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Metabolites Profiling, and Evaluation of Antifungal Activity and Biochemical Characterization of Endophytic Bacteria Isolated from Coneflower (*Echinacea Purpurea*)

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Abstract:

Background and Objectives: Echinacea purpurea is an herbal medicinal plant native to North America and commonly called purple coneflower. It has various health benefits including anti-inflammatory, antimicrobial, antiseptic, and anticancerproperties. The active substances like secondary metabolites and different essential oils are responsible for its health values. This study aims to isolate the endophytic bacteria from Echinacea purpurea and evaluate these isolates for their antifungal activities. **Material and method:** The plant leaf, root, flower petal and stem samples of Coneflower (Echinacea purpurea) were collected, sterilized, and crushed. Crushed samples were then cultured directly on the nutrient agar media. Grown isolate colonies were identified by 16S rRNA gene sequencing method followed by BLAST. Each isolate was cultured in LB medium and then antifungal compounds was extracted using ethyl acetate and tested against the target fungal strains. Metabolites profiling was done using GC-MS and biochemical tests were performed. **Result:** 3 out of 16 isolates exhibited decent antifungal activity against selected pathogenic fungal strains. These 3 isolates were Eb6c, Eb8a, and Eb13 i.e.H. rubrisubalbicans, H. huttiens and P. dispersa respectively. They shown eminent antifungal activity against Fusarium, Aspergillus and Candida species. Metabolites responsible for antifungal activity such as 1-Heptadecene, 1-Nonadecene were analysed using GC-MS.Biochemical tests detected the IAA production, phosphate solubilisation, siderophore production, N_2 fixation and ACC deaminase activity.**Conclusion:** The study revealed a diverse community of endophytes that can produce bioactive compounds like those in the host plant. Besides, the study identified specific endophytic strains showing promising antimicrobial activities. The isolates perform a great biocontrol and biofertilizer benefits to the host plant.

Keywords: Echinacea purpurea, Endophytes, Bacteria, Bioactive compounds, Antifungal.

Introduction:

Echinacea purpurea is a perennial herbaceous plant also known as purple coneflower[1]. It is native to North America. It belongs to the Asteraceae family and spread along the wild of the southeastern and Midwest of the United

States[2]. It has been used in traditional medicines for centuries to support immune health[3]. The Indian origin of Echinacea is Andrographis paniculate known as ‘‘kalmegh’’[4]. It has gained extensive attention due to its ability to treat colds and other respiratory tract infections[5].

Research has been conducted to investigate the potent health benefits of the plant. Scholars advocate that it acts as an antioxidant, stimulates the immune system and lessens inflammation[6]. It is also known as an ‘‘anti-infectious’’ agent because of its property to treat various bacterial and viral infections. The plant extract of E.purpurea shows inhibitory effects against pathogens like Candidaalbicans and Saccharomyces cerevisiae[8], Streptococcus pyogenes, Haemophilus influenzae[9], and Legionella pneumophila[10]. It is used to cure several infections like acne and ulcers. It shows promising results in reducing the symptoms of rheumatoid arthritis and other inflammatory disease. The plant has also been observed to show mosquitocidal activity[7].

Endophytes present within the plants play an important role in synthesising these bioactive metabolites. They have been found to produce secondary metabolites responsible for the plant’s medicinal properties, including its analgesic, anti-inflammatory, and immune-boosting effects[11].Bioactive secondary metabolites like chicoric acid, alkamides, caffeic acid derivatives, and polysaccharides are the main groups[12]. 10 alkamides, primarily with isobutylamide and 2-methylbutylamide components were isolated from the n-hexane extract of the roots of plant[13]. While the chloroform extract of the root yielded alkamides with nitidanin-diisovalerianate, sesquiterpene called 1β-hydroxy-4 (15),5E,10 (14)-germacratriene components[14].It was observed that aerial plant parts are not a significant source of alkamides rather they contain polysaccharides, polyacetylenes, and glycoproteins[15].

