; Lindow & Brandl, 2003; Rosenblueth & Martínez-Romero, 2006), although gradients can be observed, namely a neutral interaction can become either beneficial or harmful, depending on different intrinsic and/or external factors.

Phytomicrobiome colonizes different parts of the plant, both vertically (Fig. 1) and horizontally. The MO colonizing the aboveground parts of the plant (e.g., leaves) inhabit the phyllosphere (Schlaeppi & Bulgarelli, 2015; Whipps et al., 2008), and those colonizing the underground (e.g., plant roots) inhabit the rhizosphere (Schlaeppi & Bulgarelli, 2015). Microorganisms can be found either inside their host's tissues or on their surface and are therefore referred to as endophytic or epiphytic, respectively. Endophytic MO is associated with the host for the whole or only a part of their life cycle. The beneficial effects of phytomicrobiome on their host have been well documented and include (i) increased tolerance to abiotic stresses (Rolli et al., 2014), (ii) enhanced nutrient uptake (Van Der Heijden et al., 2015), (iii) priming plant immune system (van der Ent et al., 2009) and (iv) protection against pathogens (Ritpitakphong et al., 2016). Some authors hypothesise that the phytomicro-

biome is an extension of the plant genome (Rosenberg & Zilber-Rosenberg, 2016; Schlaeppi & Bulgarelli, 2015).

The root system is a habitat for several MO, which inhabit both the surface of the roots and internal parts of this branched underground plant organ. Henceforth, we focus on three groups of rhizosphere-associated MO associated with hyperaccumulating plants: (i) mycorrhizal fungi, (ii) dark septate endophytes, and (iii) rhizoplane MO.

Do hyperaccumulators form mycorrhizal associations?

Mycorrhiza is one of the oldest and best-studied, presumably beneficial endophytic interactions between plants, and there is robust evidence that this interaction has played an important role in the evolution of land plants (Buscot, 2015; Martin & van der Heijden, 2024; Strullu-Derrien et al., 2018). Among different types of mycorrhiza, arbuscular mycorrhiza (AM) and arbuscular mycorrhizal fungi (AMF) are also associated with hyperaccumulating plants. Nevertheless, although they colonize most land plants, some plant families are believed not to form AM associations (Sharma et al., 2023). These include Brassicaceae, which hosts many known hyperaccumulators (Johnson et al., 1997; Harley & Harley, 1987; Veiga et al., 2013). However, some recent studies show that the non-mycorrhizal status of Brassicaceae might not be universal, at least in the sense of the plant family as a whole (Trautwig et al., 2023). For example, the presence of AMF was observed in *Biscutella laevigata* (Brassicaceae), a Tl hyperaccumulating plant (Pošćić et al., 2013), both at metalliferous and non-metalliferous sites with all typical AMF structures, i.e., vesicles, coils, and arbuscules (Orłowska et al., 2002). A year later, Regvar et al. (2003) discovered that pennycresses (*Noccaea* spp.; Brassicaceae) form AMF associations too. The AMF colonization of a Cd and Zn hyperaccumulating *N. praecox*

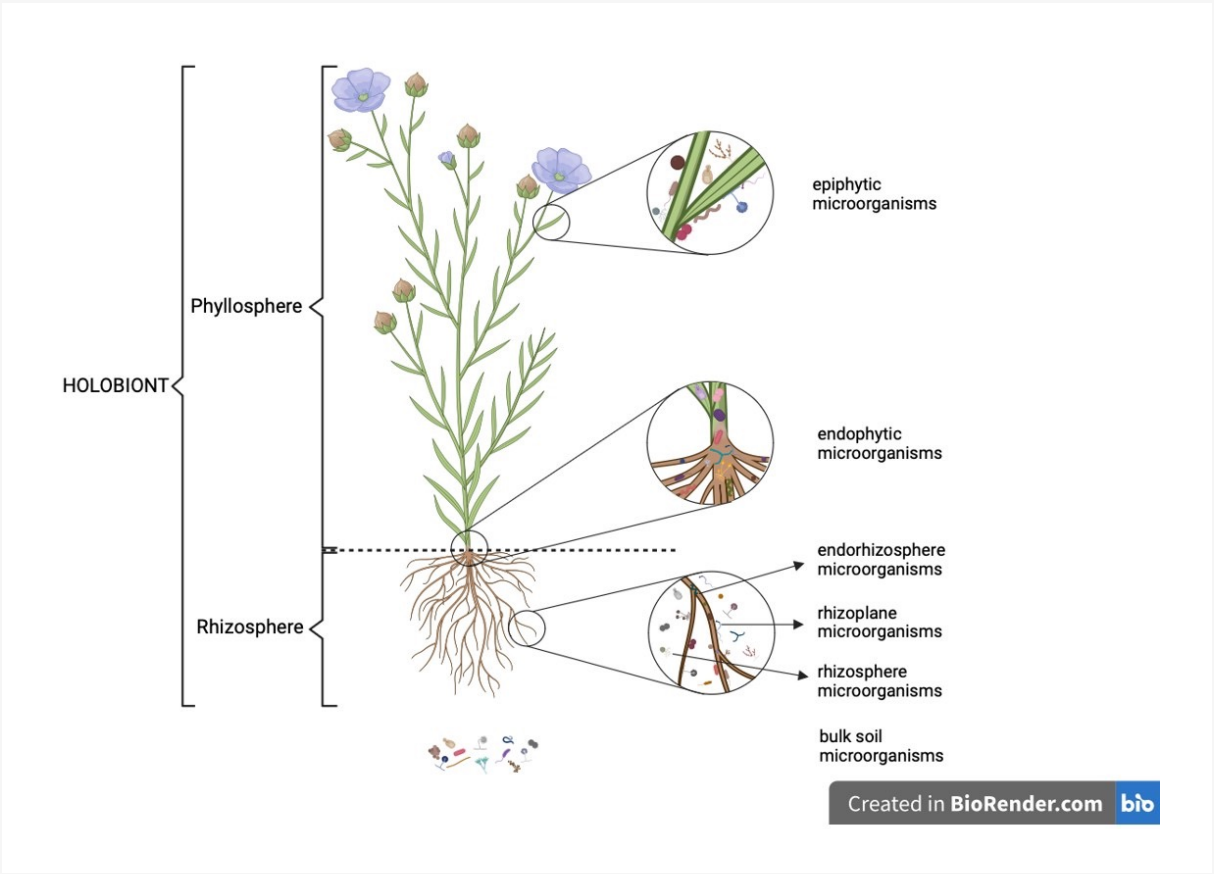

Figure 1. A representative plant with associated microorganisms.

