RESEARCH PLANT

Antimicrobial activity of emodin – secondary metabolites from Trichoderma against some human pathogenic bacteria in Wasit province, Iraq

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Abstract

Objective: To evaluate the antibacterial activity of emodin isolated from trichoderma longibrachiatum against certain pathogens in patients with bacterial disease.

Methods: The cross-sectional study was conducted at Al-Zahraa Teaching Hospital, Wassit, Iraq, from December 2021 to March 2022, and comprised tichoderma longibrachiatum taken from the Wasit University's garden soil and were subjected to plate morphology, microscopic examination, and polymerase chain reaction-internal transcribed spacer testing. Emodin was extracted by ethyl acetate with final yield of 58mg/L. Using the agar well diffusion method, emodin extract doses of 0.5µg/ml, 1µg/ml, 2µg/ml and 4µg/ml were tested against clinical pathogenic microorganisms. Data was analysed using SPSS 22.

Results: Emodin 0.5µg/ml showed high antibacterial activity against proteus mirabilis, with growth inhibition zone of 8.7mm, followed by staphylococcus epidermidis 7mm, streptococcus pyogenes 7.6mm, staphylococcus aureus 7.5mm, escherichia coli 6.7mm and klebsiella species 0.4mm. Pseudomonas aeruginosa was resistant to emodin 0.5µg/ml.

Conclusion: Emodin extract of trichoderma longibrachiatum showed high antimicrobial activity against human pathogenic bacteria.

Keywords: *Pseudomonas aeruginosa, Staphylococcus aureus, Proteus mirabilis, Escherichia coli, Trichoderma, Ethyl acetate, emodin, Agar, Mirabilis, Klebsiella, Bacterial, Polymerase*. **DOI: https://doi.org/10.47391/JPMA.IQ-28**

Introduction

Trichoderma, a genus of filamentous fungi, is common in many soils and rhizosphere. Since the 1930s, it has been a prominent topic of basic and applied mycology study. Using antagonism, parasitism, and/or antibiosis, several members of this genus are able to combat nematodes and plant pathogenic fungi.[1] Trichoderma is one of the most extensively studied filamentous fungal genera, with diverse applications in agriculture, industry, and the environment.[2] It is a prolific producer of a variety of secondary metabolites, such as non-ribosomal peptides (NRPs), peptibols, polyketides, pyrones, siderophores, and volatile and non-volatile terpenes can all be used in the food, pharmacological, agricultural, and biotechnology industries.[3-5] These are natural products that exhibit biological activity, and have an enormous social impact. Others are interested in the pharmaceutical field, especially antibiotics, some of which have adverse effects, and disease encounters with plants or animals.[6] Trichoderma secondary metabolites acting as self-regulators that have been identified and characterised include 1,3,8-Trihydroxy-6-Methyl-anthraquinone (emodin), pachybasin, 1-Octen-3-Ol (octenol), and 3-Octanone.[7,8] Emodin is a natural anthraquinones found in barks and roots of numerous plants, mold and lichen.[9] Emodin, together with physcion, aloe-emodin, chrysophanol and rehin, is the building block for a variety of purgative anthraquinone derivatives and has been widely utilised as a laxative substance since ancient times.[10] Pharmacological studies have shown that emodin has a variety of biological functions, such as anti-inflammatory,[9] anti-bacterial[11] and anti-cancer.[12-14] In vitro and in vivo investigations have shown that emodin protects the liver from harm caused by lipopolysaccharide (LPS), carbon tetrachloride (CCl4), alcohol, and high-fat diets. Although emodin has a positive effect on liver diseases, there is no comprehensive assessment of emodin's role in this regard.[15] The current study was planned to investigate the efficacy of emodin as an antibacterial agent in relation to a selection of clinical infections.