Antioxidant properties are mainly due to phenolic compounds and chicoric acid[16]. Alkamides have no impact on free radicals rather they play a supportive role by enhancing the antioxidant efficacy of chicoric acid[17]. Chicoric acid and other caffic acid derivatives have been proven to have anti-inflammatory and antioxidant properties[18]. Echinacoside and chichoric acid can also be used as natural disinfectants as they possess strong antibacterial activity[19][20].

Along with these, the plant contains other secondary metabolites like alkaloids, amides, phenolics like p-coumaric and flavonoids like quercetin[21]. These compounds are chiefly responsible for the medicinal properties of the plant.

According to literature, endophytes have also been linked to the resilience of plants against biotic and abiotic stresses, making it an appreciated topic for further research in the field of plant-microbe interaction[22]. They have also been associated with plant growth promotion and inclusive plant fitness by increasing nutrient uptake and stress tolerance and improving root development etc[23].

As the demand for traditional and plant-derived medications continues to grow, understanding the symbiotic relationship between plantsand their residing endophytes can reveal novel strategies for improved product quality and discover novel bioactive compounds with potential therapeutic applications[24]. It can offer an exciting path to harness the full potential of this herbal remedy in the growing field of interesting pharmaceutical sectors[25]. This study, therefore, aims to isolate endophytes and analyse their effects on phytopathogens and the growth and development of the plant.

2. Material and Method:

2.1. Isolation of endophytes: Plant samples were collected from Himalayan region and were surface sterilised by immersion in 70% ethanol for 1 minute, followed by treatment with sodium hypochlorite for 3 minutes. This was succeeded by rinsing with 70% ethanol for 30 seconds, followed by a final wash with autoclaved distilled water for 10 minutes, with the process being repeated three times. Sterilised samples were cut into small pieces and further plated on NAM(Nutrient Agar Media) for the growth of bacterial endophytes[26]. Pure colonies isolated were analysed via 16SrRNA for identification[27].

2.2. Test of antifungal activity

2.2.1. Phytopathogenic strains: The target phytopathogenic fungi A. triticina, C. oxysporium, F. oxysporum, P. variotii, P lilacinus, A. niger, A. fumigatus, A. flavus and C. albicans were sourced from the Microbial Type Culture Collection (MTCC) at the Institute of Microbial Technology (IMTECH), Chandigarh and maintained over PDA(Potato Dextrose Agar).

2.2.2. **Antifungal activity:** The antifungal activity of metabolite extract was analysed against pathogenic fungalstrains using the Kirby -Bauer test[32][33]. The disc diffusion assay was performed in 10 cm diameter Petri plates. The isolated sample was settled in the agar plate already inoculated by desired fungi.

2.3. Extraction of metabolites: The endophytes were cultured in LB (LuriaBroth) at 28℃ for 2 weeks and proceeded for metabolite extraction using below method.

The cultured media and ethyl acetate are mixed in a ratio of 1:1 and stirred using a magnetic stirrer for 6 hrs. The organic supernatant was separated and centrifuged at 5000 rpm for 10 minutes. The ethyl acetate layer was transferred to a clean flask and dried using a rota-evaporator at 50℃. The dry extract was then dissolved in 2 ml of ethanol. The Supernatant was centrifuged, and the solvent was dried.

2.4. Metabolite profiling: Metabolite profiling was done using GC-MS to find out the bioactive compounds present in the ethyle acetate extract of isolated endophytic bacterias.

2.5. Biochemical tests:

a. **IAA production:** IAA production was detected by inoculating the culture in LB for 3 days. It was further centrifuged at 6000rpm for 15 minutes. 2ml of the supernatant was mixed with orthophosphoric acid and Salkowaski''s reagent. IAA was measured using UV spectrophotometer at 530nm.
b. **N_2 fixation:** Nitrogen fixation was detected by measuring the nitrogenase enzyme activity using ARA (Acetylene reduction assay) assay. This assay uses the gas chromatography to measure the reduction of acetylene to ethylene by nitrogenase enzyme in a quick and effective manner.
c. **ACC deaminase activity:** ACC medium was prepared using Dworkin and foster (DF) salt minimal medium containing ACC. Fresh LB culture was inoculated with bacteria for 2 days at 30℃. The cultured bacteria were then centrifuged at 10,000rpm for 5-6 days at 5℃ and the pellet was spotted on ACC medium.