Slika 1. Reprezentativna rastlina s pripadajočimi mikroorganizmi.

was found at metal-polluted and non-polluted sites but was significantly lower or absent on the most polluted sites with the highest concentrations of Cd, Pb, and Zn (Vogel-Mikuš et al., 2005). This observation could mean there is a threshold of metal(loid) concentration that AMF can tolerate and above which mycorrhizal interactions cannot be formed. A greenhouse inoculation experiment on *N. praecox* with an indigenous AMF mixture (Vogel-Mikuš et al., 2006) indicated that AMF association is favoured during the reproductive period of the plant when the requirements for nutrients are elevated. Moreover, inoculated plants showed significant improvement in nutrient uptake and decreased uptake of Cd and Zn compared to non-inoculated plants (Vogel-Mikuš et al., 2006). Pongrac et al. (2007) later confirmed similar results under field conditions. The higher AMF colonization levels during the reproductive phase could protect the plant from potentially toxic metal(loid)s when plants invest the majority of photosynthates to seed production. The protective role of AMF was revealed by their direct effect on Cd accumulation in *N. praecox*, where the accumulation of Cd in the shoots was decreased, preventing potential over-accumulation in the seeds (Pongrac et al., 2007; Vogel-Mikuš et al., 2006). In addition, *N. praecox* (population from Žerjav, Slovenia) and a Cd, Zn, and Ni hyperaccumulating *N. caerulescens* (Ganges ecotype) were successfully inoculated with AMF monospore inocula in a controlled pot experiment. The plants were grown for six months in the commercial substrate and in heavy-metal contaminated, field-collected soil from Žerjav as described previously but without metal amendments (Pongrac et al., 2009). In addition, inoculated treatments in which 100 g of monospore AMF inoculum of *Funneliformis mosseae* or *F. caledonium* (both obtained as *Glomus mosseae* and *G. caledonium* from International Collection of Vesicular Arbuscular Mycorrhizal Fungi; reference numbers UK115 and UK301, respectively) was mixed into the commercial substrate and 100 g of N. praecox indigenous fungal mixture to the heavy-metal contaminated, field-collected soil from Žerjav. After the first three months of growth, plants were exposed to lower temperatures to induce flowering, as this developmental stage has been shown to enhance the formation of the AM symbiosis in *N. praecox* (Pongrac et al., 2007; Vogel-Mikuš et al., 2006) and plants were allowed to grow for three more months. At harvest, roots and shoots were separated, and carefully washed with deionized water, and a subsample of roots from all treatments was retained for AMF staining (Phillips & Hayman, 1970) and AMF colonization parameters (Vogel-Mikuš et al., 2006), whereas the rest of the shoots and roots were dried and weighed (dry weight). The concentrations of phosphorus (P), Zn, and Cd were determined after wet digestion using atomic absorption spectrometry (for Zn and Cd; (Pongrac, Zhao, et al., 2009)) and following the vanado-molybdate method according to Olsen & Sommers (1982) for P). In roots of inoculated *Noccaea* species AMF structures (hyphae, arbuscules, vesicles and coils) were observed (Fig. 2A). There was a stronger statistical difference between the species than between treatments observed in the dry biomass (Fig. 2B,). Only for *N. caerulescens* there was a significant decrease in root dry weight for inoculated treatments of commercial soil (Fig. 2B).

Interestingly, inoculation with *F. caledonium* resulted in the highest shoot P, Zn and Cd concentrations for both species, compared to the control commercial substrate and the *F. mosseae*-inoculated commercial substrate (Fig. 3). In field-collected soil, the inoculation with *N. praecox* indigenous inoculum increased Cd concentration in shoots only. In root Cd concentration, there was a decrease in the commercial soil-inoculated treatments, indicating an increase in the Cd translocation factors (Fig. 3). Again, in roots from the polluted site in Žerjav, AMF structures with distinct arbuscules have been observed (Fig. 4A).

Other hyperaccumulating species have also been reported to form AM. For example, in As hyperaccumulating fern *Pteris vitatta* (Pteridaceae), an increase in the translocation of As has been observed in AMF-inoculated plants compared to non-AMF-inoculated plants (Trotta et al., 2006). In an effort to bridge the gap in our understanding of the distribution and the ecology of AMF, the GlobalAMFungi database (https://globalamfungi.com) has been recently developed (Větrovský et al., 2023). It will enable us to understand better the fungal taxa that form this remarkable interaction with plants.

Dark septate endophytes associated with hyperaccumulators

Another group of endophytic fungi has been discovered and studied extensively, especially with plants growing in extreme environmental conditions, namely dark septate endophytes (DSEs). DSEs comprise a miscellaneous group of ascomycetous anamorphic groups colonizing the plants inter- and/or intracellularly (Jumpponen, 2001). They have been found to colonize about 600 plant species, classified

