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Isolation and Identification of Endophytic Fungi from Eucalyptus Plants and their Use the Plant in the Treatment of some Types of Pathogenic Bacteria and Endophytic Fungi

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Abstract

Background and Objective: Although plants and their roots contain organisms and substances that inhibit the growth of both plants and surrounding organisms, endophytic fungi and bacteria are of particular benefit and influence the absorption of substances from the soil so that the plant is more effective in resisting many diseases. **Methods:** In this study, samples were collected from the roots of the Eucalyptus plant from different locations at Al-Qadisiyah University (from 9/5/2023 to 11/29/20232) **Results:** the fungi and internal bacteria were isolated from them. After that, the leaves were taken and their inhibitory effect on some... Types of bacteria and fungi isolated from the roots, as well as on some types of pathogenic bacteria, and it was noted that they have a clear inhibitory effect on these organisms. **Conclusion:** Based on this study and previous studies, it was found that they have a role in absorbing nutrients from the soil that are necessary for the plant to be substances with an inhibitory effect on many of living organisms, as well as of the same organisms parasitic on its roots.

Keywords: Endophytic Fungi, Eucalyptus Plants, Treatment, Pathogenic Bacteria

Introduction

Humanity has always relied on nature, when it comes to finding new medicines to treat diseases (Yenn and Ibrahim.,2019). Internal fungi exist within the host plant cell and its tissues without causing damage or any secondary symptoms rather play a vital role in protecting it from pathogens harsh environmental conditions and animals (Jia et al.,2016).Studies indicate that there is a mutual interaction between endophytes and the host, where the host provides shelter and nutrition, as chemical sentinels for the functioning of endophytes (Kazenel et al.,2015).The possibility of using endophytic fungi as a source of secondary metabolites is of interest and has proven useful in the discovery of new drugs (Yan et al.,2011).The close symbiotic relationship between endophytes and host plants endows endophytes with a strong ability to produce new bioactive compounds whose carbohydrate production is fueled by the host plant (Aly, Debbab & Proksch, 2011).These bioactive compounds increase plant resistance to pathogens and herbivores, enhance growth and enhance competitive capabilities (Zhang, Song & Tan, 2006). Due to the diversity of fungal bioactive metabolites endophytic fungi are considered useful as novel drugs (Guo et al., 2008). In recent years, endophytes have been viewed as a source of secondary metabolites, including antibiotics, anticancer agents, antioxidants, and anti-inflammatories (Bungihan et al., 2011 ;Gutierrez Gonzalez & Ramirez, 2012).

Endogenous bacteria are a common type in all types of vascular plants, and it is believed that all plants contain endobacteria because of their properties (absorbing nutrients, promoting plant growth, and are also considered good biofertilizers for plants, endogenous bacteria can also be considered internal colonizers of all parts of the plant, as they can form a set of interactions, including nutritional and symbiotic interactions. They also support the host by producing various natural materials for use in agriculture, industry, as well as medicine., (Khan et al.,2020).