3. Results:

3.1. Isolation of endophytes: Overall,16 bacterial endophytes were isolated from the plant E. purpurea based on morphological (size, circular, irregular, pigmentation) and elevation (flat, convex, raised etc.) characteristics. .

3.2. Purification of endophytes: The endophytes grown out of sample tissue were purified by subculturing over Luria Agar. Out of 16 isolates, only 3 exhibited antifungal activity against at least one of the tested phytopathogenic fungal strain. (Fig. 1). These 3 isolates were Eb6c, Eb8a, and Eb13 i.e.H. rubrisubalbicans, H. huttiens and P. dispersa respectively when analysed via 16SrRNA analysis.

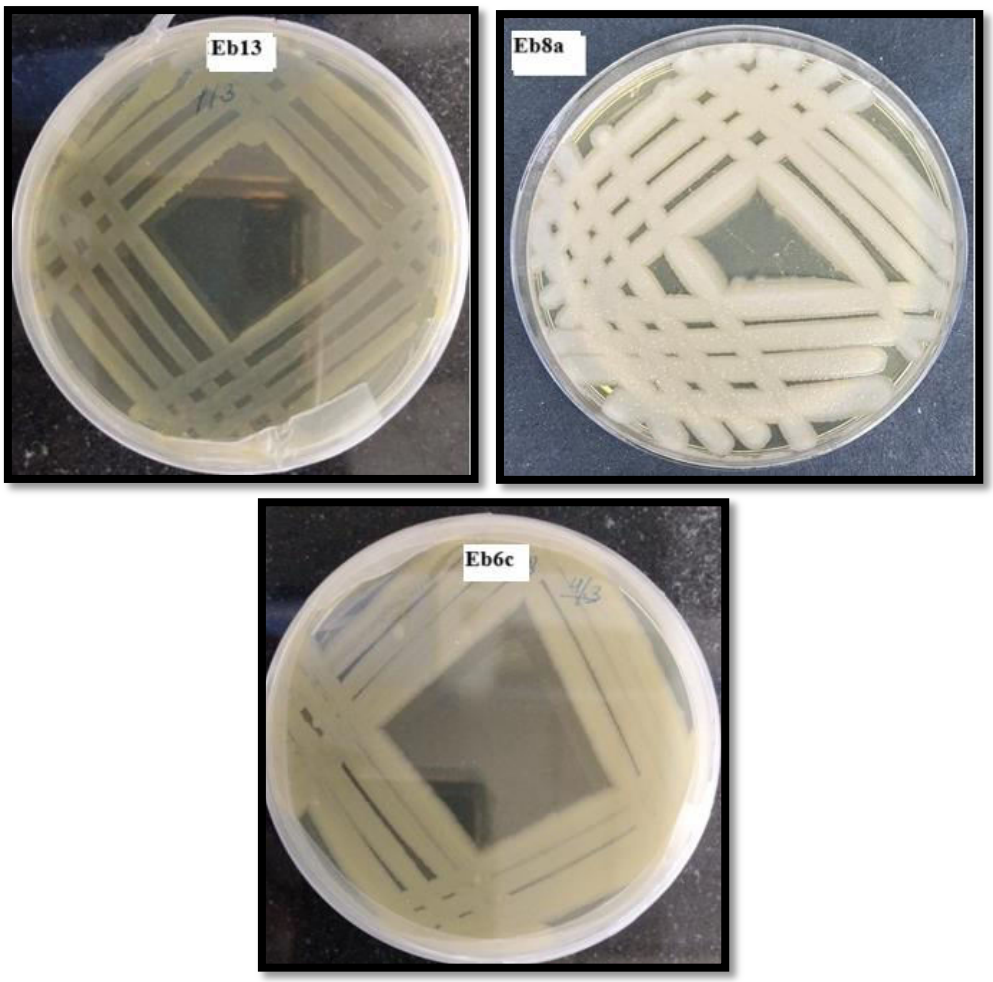

Fig.1: Bacterial isolates Eb13, Eb8a, and Eb6c.