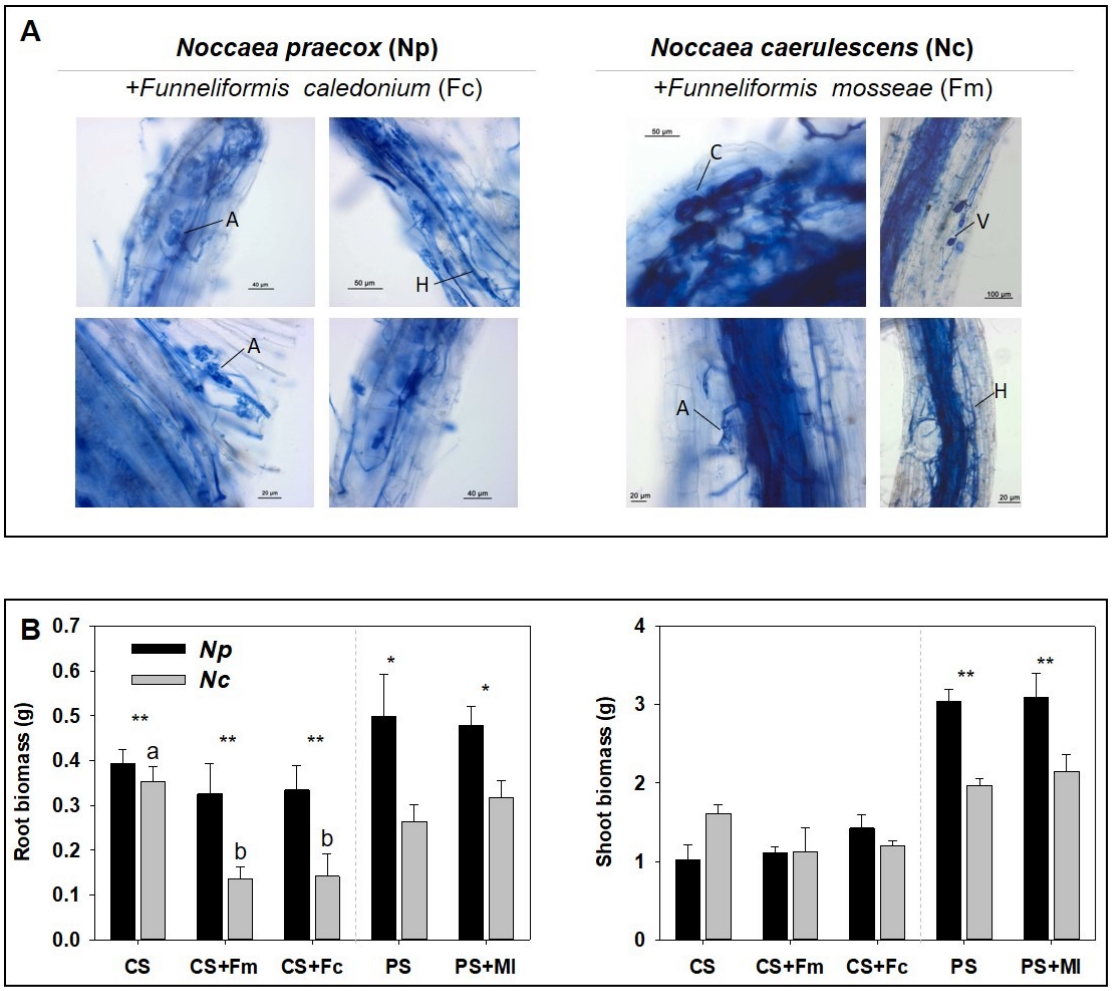

Figure 1. Arbuscular mycorrhizal (AM) structures (hyphae, H; arbuscules, A; coils, C and vesicles, V) in roots of *Noccaea praecox* (Np) and *N. caerulescens* (Nc) inoculated by AM fungi *Funneliformis mosseae* (Fm) or *F. caledonium* (Fc) in a pot experiment (A) and root and shoot biomass of six months old plants (B) grown in commercial soil (CS) and inoculated with *F. mosseae* (CS+Fm) or *F. caledonium* (CS+Fc) or grown in heavy-metal polluted, field-collected soil (PS) and inoculated with Np-indigenous AM inoculum (PS+MI). Different letters above columns present statistically significant differences between treatments (at $p<0.05$) for CS and PS separately, and asterisks indicate statistically significant differences between species (*, $p<0.05$; **, $p<0.01$;) as determined by two-way ANOVA and Holm-Sidak post-hoc test. Shown are means (n=3) + standard errors.

Slika 1. Arbuskularne mikorizne (AM) strukture (hife, H; arbuskule, A; tuljave, C in vezikule, V) v koreninah *Noccaea praecox* (Np) in *N. caerulescens* (Nc), inokuliranih z AM glivami Funneliformis mosseae (Fm) ali *F. caledonium* (Fc) v lončnem poskusu (A) ter biomasa korenin in poganjkov šest mesecev starih rastlin (B), gojenih v komercialni zemlji (CS) in cepljenih z glivami *F. mosseae* (CS+Fm) ali *F. caledonium* (CS+Fc) ali gojenih v s težkimi kovinami onesnaženi, na polju zbrani zemlji (PS) in cepljenih z inokulumom Np-indigenous AM (PS+MI). Različne črke nad stolpci predstavljajo statistično pomembne razlike med obravnavami (pri $p<0,05$) ločeno za CS in PS, zvezdice pa označujejo statistično pomembne razlike med vrstami (*, $p<0,05$; **, $p<0,01$;), kot je bilo določeno z dvosmerno ANOVA in Holm-Sidakovim post-hoc testom. Prikazana so povprečja (n=3) + standardne napake.

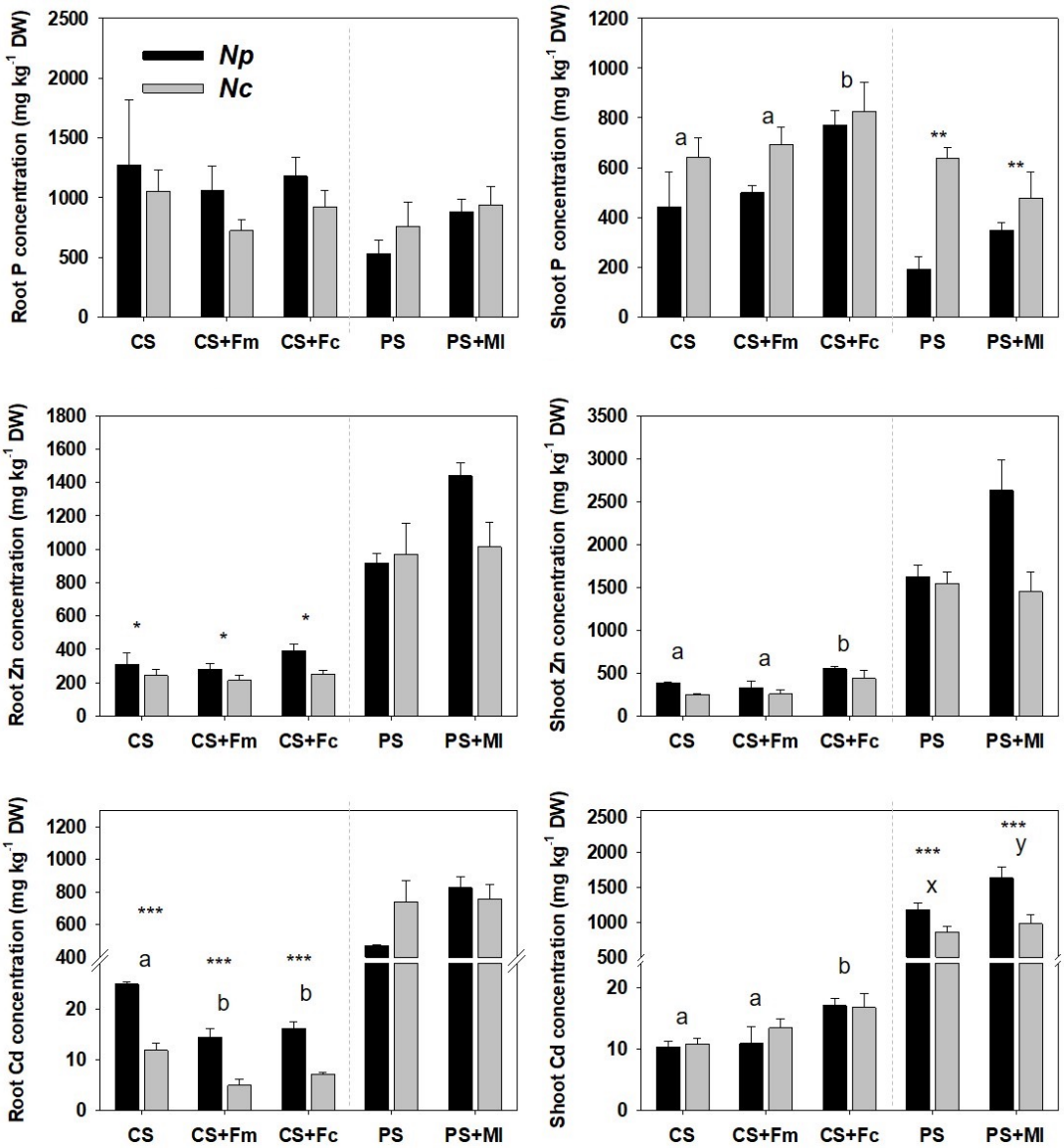

Figure 3. Concentration of phosphorus (P), zinc (Zn) and cadmium (Cd) in roots and shoots of *Noccaea praecox* (Np) and *N. caerulescens* (Nc) grown in commercial soil (CS) and inoculated with monospore culture of arbuscular myorrhizal fungi *Funneliformis mosseae* (CS+Fm) and *F. caledonium* (CS+Fm) or grown in heavy-metal polluted, field-collected soil (PS) and inoculated with Np-indigenous mycorrhizal inoculum (PS+MI). Different letters above columns present statistically significant differences between treatments (at $p<0.05$) for CS and PS separately, and asterisks indicate statistically significant differences between species (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$) as determined by two-way ANOVA and Holm-Sidak posthoc test. Shown are means (n=3) + standard errors. DW, dry weight.