In this study, Eucalyptus camaldulensis was used for investigation is a tree belonging to the genus Eucalyptus from Myrtaceae family ,Eucalyptus leaves and their essential oils have been used by indigenous peoples for centuries as a traditional herbal remedy, and because of their antiseptic, anti-inflammatory, and antipyretic properties they have been used in everyday life (Silva et al., 2003). The E. camaldulensis plant has been used in Australia in medicines to treat gastrointestinal symptoms (including diarrhoea, colic, and dysentery), respiratory ailments (colds, sore throats, laryngitis, coughs, asthma, pharyngitis, and sore throats), and to stop bleeding. cuts and open wounds, and its decoctions are also used to relieve spasms, muscle pain, joint pain, and toothache (Sabo and Knezevic.,2019). Plants continue to encounter microbes in unfavorable environments that colonize their tissues in search of plant-derived nutrients. Some of these microbes live parasitically, while plant growth and stress tolerance benefit others (Smith and Read, 2008; van der Heijden et al., 2015) Therefore, plants require an adaptive and multifaceted immune system to allow them to resist disease-causing microbes while allowing the growth of beneficial species (Hacquard et al., 2017). Sensing and filtering mechanisms continue to be a barrier for plant breeders in selecting plants that are tolerant of (harsh environmental conditions and frequent disease outbreaks) (Kroll et al., 2017). It is known that the Eucalyptus plant has good medicinal properties in treating many diseases ,This and previous studies provide valuable insight into the diversity of endophytic fungi and their symbiotic benefits for each plant,The presence of endophytic fungi in different places of the plant is due to the symbiotic relationship between the fungus and the plant, where the endophytic fungus obtains the nutrients necessary for its continuity and quality, in addition to helping the plant absorb materials from the soil if it is present in the roots. It also secretes toxins to kill all types of organisms surrounding the plant’s roots (Deepthi et al.,2018). Then, grow other samples of roots on the medium (nutrient medium) for bacterial growth. We had only three isolates of pseudomonas, which were diagnosed based on some chemical tests, as they were hemolytic, positive for catalase and oxidin tests, and negative for gram stain (Gilardi.,2020). And one isolate of Bacillus sp, the bacteria were then purified and characterized based on Gram stain (Duan et al., 2013).

Staphylococcus aureus is the most widespread and well-known type of pathogenic bacteria.The severity of its illnesses begins with skin diseases, blood poisoning, and respiratory infections(Cheung et al.,2021).It usually causes death in people in chronic cases, that caused by acquired immune deficiency syndrome (AIDS),viral hepatitis and tuberculosis combined (Van et al .,20112)

One of the common and chronic diseases among people is tooth decay, as it poses a great threat to health (Zheng et al.,2023). Tooth decay is due to the presence of S.mutans bacteria, as the acidity level in the mouth approaches pH 7, but when consuming acidic foods and drinks, the percentage may decrease, and thus the tooth enamel is affected, which encourages the growth of tooth decay bacteria, which have the ability to manufacture large quantities Glucan is important in bacterial biofilm formation(Lemos et al.,2019).

Klebsiella pneumoniae is a type of non-motile Gram-negative bacteria that has widespread colonization among people with weak immune systems, such as the elderly, diabetics, and children (Ripabelli et al.,2020). Urinary tract infections are the most common type of infection in hospitals (Karimi et al.,2021). The excessive use of some types of antibiotics has led to difficulties in treating Klebsiella pneumonia (Tella et al.,2019), and this has been proven by some previous studies, where the bacteria showed high resistance against a wide range of antibiotics due to their ability to form biofilms (Vuotto et al.,2014).

The study aimed to:

1 - Isolation and identification of some organisms from the roots of the Eucalyptus plant

2 - Isolation and diagnosis of some types of pathogenic bacteria.

3 - Evaluation of the inhibitory effect of eucalyptus leaf extract on some types of bacteria and fungi under studied laboratory conditions.

Materials and Methods

Collect plant material

In this study, samples of the roots and leaves of the eucalyptus plant were collected from Various places at Al-Qadisiyah University, (from 5/9/2023 to 29/11/2023). The plant material was transported to the laboratory in sterile bags and processed within a few hours after sampling, then the roots were taken and cut into small samples on a sterile glass plate (Kosari et al.,2008) .

Isolation and diagnosis of endophyte fungal and internal bacteria

Forty-seven samples of eucalyptus roots were taken from different places at Al-Qadisiyah University, some of them were grown in fungal growth medium (PDA) and incubated at 25°C for 7 days. 18 isolates of Pythium fungus were found. The appearance of the Pythium colony under the microscope is flat, colorless or light brown with fine rays at the edges (Gurnani et al., 2022). Only 3 isolates of Rhizoctonia were identified based on their morphological characteristics, Internal isolates were identified by coloring the slides containing the samples, using lactophenol cotton blue reagent and then examined under a microscope, Pattern and color of the colony and medium, surface texture, aerial fungi, root shape and production, coloring of the medium, size and color of conidia (Al-Ithawi and Al-Taie et al., 2022).