3.3. Antifungal activity of isolates: The isolated bacterial endophytes were analysed for antifungal activity against certain phytopathogenic fungal strains like F. oxysoprum, C. albicans, A. fumigates and A. niger etc. (Fig.2) (Table1) The MIC(Minimum Inhibitory Concentration) was calculated using Risazurin based Microbroth Dilution Assay. Graph showing Zone of Inhibiton and MIC(Minimum Inhibitory Concentration) (Fig.3)(Fig.4) shows Eb13 as an excellent biocontrol agaent.

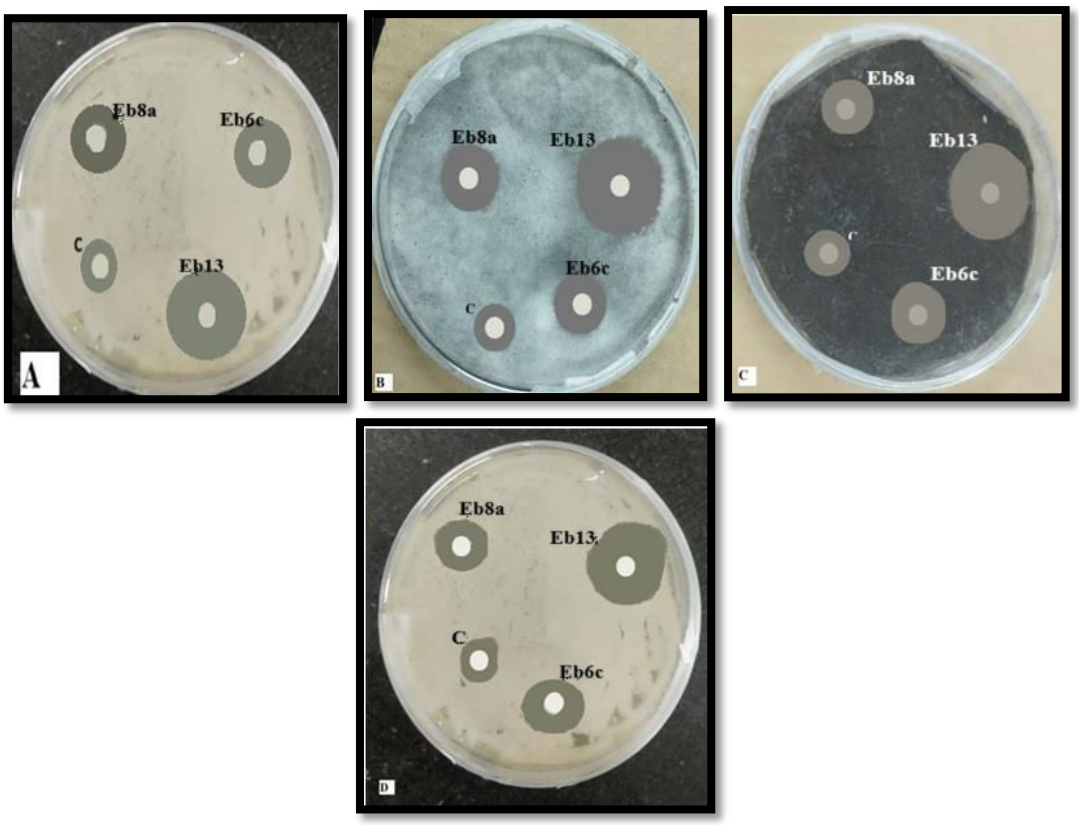

Fig.2: Antifungal activity of bacterial isolates against fungal phytopathogens. (A) shows antifungal activity of Eb13, Eb8a, and Eb6c against F. oxysporum (B) against A. fumigatus, (C) against A. niger, and(D) against C. albicans.