Slika 3. Koncentracija fosforja (P), cinka (Zn) in kadmija (Cd) v koreninah in poganjkih *Noccaea praecox* (Np) in *N. caerulescens* (Nc), gojenih v komercialni zemlji (CS) in inokuliranih z monosporno kulturo arbuskularnih mioriznih gliv *Funneliformis mosseae* (CS+Fm) in *F. caledonium* (CS+Fm) ali gojene v s težkimi kovinami onesnaženi, na polju zbrani zemlji (PS) in inokulirane z mikoriznim inokulumom Np-avtohtonih mikoriz (PS+MI). Različne črke nad stolpci predstavljajo statistično značilne razlike med tretiranji (pri $p<0,05$) ločeno za CS in PS, zvezdice pa označujejo statistično značilne razlike med vrstami (*, $p<0,05$; **, $p<0,01$; ***, $p<0,001$), določene z dvosmerno ANOVA in Holm-Sidakovim posthoc testom. Prikazana so povprečja (n=3) + standardne napake. DW, suha masa.

into 320 genera and 114 families (Jumpponen & Trappe, 1998). DSEs benefit their host by facilitating carbon, nitrogen, and phosphorus uptake (Vergara et al., 2018; Yakti et al., 2018). Furthermore, they can induce the release of phytohormones like auxins (Berthelot et al., 2016) and protect plants against abiotic stress (Wang et al., 2016). It is known that DSEs colonize extreme habitats, like high salinity sites (Sonjak et al., 2009), dry habitats (Barrow, 2003), and metal-enriched soils (Ban et al., 2012; Likar & Regvar, 2009, 2013). Although most studies have debated the beneficial role of DSEs (Ban et al., 2012; Likar & Regvar, 2009, 2013; Newsham, 2011), some studies proposed that DSE taxa can form parasitic associations with their host (Wilcox & Wang, 1987).

In metal-enriched soils, DSEs have been shown to positively impact plant fitness. A particular plant species where DSEs frequently colonize the roots, especially in highly metal-contaminated soil, is goat willow (*Salix caprea* L.; Salicaceae). The study by Likar & Regvar (2013) revealed smaller Cd and Zn leaf concentrations, larger chlorophyll concentrations and transpiration in DSE-inoculated *S. caprea* grown in metal-enriched soils compared to non-DSE-inoculated plants. It has been proposed that the binding of Cd and Zn to melanin in DSE may, through limiting the translocation of heavy metals, contribute to these observations (Potisek et al., 2021) in accordance with reports by Ban et al. (2012). These observations suggested that DSE associations are favorable for *S. caprea* under metal-enriched conditions. Furthermore, a larger diversity of DSE was found at sites with higher metal concentrations compared to low soil-metal concentrations (Likar & Regvar, 2009).

Although several studies of DSEs were performed on non-hyperaccumulators (Ban et al., 2012; Barrow, 2003; He et al., 2019; Likar & Regvar, 2013; Stoyke & Currah, 1991), little is known about the associations between DSEs and hyperaccumulators. In a recent study DSE strains isolated from the roots of poplar growing at different trace element contaminated sites were used to successfully inoculate *N. caerulescens* (Yung et al. 2021). Moreover, in the highly contaminated soil, a specific strain of DSEs significantly increased the root biomass of *N. caerulescens* compared to the non-inoculated plant without affecting the nutrient status of the plant (Yung et al., 2021). The positive effect of DSE strains was also observed in the accumulation of Zn and Cd, which was larger in the roots in highly contaminated soil with inoculation of DSE (30% and 90% more, respectively) compared to non-DSE-inoculated plants (Yung et al., 2021). The DSEs were also observed in roots of *N. praecox*, a Cd and Zn hyperaccumulating plant, when grown on a highly contaminated site in Žerjav (Slovenia) (Fig. 4B) – an area still affected by the past mining and smelting activities (Bočaj et al., unpublished; Pongrac et al., 2009). DSEs may be important partners for the host, but more focused research is required, ideally by using DSE inoculum and performing controlled experiments to evaluate effects in different plants in different soils.

Are, therefore, DSEs mycorrhizal or not? Jumpponen (2001) claimed that at least under some conditions, DSE should be treated as a mutualistic partner, in agreement with Treu et al. (1996). By contrast, Brundrett (2006) disputes this by pointing out that in contrast to mycorrhizal symbiosis, the relationship between host plant and endophyte (e.g., DSE) lacks three features typical for arbuscular mycorrhiza: a cellular interface where specialized structures (e.g., arbuscules in AM) occur, but the same is true for ectomycorrhizal fungi. The synchronization of the development between the plant host and fungi and the benefits for both partners in the interaction are those that count. This demonstrates that there is a thin and often unclear line between beneficial or harmful, mycorrhizal or not.

Rhizosphere epiphytic microorganisms may also play an important role in hyperaccumulation

Sometimes overlooked in comparison to AMF and DSEs is the rhizosphere MO, one of the most complex microbial communities on the earth with a large number and rich diversity (Mendes et al., 2013) because they play a crucial role in plant growth and development, nutrient acquisition, pest prevention, and yield improvement (Mendes et al., 2011). The diversity and complexity of the soil as a substrate and a habitat are reflected in different microbial communities between bulk soil and rhizosphere MO, as well as between rhizosphere and rhizoplane (i.e., the external surface of the root, including closely adhering soil particles). The involvement of rhizosphere and rhizoplane MO in the ability of plants to hyperaccumulate metal(loid)s have been only poorly investigated so far. In a Cd and Zn hyperaccumulating *Sedum alfredii* (Crassulaceae), the diversity of four spatial compartments: bulk soil, rhizosphere, rhizoplane, and endosphere revealed that regardless of the soil type or genotype of *S. alfredii*, diversity diminished from rhizosphere to rhizoplane and