Then, grow other samples of roots on the medium (nutrient medium) for bacterial growth. We had only three isolates of Bacillus pseudomonas and one isolate of Bacillus sp. The bacteria were then purified and characterized based on Gram stain (Duan et al., 2013).

Isolation and diagnosis of some type of pathogenic bacteria

Staphylococcus aureus: 35 samples were taken from acne cysts and some skin pimples using a swab, then the sample was distributed on the medium of mannitol salt agar, then incubated at a temperature of (37) degrees Celsius for (18-24) hours, and only 20 isolates belonging to the aureus bacteria appeared. Staphylococcus aureus, and it was diagnosed based on some morphological characteristics, as these bacteria are gram-positive, and the most important characteristic of them is that they ferment the menthol present in the menthol salt agar medium and turn it yellow, and this is what distinguishes it from the types of staphylococci, which is that it does not change the color of the medium (Salih and Shaheed, 2022). After their growth (Tigabu & Getaneh et al., 2021) (Islam et al., 2016).

Streptococcus mutans: 10 samples were taken from the infections of people suffering from tooth decay and gum disease. We obtained only 7 isolates of the Streptococcus mutans bacteria responsible for tooth decay. They were taken using a piece of cotton and cultured on Mitis - Salivarius Bacitracin Agar (MSB), where they were added. Potassium tellurite onto MSB agar and bacteria were diagnosed based on the morphological appearance of the colony (Al-Taei,2020) and these bacteria were also diagnosed based on morphological characteristics and biochemical tests such as Vitek 2 (Saleh & Al-

Ahmer et al., 2021). This system is one of the most important modern systems in diagnosing bacteria, with results reaching 99% accuracy (Jassam et al., 2022).

Klebsiella pneumoniae: 15 samples were taken from the urine of people who had urinary tract infections, and we found only 3 isolates of Klebsiella bacteria, which are facultative anaerobic bacteria that infect the urinary tract and cause serious infections in humans. They are diagnosed based on the shape and color of the colony (Nogueira et al., 2024, Al-Harmooshee and Shaheed, 2020), as the most important A characteristic of their colonies when they grow on solid media, such as MacConkey solid medium, is that they are large and sticky, and the degree of mucility depends on the amount of carbohydrates in the medium. These bacteria appear smooth and pink in color due to their ability to ferment lactose and produce acid (Dubey et al.,2013; Wang et al.,2012; Chiu et al.,2013).

Use the plant in the treatment pathogenic bacteria

- Preparation of plant material and extracts

After collecting the fresh leaves and cleaning them with distilled water, the leaves are ground with the addition of distilled water and drying them, then filtered with gauze, and the extract is taken to filter using filter papers, then dried using an air oven for 5 days until a powder is obtained, and then the dried material is collected in a box and stored in a dark glass container until used, at a temperature of 4 degrees Celsius.

-Using different concentrations of plant extract on some bacteria and fungi isolated from the roots, as well as pathogenic bacteria

Concentrations (500, 600, 700, 800 mg) of the previously prepared plant material extract were used, with distilled water added to it. After that, three replicates were made for each concentration and three holes for each dish in the dishes containing Mueller-Hinton agar medium fortified with some types of bacteria isolated from the roots of the Eucalyptus plant. 1 ml of each concentration was used and placed in each hole, then the dishes were prepared in the same medium,with the vancomycin antibody added as a positive control.

Then the experiment was repeated by adding 1 ml of each concentration before pouring the PDA medium into the pouring dishes. After pouring the medium and solidifying, the dishes were inoculated with endophytic fungi isolated from the roots, and anti-tetracycline was applied to some of the dishes as a positive control agent.

Then dishes inoculated with bacteria and fungi were left separately without treatment as a negative control.

As for the pathogenic bacteria that were isolated from the body, the experiment was repeated at the same concentrations to determine the inhibitory effect of the extract on them (as these concentrations were used at 1 ml for each hole of the dishes containing Mueller-Hinton Agar medium and inoculated with Staphylococcus aureus, Streptococcus mutans, and Klebsiella pneumoniae bacteria on Three replicates for each concentration and each bacterium separately).