Table 1: Antifungal activity of 3 endophytic bacterial strains against phytopathogens (Mean Diameter zone of inhibition (mm) ± Standard deviation (SD)

Fungal strains	**Control(Ethyl Acetate)**	**Eb13(1µg/ml)**	**Eb8a(1µg/ml)**	**Eb6c(1µg/ml)**
F. oxysporum	8 ± 0.2	25±0.2	19±0.2	20±0.2
A.fumigatus	8.4± 0.2	23±0.2	12±0.3	15±0.3
A.niger	8.2± 0.3	21±0.3	16±0.2	18±0.2
C.albicans	7.8± 0.2	28±0.2	25±0.2	23±0.3

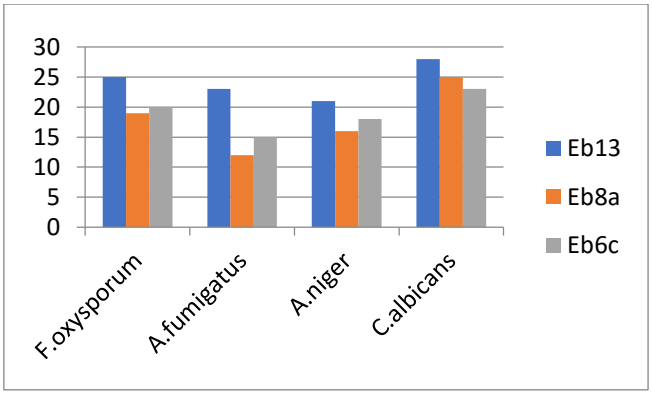

Fig.3. Graph showing Zone of inhibition

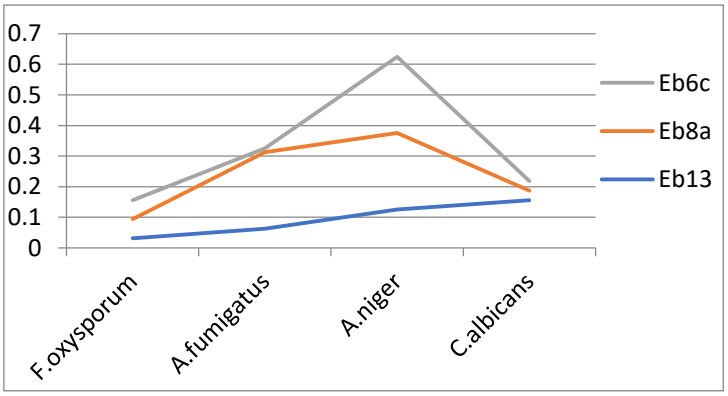

Fig.4. Graph showing MIC (Minimum Inhibitory Concentration) value

3.4. Metabolites profiling of bacterial extract: Metabolites profiling was done by GC-MS spectroscopy which reveals the presence of compounds like 1-Heptadecene, 1-Nonadecene, 6-[12(Z)-Nonadecenyl] salicylic acid (2TMS), 1-Pentadecene which are responsible for antifungal activities performed by endophytic bacteria.

3.5. Biochemical Tests: The results of biochemical tests are shown in the table

Table 2. Biochemical characterization of various endophytic isolates

Biochemical Test	EB6c (H. rubrisubalbicans)	EB8a (H. huttiense)	EB13 (P. dispersa)
IAA production	+ve	+ve	+ve
Nitrogen fixation	+ve	+ve	+ve
ACC deaminase activity	+ve	+ve	+ve
Siderophore	+ve	+ve	+ve