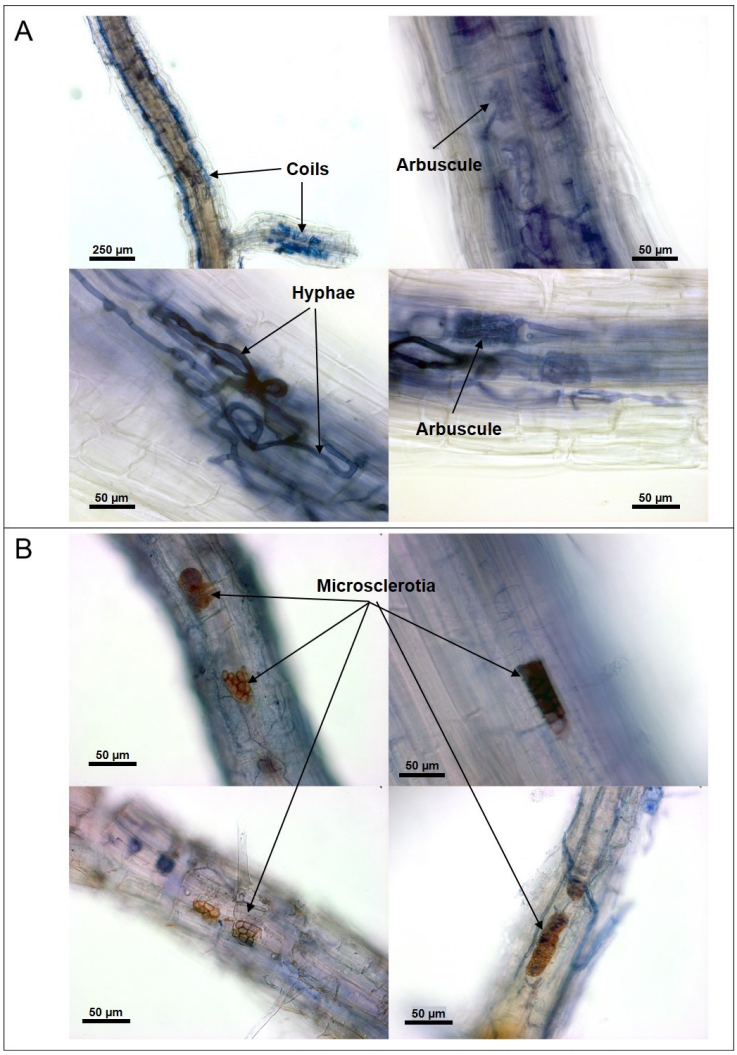

Figure 4. Representative structures of arbuscular mycorrhizal fungi (A) and dark septate endophytes (B) in roots of *Noccaea praecox* collected in Žerjav (Slovenia) in 2023. Roots were stained following the standard Tripan blue staining protocol (Philips & Haymann, 1970).

Slika 4. Reprezentativne strukture arbuskularnih mikoriznih gliv (A) in temnosemenskih endofitov (B) v koreninah *Noccaea praecox*, nabranih v Žerjavu (Slovenija) leta 2023. Korenine so bile obarvane po standardnem protokolu barvanja s Tripan blue (Philips & Haymann, 1970).

endosphere (Luo et al., 2017). By contrast, the diversity increased from the bulk soil to the rhizosphere, which was confirmed by Xiao et al. (2021) and later by Kushwaha et al. (2022), claiming that the diversity is larger close to the roots than in nearby soil. This may be attributed to plant exudates, microbial chemotaxis towards exudates and nutrients (García-Salamanca et al., 2012), and changes in pH caused by plant exudates (Fan et al., 2017).

Interestingly, the taxa richness of MO was reported to differ between hyperaccumulators and non-hyperaccumulators. For example, comparing several Se-hyperaccumulators and non-hyperaccumulators from the same family sharing the same growing site revealed substantial differences in rhizosphere communities, with hyperaccumulators harboring a larger number of rhizobacterial species (Cochran et al., 2018). Similarly, a study by Martos et al. (2021) showed that in hyperaccumulators from the Brassicaceae an enrichment in previously described metal-tolerant bacteria and bacteria involved in nitrogen cycling was found compared to non-hyperaccumulating species (Martos et al., 2021). Unfortunately, these studies have so far been conducted on confamilial species and therefore, further in-depth species-specific investigations are required before meaningful conclusions can be made.

The importance of soil MO communities for hyperaccumulation was studied by Muehe et al. (2015), who

compared the microbial community of *A. halleri* growing on metal-contaminated and gamma-irradiated soil, the latter having decreased diversity and species richness. Although no differences in aboveground biomass were observed during the experiment between treatments, *A. halleri* grown in untreated soil accumulated significantly more Cd and Zn (100% and 15%, respectively) than when grown in gamma-irradiated soil (Muehe et al., 2015). Mechanisms by which MO improve accumulation may lie in their ability to improve the bioavailability of metal(loid)s (Audet & Charest, 2007). Whiting et al. (2001) used sterile soil where Zn was initially unavailable in water-soluble forms. Inoculation of *N. caerulescens* seeds with bacteria increased the solubility of Zn in the rhizosphere, resulting in a higher concentration of water-extractable Zn compared to axenic conditions (Whiting et al., 2001). In highly contaminated sites where hyperaccumulation could cause toxicity to plants, MO can also play an important role in reducing the bioavailability of metal(loid)s. Such an effect was shown for the Ni accumulation in *N. caerulescens* whose inoculation with Ni-resistant bacteria did not result in the increase in Ni uptake, but it reduced the bioavailability of Ni in the serpentine soil and promoted plant growth compared to the axenic control (Aboudrar et al., 2013).

All the above findings indicate the significant importance of soil MO on hyperaccumulation by altering metal(loid) bioavailability either positively or negatively.

Biological cleaners of metal(loid)s pollution and predictions

The use of hyperaccumulators to sustainably and in an environmentally friendly way remove toxic metal(loid) concentrations from the soil with commercial viability has been demonstrated for Ni only because plants with sufficient biomass and Ni hyperaccumulation capacity have been identified. For other metal(loid)s, the main constraint remains the small aboveground biomass of hyperaccumulators and/or limited accumulation capacity. Therefore, efforts into the discovery of new hyperaccumulators or enhancing biomass and hyperaccumulation capacity of known hyperaccumulators are essential if we are to remove other metal(loid)s using plants. According to Ernst (2005), an ideal plant for phytoextraction would have deep and well-branched root systems with AMF associations to take up as much metal(loid)s as possible, have efficient root-to-shoot translocation with binding capacity in the roots smaller than in the shoots, without any biomass penalties. In addition, herbivores should not be attracted to these plants to prevent transmission through food chains, and harvesting should be easy and possible with conventional agricultural methods (Ernst, 2005). There is preliminary evidence showing that MO can affect several of these traits, therefore it may be viable to tailor MO associated with hyperaccumulators. However, our understanding of MO and their role in hyperaccumulation remains scarce. In this review, we underlined the indispensable role of MO for their hyperaccumulating hosts. Current knowledge of the link between functional profiles and ecological functions in the MO communities is still scarce, and several questions, including the AM status of hyperaccumulators from the Brassicaceae, remain open. With the advances in analytical methods, such as cheaper and high-throughput next-generation sequencing and metabolomics, we can expect ground-breaking advancements in this novel field of research. Furthermore, with a better understanding of the hyperaccumulating plants and their interactions with MO, phytoremediation efforts may be improved and developed to the point that it becomes applicable to a variety of environmental remediation strategies.

Author Contributions

Conceptualization, M.R. and P.P.; investigation, V.B.; writing—original draft preparation, V.B.; writing—review and editing, M.R. and P.P.; supervision, P.P.

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Conflicts of Interest

The authors declare no conflict of interest.

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ABS

Short Note

Growth and development of *Hyssopus officinalis* L. (Lamiaceae) under the conditions of Karshi oasis

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Abstract

The article describes some bioecological features of the hyssopus (*Hyssopus officinalis* L.) species introduced to the Karshi oasis of Uzbekistan. The growth and development of the plant, introduced for the first time to the conditions of the Karshi oasis, were studied. In the present study, information about the stages of ontogenesis of the hyssopus and their description is given, and the period and stages of ontogenesis are highlighted. The results of the study showed that the studied species goes through the stages of seedlings, juvenile, immature and virginal in the first year of ontogenesis. The juvenile stage lasted 14-16 days, and the virginal stage lasted 132-138 days. It was revealed that seed germination was 82% in laboratory conditions and 76% in field conditions, so it was recommended to sow seeds in open areas in March. It has been determined that in the first year of life, the plant develops branches up to the third order, and it enters the generative phase in the second year of life. In the conditions of the Karshi oasis, it was observed that the hyssopus plants did not enter the subsenile stage in the three experimental years and also in eight years from the original plant from which seeds used in this experiment derived.