Results and Discussion

Isolation and diagnosis of endophytic fungi

Table (1)shows the fungi and bacteria isolated from the roots of Eucalyptus plants

N	Isolation places	Number of samples	Isolated fungi	number	Isolated bacteria	number
1	Science Park	20	Pythium	6	pseudomonas sp	3
2	Club garden	15	Rhizoctonia	2	Bacillus sp	1

3	Presidential Garden	16	Pythium	7		
4	Law Garden	11	Pythium	5		
			Rhizoctonia	1		

Table No. 1 shows the samples obtained from different places in the gardens of Al-Qadisiyah University. 20 samples of Eucalyptus roots were taken from the garden of the College of Science. 6 isolates of Pathium fungus and 3 isolates of Pseudomonas bacteria were obtained from the garden of the Presidency of Al-Qadisiyah University. 15 samples were given two isolates of Rhizoctonia fungus and one isolate of Bacillus sp. From the Deanship of the College of Science, Al-Qadisiyah University, 16 samples were taken, which yielded only 7 isolates of the Bacillus fungus. Finally, 11 samples were taken from the garden of the College of Law, obtaining 5 isolates of the Pathium fungus and one isolate of the Rhizoctonia fungus. This is not consistent with (Al-Taie et al.,2020), as it was found that the dominant fungus is Rhizoctonia with a percentage of 81.4% in the soil, followed by Pathium with a percentage of 33.8%.

Isolation and diagnosis of Some types of bacteria

Table (2)shows bacteria isolated from the human body

N	Isolation places	Number of samples	Type of bacteria	Number
1	face	35	Staphylococcus aureus	20
2	teeth	10	Streptococcus mutans	7
3	Urinary Tract	15	Klebsiella pneumoniae	3

The table above shows some types of pathogenic bacteria isolated from the human body. 35 samples were taken from the face, from which we obtained 20 isolates of Staphylococcus aureus bacteria. Likewise, 10 samples were taken from the oral cavity and decayed teeth, from which we obtained only 7 isolates of Streptococcus mutans bacteria, and finally 15 samples were isolated from the urinary tract, and only three isolates of Klebsiella pneumoniae bacteria were obtained.

Testing the effect of eucalyptus leaf extract on inhibiting the growth of some types of bacteria and endophytic fungi

The effect of the extract on Staphylococcus aureus bacteria can be observed by reading the inhibition percentage of each concentration compared to the antibiotic, where the inhibition percentage was 500 (0.9), the inhibition percentage was 600 (1.4), and the inhibition percentage was 700 (1.9). The inhibition percentage for concentration 800 was (2.3), which is the highest and best percentage in inhibiting bacterial growth compared to the rest of the concentrations. While the antibiotic inhibition rate in the dishes was 2 cm, this percentage was within the framework of antibiotic inhibition according to the CLIS system, where inhibition rates should range between 0.5-2. The inhibition rate for the concentration of 800 was higher and better in inhibiting the growth of staphylococcal bacteria compared to the antibiotic vancomycin, and this does not agree with (Abu Bakr, 2010), where the inhibition rate for the Eucalyptus plant extract was 12 mm. This is not consistent with this study, where the best inhibition rate was 2.3. millimeter. This is consistent with) Reda et al., 2017)who showed that methanol present in the leaves of the Eucalyptus plant has an inhibitory effect on Staphylococcus aureus bacteria. The percentages of inhibition of the extract against Streptococcus mutans bacteria

were (0.7, 1.2, 1.7, 2), respectively. The highest rate of inhibition was 800, which is the best rate of inhibition for bacteria compared to the antibiotic, which had an inhibition rate of 1.9 for Streptococcus mutans bacteria.The percentages of inhibition of the extract against Klebsiella pneumoniae bacteria were respectively (0.9, 1.3, 1.7, 2.1) as the highest percentage of inhibition was for the concentration of 800, which is the best percentage for inhibiting bacteria, which was (2.1) compared to the antibiotic whose inhibition percentage was 2. The percentages of inhibition of the extract against the bacteria pseudomonas sp were respectively (0.5, 1, 1.3, 1.7), while the percentage of inhibition of the antibiotic was (1), which is a percentage less than the percentage of inhibition of the extract for the two concentrations (800, 700), but equal to the inhibition of the concentration (600).
The percentages of inhibition of the extract against Bacillus sp bacteria were respectively (1, 1.4, 1.8, 2), while the percentage of inhibition of the antigen was (1.5), which is a lower percentage than the percentage of inhibition of the extract for the two concentrations (800, 700).