PO_4^- solubilization	+ve	+ve	+ve

Discussion

The objective of present study was to isolate the endophytes from Himalayan medicinal plant E. purpureae and further evaluate the endophytes for antimicrobial activity. Total 16 endophytic bacteria were isolated from roots, stem, leaves and flower petals. The flowering stage was considered as this stage is most nutritious and provide more favourable condition to the endophytes to flourish. More endophytes were found in shoot as compared to roots indicating more favourable conditions are provided by stem than roots to harbour the endophytes. Surface sterilization was done to remove extras and cultured isolates were examined for morphological and molecular characterization. Whitish and yellow creamy colonies were observed. 16SrRNA sequencing followed by BLAST identified the selected isolates EB6c, EB8a, EB13 as Herbaspirillium rubrisubalbicans, H. huttiens and P. dispersa respectively. While the 16S rRNA sequencing analysis was effective in detecting microorganisms at the genus and species levels, it be deficient in the capability to discriminate between subspecies and strains, making it inappropriate for evaluating intra-species relationships. The bacterial isolates underwent antimicrobial screening against pathogenic fungal strains. It was observed that Herbaspirillium rubrisubalbicans shows antifungal activity against Alternaria, Cladosporium oxysporium, Aspergillus niger, Paecilomyces variotii and Fusarium. H. huttiens profoundly shows antifungal activity against Aspergillus niger, Pythium, Paecilomyces variotii (weak activity), Cladosporium oxysporium, Aspergillus flavus and Fusarium. P. dispersa has been found active against Alternaria, Paecilomyces variotii, Candida, Aspergillus niger and flavus bothand Ceratocystis fimbriata. The bioactive metabolites responsible were identified as 1-Heptadecene, 1-Nonadecene, 6-[12(Z)-Nonadecenyl] salicylic acid (2TMS), 1-Pentadecene when analysed through GC-MS analysis. Various biochemical tests performed showed the production of IAA by endophytes, which acts as a positive plant growth regulator. IAA production detects the presence of L-tryptophan. Siderophore production makes the free Fe^{3+} unavailable to phytopathogens protecting plant health. Ability to solubilize phosphates enables the host plant acquire more phosphorus in soluble form. ACC deaminase activity modulates the level of ethylene during salinity stress. HCN produced by Herbaspirillium is lethal to phytopathogens. These endophytic bacteria can grow on a media lacking N_2, hence able to fix N_2 and convert it to ammonia. So, the results indicate that the endophytic bacteria inhabiting the plant E. purpurea are responsible for its medicinal properties to a great extent. They protect the plant from phytopathogens producing secondary metabolites, fix the nitrogen to usable

form and also produce the plant growth promoting phytohormones.The bioactive metabolites produced are having antimicrobial properties to increase plant defence mechanism and resistance to disease. Besides, endophytes also play crucial role in phytohormone as well as siderophore production to enhance the plant growth and development and iron uptake efficacy in plants.

Conclusion:

In this study, endophytic bacteria were isolated from different parts of Echinacea purpurea, including stems, roots, leaves etc. The study revealed a diverse endophytic community having the potential to produce bioactive compounds like those found in the host plant. Besides, the study identified specific endophytic strains that effectively checked the growth of fungal phytopathogens like Fusarium, Candida, and Aspergillus etc., indicating their potential aids to the medicinal properties of Echinacea purpurea. The endophytes and the bioactive compounds produced may provide analternative source of disease control in the plant. It was found that endophytic bacterial isolates from the plant E. purpurea can inhibit the growth of various phytopathogens, promote plant growth and fix nitrogen showing the biocontrol as well as biofertilizer properties of endophytes. The antifungal compounds identified can be used as a biocontrol agent. Further studies are required to find the range of bacterial and fungal phytopathogens that can be inhibited by these endophytes. There cytotoxicity should be checked before their application in pharmaceuticals.

Abbreviations:

NAM -Nutrient Agar Medium
PDA - Potato Dextrose Agar
GC-MS - Gas Chromatography-Mass Spectroscopy
Spp. - Species
E. - Echinacea
H. - Herbaspirillium
P. - Pantoea
S. - Saccharomyces
C. - Candida
A. – Aspergillus
F. - Fusarium
UV - Ultraviolet

Conflict of Interest : The authors declare no conflict of interest.

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Reference:

1. Haneenulla, S. Dixit, S. Purkayastha, and V. Kumar, "Echinacea Purpurea: A Review On Bioactive Constituents And The Pharmacological Activity (Purple Cone Flower)," 2022.
2. M. M. Karhib, R. A. El-Sayed, N. F. Ghanem, and F. M. El-Demerdash, "Nephroprotective role of Echinacea purpurea against potassium dichromate-induced oxidative stress, inflammation, and apoptosis in rats," Environ Toxicol, vol. 37, no. 9, pp. 2324–2334, Sep. 2022,
3. C. Burlou-Nagy et al., "Echinacea purpurea (L.) Moench: Biological and Pharmacological Properties. A Review," Plants, vol. 11, no. 9, May 2022,
4. H. Devi and A. Tirkey, "Assessment of Genetic Diversity of Indigenous Germplasm Accession of Kalmegh [Andrographis paniculata (Burm. F.) Nees]," 2017.
5. E. Kakouri, M. Talebi, and P. A. Tarantilis, "Echinacea spp.: The cold-fighter herbal remedy?," Pharmacological Research - Modern Chinese Medicine, vol. 10, p. 100397, Mar. 2024.
6. H. Verma, M. Negi, B. Mahapatra, A. Shukla, and J. Paul, "Evaluation of an emerging medicinal crop Kalmegh [Andrographis paniculata (Burm. F.) Wall. Ex. Nees] for commercial cultivation and pharmaceutical & industrial uses: A review," 2019.
7. R. A. Chudak, "Echinacea pallida extract effect on quils meat quality," Ukr J Ecol, vol. 9, no. 2.
8. S. Garzoli, F. Maggio, V. Vinciguerra, C. Rossi, M. G. Donadu, and A. Serio, "Chemical Characterization and Antimicrobial Properties of the Hydroalcoholic Solution of Echinacea purpurea (L.) Moench. and Propolis from Northern Italy," Molecules, vol. 28, no. 3, Feb. 2023.
9. C. Burlou-Nagy et al., "Determination of the Bioactive Compounds from Echinacea purpurea (L.) Moench Leaves Extracts in Correlation with the Antimicrobial Activity and the In Vitro Wound Healing Potential," Molecules, vol. 28, no. 15, Aug. 2023.
10. P. Ciganović, L. Jakupović, P. Momchev, L. Nižić Nodilo, A. Hafner, and M. Zovko Končić, "Extraction Optimization, Antioxidant, Cosmeceutical and Wound Healing Potential of Echinacea purpurea Glycerolic Extracts," Molecules, vol. 28, no. 3, Feb.
11. V. Goel et al., "A proprietary extract from the echinacea plant (Echinacea purpurea) enhances systemic immune response during a common cold," Phytotherapy Research, vol. 19, no. 8, pp. 689–694, Aug. 2005,
12. M. Yang et al., "Chicoric Acid: Natural Occurrence, Chemical Synthesis, Biosynthesis, and Their Bioactive Effects," Front Chem, vol. 10, Jun. 2022.

13. M. O. Thomsen, L. P. Christensen, and K. Grevsen, "Harvest Strategies for Optimization of the Content of Bioactive Alkamides and Caffeic Acid Derivatives in Aerial Parts and in Roots of Echinacea purpurea," J Agric Food Chem, vol. 66, no. 44, pp. 11630–11639, Nov. 2018.
14. N. Petkova et al., "Chemical Composition of Different Extracts from Echinacea purpurea (L.) Moench Roots and Evaluation of Their Antimicrobial Activity," ChemEngineering, vol. 7, no. 5, Oct. 2023.
15. L. Micheli et al., "Echinacea purpurea against neuropathic pain: Alkamides versus polyphenols efficacy," Phytotherapy Research, vol. 37, no. 5, pp. 1911–1923, May 2022.
16. L. Ravazzolo et al., "C3H Expression Is Crucial for Methyl Jasmonate Induction of Chicoric Acid Production by Echinacea purpurea (L.) Moench Cell Suspension Cultures," Int J Mol Sci, vol. 23, no. 19, Oct. 2022.
17. X. jing Lin et al., "Correlation between Polyphenol Contents and Antioxidant Activities in Different Echinacea Purpurea Varieties," Curr Med Sci, vol. 43, no. 4, pp. 831–837, Aug. 2023.
18. R. G. L. Vieira et al., "In vitro studies of the antibacterial activity of Copaifera spp. oleoresins, sodium hypochlorite, and peracetic acid against clinical and environmental isolates recovered from a hemodialysis unit," Antimicrob Resist Infect Control, vol. 7, no. 1, Jan. 2018,
19. Y. Chang et al., "Antibacterial Activity of Chrysanthemum buds Crude Extract Against Cronobacter sakazakii and Its Application as a Natural Disinfectant," Front Microbiol, vol. 11, Feb. 2021.
20. J. Kim, S. Park, Y. K. Shin, H. Kang, and K. Y. Kim, "In vitro antibacterial activity of macelignan and corosolic acid against the bacterial bee pathogens Paenibacillus larvae and Melissococcus plutonius," Acta Veterinaria Brno, vol. 87, no. 3, pp. 277–284, 2018.
21. R. Belal, M. Eldesouky, A. Elmasry, B. Weheda, and S. Aggag, "Gene Expression Profile of some Antioxidant-Related Genes in Five Species of the Family Asteraceae in Egypt," Journal of Agricultural Chemistry and Biotechnology, vol. 14, no. 5, pp. 57–64, May 2023.
22. A. Mahjoub, W. Mnafgui, N. Maiza, N. Ressaissi, N. Ludidi, and M. Badri, "Harnessing the potential of endophytes: Sustainable solutions for enhancing forage crop resilience and soil fertility," Journal Of Oasis Agriculture And Sustainable Development, vol. 5, no. 2, pp. 48–56, Oct. 2023.
23. A. Verma et al., "Fungal Endophytes to Combat Biotic and Abiotic Stresses for Climate-Smart and Sustainable Agriculture," Front Plant Sci, vol. 13, Jul. 2022.
24. P. F. Arifin, T. I. Y. ASSA, A. Nora, and R. Wisastra, "Evaluating The Antioxidant Activity Of Echinacea purpurea (L.) Moench Ethanol Extract," Indonesian Journal of Biotechnology and Biodiversity, vol. 6, no. 2, pp. 39–44, Aug. 2022,.