Keywords

medicinal hyssop, seeds, ontogenesis, growth, stage, development, bloom

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Rast in razvoj *Hyssopus officinalis* L. (Lamiaceae) v pogojih oaze Karshi

Izvleček

V članku so opisane nekatere bioekološke značilnosti vrste *Hyssopus officinalis* L. , ki je bila vnešena v oazo Karshi v Uzbekistanu. Preučena sta bila rast in razvoj rastline, ki je bila prvič vnesena v razmere oaze Karshi. V pričujoči študiji so podani in izpostavljeni podatki o fazah ontogeneze ožepka. Rezultati študije so pokazali, da gre preučevana vrsta v prvem letu ontogeneze skozi stadije kalic, mladostnih, nezrelih in deviških rastlin. Mladostni stadij je trajal 14-16 dni, deviški stadij pa 132-138 dni. Ugotovljeno je bilo, da je bila kaljivost semen v laboratorijskih pogojih 82%, v poljskih pa 76%, zato je bilo priporočeno, da se semena sejejo na prosto marca. Ugotovljeno je bilo, da rastlina v prvem letu življenja razvije veje do tretjega reda, v generativno fazo pa preide v drugem letu življenja. V oazi Karshi rastline niso vstopile v fazo podrasti, kar velja tako za sejane rastline (po treh letih) kot tudi za izvorne rastline (po osmih letih), na katerih smo nabrali semena za poskus.

Ključne besede

medicinski svišč, semena, ontogeneza, rast, faza, razvoj, cvetenje

Introduction

In the Republic of Uzbekistan, great attention is paid to increasing the number of pharmaceutical enterprises that serve to provide the population with quality pharmaceutical and medicinal plant raw materials, and certain results are achieved. Based on this point, the introduction of medicinal plants in the arid regions of our republic and the study of their growth and development under the conditions of introduction are of great scientific and practical importance.

Hyssopus officinalis L. – hyssop officinalis is an evergreen plant belonging to the mint family (Lamiaceae). One of the most valuable medicinal species is due to the accumulation of a large amount of essential oil (0,90-1,98% in leaves). The essential oil contains α- and β-pinenes, 1-pinocamphone, 1-pinocampheol and its acetic esters, aldehyde, camphene, seniolum, acetic acid, alcohol compounds and sesquiterpenes. Since ancient times, many peoples considered hyssop medicinal as a sacred herb, and it was widely cultivated as a medicine, essential oil and spice in the national economy (Fathiazad F. and Hamedeyazdan S., 2011; Libus et al., 2004; Mitic, Dordevic, 2000; Nasriddinova, 2020).

In pharmaceuticals, the essential oil obtained from the plant is mainly used to improve the smell of ointments and other medicines applied to the surface, and due to its antiseptic properties, it is used as a remedy for burns. Tinctures and herbal infusions are used as a remedy for shortness of breath, bronchitis, bronchial asthma and other respiratory diseases. Includes sedatives and dietary supplements for the treatment and prevention of respiratory diseases. Hyssop oil is widely used in the food industry (Libus et al., 2004; Gaspar-Pintiliescu A. et al., 2022).

The species *H. officinalis* is not found in the natural flora of Uzbekistan. The plant was first cultivated in 1930-1934 by Kudryashov (1936) and was introduced by the Central Asian State University (now UzMU) in the conditions of the Botanical Garden, where an introduction test was conducted.

First, Khodjaev Kholmatov (1965) planted and introduced seedlings from seeds brought from VILAR (All-Russian Research Institute of Medicinal and Aromatic Plants) into the Botanical Garden of the Academy of Sciences of the Republic of Uzbekistan, and a number of studies were conducted on the plant under these conditions. In particular, Khodjaev studied the bioecological features, Khodjimatov and Ramazanova (1975) studied the biological properties, and Toshmatova (1981) studied the biology of flowering and developed propagation methods. Murdakhaev (1992) studied its phenology and productivity and noted that this is an evergreen plant since it is not damaged in winter and does not shed its leaves in open ground.

The purpose of the research is to introduce the species *H. officinalis* into the conditions of the Karshi oasis and to study the characteristics of growth and development at different stages of ontogenesis.

Materials and Methods

Hyssop officinalis is a perennial evergreen plant with a pungent odour, 50-80 cm high. Its homeland is the Mediterranean countries (Western Europe, Crimea, Caucasus, Iran) and Central Asia (Southern Kazakhstan and Kyrgyzstan). In nature, it grows on mountain slopes in dry places, on small rocky cliffs, among trees and bushes in the foothills and middle parts of the mountains. The border of vertical distribution is 1400 m above sea level (Kudryashov, 1936; Kalinichenko, 2013).

Murdakhayev (1992) included this plant in the evergreen group since it remains green without shedding its leaves even during the winter months in Tashkent.

H. officinalis, first introduced to the Karshi Oasis in 2014, was grown from seeds brought from the Botanical Garden of Latvia. The plants were grown on the experimental plot of the Department of Botany of the Karshi State University. Initially, the plants grew well in the new conditions and had the following morphological characteristics: Root - arrow-root. Stem erect, 4-sided, the main part woody, branched. Leaves glabrous or lanceolate with short bands, opposite on the stem. Flowers dark blue, two-lipped, collected in inflorescences of a raceme 20-22 cm long. The fruit consists of an ovoid nut. Seed long-ovoid.

Experiments were conducted in laboratory and field conditions to determine the germination characteristics of *H. officinalis* seeds grown in the Karshi oasis. The seeds for the experiment were collected from plants grown in the scientific experimental section of the Department of Botany of Karshi State University. The quality of seeds was studied according to the recommendations of some researchers (Firsova, 1959; Levina, 1981). To determine the quality indicators of seeds, the size (length and width) of seeds one pc., the weight of 1000 seeds, seed purity and germination were determined.

One thousand seeds were measured in 3 replicates to determine the weight of the seeds. To determine the size of the seeds, the height and width of 10 seeds were measured 3 times in repetition. The results were analyzed using the mathematical statistics method of Zaitsev (1991) and the Microsoft Excel program (2010). The experimental data were expressed as mean ± standard error of the mean (SE).

When determining the purity of seeds, their full ripeness, integrity, and presence of straw impurities were checked. For this, three samples of 5.0 g each were selected from the seeds, and each seed sample was examined separately using a magnifying glass, with whole seeds being allocated to one group and straw and broken fragments of seeds between the seeds to another. Each of the separate parts of the seeds was weighed separately, their weight was determined, and the percentage content was determined in relation to the weight of the total sample (5.0 g). According to the results obtained, the selected suitable seeds made up 98.2% of the total weight, while the amount of straw and broken fragments of seeds among the seeds was very insignificant - 1.8%.