Table (3) showing the different concentrations of the plant extract with the zones of inhibition for the isolated bacteria

Concentrations	500	600	700	800
Inhibition zone Staphylococcus aureus	0.9	1.4	1.9	2.3
vancomycin antibody	2			
Inhibition zone Streptococcus mutans	0.7	1.2	1.7	2
vancomycin antibody	1.9			
Inhibition zone Klebsiella pneumoniae	0.9	1.3	1.7	2.1
vancomycin antibody	2			
Inhibition zone pseudomonas sp	0.5	1	1.3	1.7
vancomycin antibody	1			
Inhibition zone Bacillus sp	1	1.4	1.8	2
vancomycin antibody	1,5			

Table (4) It shows the different concentrations of the plant extract and their effect on the radial growth of fungi

Concentrations	500	600	700	800
Pythium sp	3.6	2.4	1.3	.,3
Tetracycline	.,8			
Rhizoctonia sp	3.8	3.2	1.5	1
Tetracycline	1.5			

The results shown in Table (4) showed the effect of the plant extract on the fungus Pythium *spp* at three concentrations compared to the antibiotic Tetracycline. The average diameter of the radial growth inhibition zone for the concentration of 500 for the extract was 3.6, which is a ratio different from the average diameter of the radial growth zone for the negative control and in the case of the concentration of 600 for the extract. The average diameter of the radial growth inhibition zone was 2.4, which is a ratio different from the average diameter of the radial growth zone for the negative control, as the average diameter of the negative growth zone was 6. As for the concentration of 700 for the extract, the average diameter of the radial growth inhibition zone was 2.4, while the concentration of 800 for the extract was more effective than the three concentrations. The previous one, as well as the antibiotic in the process of inhibiting growth, as the average diameter of the radial growth zone reached 0.3, which is the highest percentage of inhibition for the fungus Pythium sp. As for the Rhizoctonia sp fungus, the average diameter of the radial growth zone of inhibition for the concentration 500 for the extract was 3.8, which is a percentage different from the average diameter of the radial growth zone for the control. At a concentration of 600 for the extract, the average diameter of the radial growth inhibition zone was 3.2, which is a ratio different from the average diameter of the radial growth zone for the negative control, as the average diameter of the negative growth zone was 6. At a concentration of 700 for the extract, the average diameter of the radial growth inhibition zone was 1.5, which is equal to the average diameter of the extract. The positive inhibition zone (tetracycline antibiotic), while the concentration of 800 of the extract was more effective than the previous three concentrations, as well as the antibiotic in the process of inhibiting growth, as the average diameter of the radial growth zone reached 1.

Conclusion

In this study, the presence of endophytic fungi and bacteria was reported in the roots of the Eucalyptus plant isolated from different places at Al-Qadisiyah University. Based on this study and previous studies, it was found that they have a role in absorbing nutrients from the soil that are necessary for the plant to be substances with an inhibitory effect on many of living organisms, as well as of the same organisms parasitic on its roots.

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Ethical Considerations: The study was approved by the Research Ethical Committees of Al-Qadisiyah University. Informed consent was obtained from all participants and/or their legal guardians.

Authors' Contributions: Conceptualization, Zahraa Essa Radhi, Lamya Hussein Musa Mazine and Ashraf Ayyal Mutar Alrashedi funding acquisition, Zahraa Essa Radhi, Lamya Hussein Musa Mazine and Ashraf Ayyal Mutar Alrashedi; writing-original draft preparation, Zahraa Essa Radhi; writing—review and editing, Lamya Hussein Musa Mazine; project administration. All authors have read and agreed to the published version of the manuscript.

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