25. N. Akhtar, A. K. Wani, D. S. Dhanjal, and S. Mukherjee, "Insights into the beneficial roles of dark septate endophytes in plants under challenging environment: resilience to biotic and abiotic stresses," World J Microbiol Biotechnol, vol. 38, no. 5, May 2022,.
26. L. and B. Alaylar, "Isolation and Characterization of Culturable Endophytic Plant Growth Promoting Bacillus Species from Mentha longifolia L.," Turkish Journal of Agriculture and Forestry Sciences, 2021,.
27. L. E. de O. Costa, M. V. de Queiroz, A. Chaer Borges, C. A. de Moraes, and E. F. de Araújo, "Isolation and characterization of endophytic bacteria isolated from the leaves of the common bean (Phaseolus vulgaris)," Brazilian Journal of Microbiology, vol. 43, no. 4, pp. 1562–1575, 2012,.
28. U. C. Silva et al., "Genomic and Phenotypic Insights Into the Potential of Rock Phosphate Solubilizing Bacteria to Promote Millet Growth in vivo," Front Microbiol, vol. 11, Jan. 2021.
29. M. Rajkumar, N. Ae, M. N. V. Prasad, and H. Freitas, "Potential of siderophore-producing bacteria for improving heavy metal phytoextraction.," Trends Biotechnol, vol. 28, no. 3, pp. 142–149, 2010.
30. Y. Yanti, H. Hamid, Reflin, and Yaherwandi, "Biochemical characterizations of selected indigenous endophytic bacteria potential as growth promoters and biocontrol agents on tomato," IOP Conf Ser Earth Environ Sci, vol. 757, no. 1, May 2021.
31. N. V. Kladar et al., "Biochemical Characterization of Helichrysum italicum (Roth) G.Don subsp. italicum (Asteraceae) from Montenegro: Phytochemical Screening, Chemotaxonomy, and Antioxidant Properties," Chem Biodivers, vol. 12, no. 3, pp. 419–431, 2015.
32. N. S. Nysanth, · S L Sivapriya, · Chitra Natarajan, and · K N Anith, "Novel in vitro methods for simultaneous screening of two antagonistic bacteria against multiple fungal phytopathogens in a single agar plate," 3 Biotech, vol. 12, p. 140, 123AD.
33. C. Giuliano, C. R. Patel, and P. B. Kale-Pradhan, "A Guide to Bacterial Culture Identification And Results Interpretation," vol. 44, no. 4, 2019.