Seed germination in the laboratory (Laboratory experiments were conducted in the laboratory of "Plant Physiology" of the Department of Botany of KarSU) conditions were determined by sowing 100 seeds in a Petri dish on filter paper (d=9,0 cm; FM-III) moistened with distilled water, and 3 times in a thermostat at different temperatures (+17°C, +22°C, +24°C). Every day at the same time, the thermostat door was opened, and the air was changed. The lid of the Petri dish was opened for 2-3 minutes to saturate the seeds with oxygen. When the humidity in the dish decreased, and the edges of the filter paper began to dry out, water was added with a pipette. Newly collected seeds of this year and seeds collected in previous years and stored for different periods were used.

The irrigated lands of the Karshi oasis, located at an altitude of 381.88 m above sea level, are composed of pale grey soils with a relatively low humus content (up to 0.8-1.7%). Field experiments were also conducted on the experimental plot of the Department of Botany of the Karshi State University, located in the Karshi oasis. The seeds were sown in the experimental field, consisting of light grey soil, in the first ten days of March (10.03) to a depth of 0.5-1 cm in 3 replicates of 100 seeds.

We studied the ontogenesis of plants according to Rabotnov (1960) and the morphological features according to I.G. Serebryakova (1962).

Results

Laboratory Experiments

To study seed germination in the laboratory, the seeds were placed in batches of 100 in a Petri dish lined with moistened filter paper. The thermostat maintained the temperature at +17 °C, +22 °C and +24 °C. It was found that the optimum temperature for seed germination is +22°C.

After 3-4 days, the embryonic root, hypocotyl, and then yellow-green cotyledons grew from the micropyle of the seeds and began to germinate. The seed germination was 82% (Table 1).

Field Experiments

To study the germination of seeds in the field, in the spring, the seeds planted in the soil at a depth of 0,5-1 cm in 3 replicates of 100 seeds began to germinate on the surface after 8 days. The seed coat remained underground, and seed germination was determined to be underground. The sprouts are very tender. After the seedlings form 4-6 pairs of leaves, they are carefully transplanted to a permanent place. The germination of seeds in the field was up to 76% (Table 1).

In the course of our research, the growth and development of *H. officinalis* in different periods and stages of ontogenesis were studied. Plant ontogenesis was divided into the following periods and stages: latent (*se* - seeds), virginal (*p* - seedling, *j* - juvenile, *im* - immature, *v* - adult virgin), and generative (*g* - generative phase).

Latent period. The seed of the *H. officinalis* species is an oblong-ovoid triangular nut. The colour is light or dark brown, and the surface is smooth. In the observed years, the size and weight of the seeds in the first years were somewhat smaller, and in the following years, they retained their natural size. The length of the seeds is 2,01-2,02 mm, the width is 1,02-1,04 mm, the weight of 1000 seeds is 1,20-1,32 g (Table 1 and Figure 1).

The dormant period of the seeds is short (B1) (Nikolaeva et al., 1985) but retains germination for up to 5-6 years. In the first 3 years, the germination of seeds is 82%. After 4-5 years, 36-58%, and in subsequent years they completely lose fertility.

H. officinalis seeds were sown on open ground in the first ten days of March under the conditions of the introduction of the Karshi oasis.

Virginal period: *Seedling stage* – seeds sown in the first ten days of March under field conditions germinate in 8-12 days. The seed coat remained underground. The size of the cotyledons is 1,7x1.2 mm. The hypocotyl is pink, 3,9 mm long. The length of the main root is 1,5 cm. The seedling stage lasted 5 days.

Juvenile stage – true leaves begin to form when the plant is 5 days old, and the plant enters the juvenile stage. At this time, the flat kidney-shaped cotyledon reaches the size of 2,3x1,5 mm. The length of the main root is 2,7 cm, and it forms up to 6-7 lateral roots. At the age of 10 days, its height reaches 1.8 cm, and it forms 3-4 pairs of true leaves (Figure 1). The juvenile stage lasted 15 days.

Table 1. Results of laboratory and field experiments on *H. officinalis* seed germination in the Karshi oasis.

Tabela 1. Rezultati laboratorijskih in poljskih poskusov kalitve semen *H. officinalis* v oazi Karshi.

Laboratory conditions			Field conditions	
Temperature (°C)	duration of seed germination (days)	germination rate (%)	years	germination rate (%)
16-17	4–12	57	2018	68
21-22	3–9	82	2019	74
24-25	3–8	75	2020	76

Table 2. The obtained results on the size and weight of seeds collected from the species *H. officinalis* grown in the conditions of the Karshi oasis (mean ± SE).

Tabela 2. Dobljeni rezultati o velikosti in masi semen, zbranih iz vrste *H. officinalis*, gojene v pogojih oaze Karshi (povprečje ± SE).

Observed years	Seed size, mm		Weight of 1000 seeds, g
	length	width	
2018	2.01±0.040	1.02±0.041	1.20±0.040
2019	2.02±0.044	1.04±0.032	1.32±0.022
2020	2.02±0.041	1.03±0.035	1.32±0.021

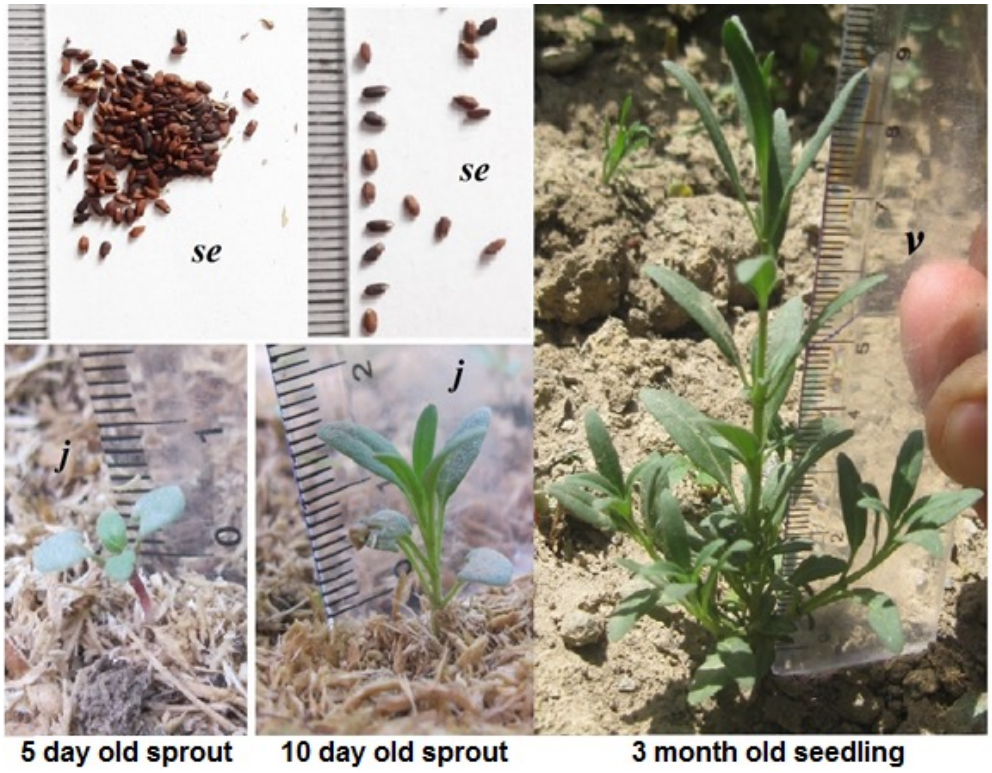

Figure 1. Period and stages of ontogenesis of *H. officinalis*: c – seeds, j – juvenile stage plants, v – adult plants in the virgin stage

Slika 1. Obdobje in faze ontogeneze *H. officinalis*: c - semena, j - mladostne rastline, v - odrasle rastline v deviški fazi

The *immature stage* is when the plant is 20 days old, its height reaches 6.6 cm, and 7-8 pairs of true leaves appear. During this period, the plant begins to form second-order branches. The cotyledon plate, 3,2 mm long and 2,3 mm wide, stops growing and begins to turn yellow. The length of the main root is 8 cm, in which the lateral roots of the second order begin to develop.

At the age of one month, the plants demonstrate rapid growth and development and reach a height of 9,6 cm. They form 12-15 pairs of true leaves and second-order branches. At this time, the cotyledons begin to fall off. They live in the plant for 35-40 days, the size is 5,1x3,8 mm. Lateral roots of the third order begin to form in the root system.

In a 3-month-old plant, the growth of the main branches and branches of the second order accelerates. In this case, the length of the main branch of the first order is 9.5 cm, the length of the branches of the second order is 4.6 cm, and 25-30 pairs of leaves are formed in them. The immature stage is 77 days. At this time, branches of the third order begin to form, and the plant enters the adult virgin stage (Figure 1).

According to the literature, in the conditions of southern Uzbekistan during the extreme period of summer, the branches of most trees and shrubs stop growing. The fact that after this period, they begin to grow again has been proven by studies conducted by scientists (Yaziev, 2001). This pattern was also noted in our observations in the conditions of the Karshi oasis in the species *H. officinalis*, which is a semi-shrub form in life form.

The seedlings stop growing in the second half of June and start growing again at the end of August. At this time, the plant is 6 months old, and its height is 11,9 cm. Warm and cool temperatures in the autumn months ensure rapid growth of the plant. Low temperatures in December stopped the growth of *H. officinalis* seedlings.

It is noted in the literature that the height of virginal plants (1-year-old) was 45-60 cm in Tashkent and 25-55 cm

in Leningrad (Tashmatova, 1981). In the Karshi oasis, their height was 63,1 cm, the number of second-order branches in the plant reached 11-12 pairs, and the number of third-order branches was 6-8 pairs. At this age, the plant did not develop generative organs. At the end of the first year of vegetation, the virgin period ended, and in the Karshi oasis, it was 138 days.

H. officinalis seedlings overwintered in the open air. No cases of cold damage were observed in them. In early spring - from the second ten days of February, plants begin to grow. The development of generative organs was observed in the plant in late April - early May.

Generative period – It is noted in the literature that in Tashkent conditions, the plant blooms in the first year (Tashmatova, 1981). In our studies, the transition to the generative period was noted in the second year of plant vegetation in the conditions of the Karshi Oasis. At first, inflorescences are formed on the main branches and second-order branches. The number of flowers in the second year of vegetation reaches 32-38, in the third year - 74-90, and in the fourth year - 72-86.

One flower in the inflorescence lasts 2-3 days, one inflorescence lasts 25-30 days, and one plant blooms 97-105 days. It was established that the flowers of *H. officinalis* belong to the day group.

The budding phase coincided with the end of April - the beginning of May. Full flowering began 12-15 days after the beginning of the opening of flowers and continued this period in May- June.

It was noted that the flowering phase lasts from May to August, and the fruiting process lasts from the third decade of May to the first ten days of August. In May-August, buds, flowers, and fruits can be found in one plant at the same time. The generative period was 97-105 days.

In our phenological observations in 2014-2023 in the Karshi Oasis, the senile period of the *H. officinalis* species was not noted. According to the literature, the ageing period of this species is observed at the age of 10 years (Gladysheva, 2016.).

Discussion

Research on the introduction of *H. officinalis* and the study of its bioecological features in Uzbekistan is insufficient. Very few introduction studies conducted in these areas were done many years ago.

The germination rate of *H. officinalis* seeds, introduced in Tashkent, in field conditions, is 61%. Under these conditions, the plant enters the flowering phase in the second year of vegetation and blooms from the second decade of May (Kudryashov, 1936). Research in the following years showed that in the Tashkent Botanical Garden, *H. officinalis* enters the flowering phase in the first year of vegetation (Toshmatova, 1981). The germination rate of *H. officinalis* seeds, introduced in the Karshi oasis, in field conditions was 76%. Under these conditions, the plant entered the generative period in the second year of vegetation.

In the first year of vegetation, the height of *H. officinalis* in Tashkent conditions was 45-60 cm, in Leningrad conditions - 25-55 cm, and in the Karshi oasis - 60-65 cm.

Observations carried out in the laboratory "Introduction of medicinal plants" of the Tashkent Botanical Garden showed that in 2023, the vegetation processes of *H. officinalis* began 7-10 days earlier (02.23.2023) than in 2022 (03.02.2022). In the conditions of the Karshi oasis, it was noted that vegetation began in the first half of February 2023.

It is obvious that the conditions of the Karshi oasis are favourable for the *H. officinalis* species, and the plant begins vegetation much earlier than in other regions. The height has also increased relative to plants grown in other regions and, accordingly, forms many branches. As a result, a lot of biomass is produced.

Conclusions

Based on the research results, the species *H. officinalis* was introduced for the first time in the conditions of the Karshi oasis. It was found that the seeds of the *H. officinalis* species grown in the new conditions have a short dormant stage, remain viable for up to 3 years, and the optimal temperature for their germination is +20-22 °C. Seed germination was 82% in laboratory conditions and 76% in the field.

It has been established that in the first year of its ontogenesis in the conditions of the Karshi oasis, *H. officinalis* goes through the stages of seedlings: juvenile, immature and virginal. The seeds of plants sown in the field, after 8-12 days, germinated and formed cotyledons, and the seedlings stage lasted 5-6 days. The juvenile stage lasted 14-16 days, and the virginal stage lasted 132-138 days. It was found that seedlings wintered in the open air are not damaged by frost. It was observed that the height of a

well-developed annual plant was higher than that of plants of the same age grown under other conditions.

It has been established that the *H. officinalis* species enter the generative period in the second year of its ontogenesis in the conditions of the Karshi oasis and form up to 25-30 inflorescences. In the third year of vegetation, the number of inflorescences reaches 74-90, and in the fourth year, 72-86. It was noted that one flower in the inflorescence lasts 2-3 days, one inflorescence - 25-30 days, and one plant blooms 97-105 days.

Based on the research results, it is recommended that the seeds of the species *H. officinalis* be sown in open fields in the first or second decade of March.

Author Contributions

M.N. carried out all research work and writing. The author has read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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Acta Biologica Slovenica 2025 Vol. 68 | Št. 2