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Materials and Methods

The cross-sectional study, conducted at Al-Zahraa Teaching Hospital, Wassit, Iraq, from December 2021 to March 2022 was approved by the Research Ethics Committee of Wasit Directorate General of Health and the Ethics Review Board of AL-Zahra Teaching Hospital on 27. November, 2021. The patient's consent was obtained for providing the samples for analysis.

The study was conducted on tichoderma longibrachiatum samples taken from the Wasit University's garden soil which were subjected to plate morphology, microscopic examination, and polymerase chain reaction-internal transcribed spacer (PCR-ITS) testing.

Czapek's media with some modifications assessed the production of inhibitory substances of crude extracts. Trichoderma isolates were cultivated for 7 days on potato dextrose agar (PDA), and four blocks of trichoderma agar culture 10mm in diameter were added to the Czapek's modified media (500ml) in flasks. They were incubated in the dark and kept unshaken for 14 days at mean temperature of 27±2°C. The organic solvent was added to remove anti-fungal compounds from Czapek's modified media. Ethyl acetate (Et OAc) 500ml was placed in a shaker set at 121 revolutions per minute (rpm) overnight. The extraction took a fixed time of 24 hours to finish. A revolving evaporator (Gallen Hamp, England) at 37°C was used to extract antifungal extracts and the Et OAc boiling point of 77.1°C was taken as the reference point.[16]

Emodin extracts of trichoderma isolate in vitro antimicrobial activity were studied against pathogenic bacteria *proteus (P.) mirabilis, staphylococcus (S.) epidermidis, staphylococcus (S.) aureus, streptococcus (S.) pyogenes, escherichia (E.) coli, pseudomonas (P.) aeruginosa and klebsiella (K.) species*. Using the agar well diffusion method, emodin extract doses of 0.5µg/ml, 1µg/ml, 2µg/ml and 4µg/ml were tested against clinical pathogenic bacteria.

Data was analysed using SPSS 22. Descriptive and inferential statistics were employed. P<0.05 was considered significant.

Results

Emodin 0.5µg/ml showed high antibacterial activity against P. mirabilis, with emodin inhibition zone (EIZ) of 8.7mm, followed by *S. epidermidis* 7mm, *S. pyogenes* 7.6mm, *S. aureus* 7.5mm, *E. coli* 6.7mm and *klebsiella species* 0.4mm. *P. aeruginosa* was resistant to emodin 0.5µg/ml.

EIZ was found to significantly different at differ emodin concentrations (p=0.000001). EIZ at 0.5µg/ml concentration was non-significantly lower than EIZ at 1µg/ml (p>0.05), but EIZ at 0.5µg/ml was significantly lower than at (2 µg/ml, 4µg/ml (p<0.05) (Table). *E. coli* showed lower EIZ at 0.5µg/ml, and higher EIZ was recorded at 4µg/ml (Figure 1A). Findings indicated a bivariate nonlinear, quadratic and significantly strong positive correlation between concentration level and inhibition zone for E. coli (r=0.995, p=0.000001).

For klebsiella species, the inhibition zone was lower with emodin concentration 0.5µg/ml, and higher with the concentration of the disc (Figure 1B). There was a bivariate nonlinear, cubic and significantly strong positive correlation between concentration level and inhibition zone for *klebsiella species* (r=0.965, p=0.000001).

Lower inhibition zone was seen in *P. aeruginosa* with emodin concentration of 1µg/ml, and higher with 4µg/ml. At 0.5µg/ml concentration and, disc there were zero inhibition zones (Figure 1C). There was a bivariate, nonlinear, cubic and significantly strong positive correlation between concentration level and inhibition zone for *P. aeruginosa* (r=0.984, p=0.000001).

The inhibition zone for *S. aureus* was seen to be lower with emodin concentration 0.5µg/ml, and higher with the disc (Figure 1D). There was a bivariate, linear and significantly strong positive correlation between concentration level and inhibition zone for *S. aureus* (r=0.976, p=0.000001).

For *S. epidermidis*, the inhibition zone was lower mean with emodin concentration 0.5µg/ml, and higher with 4µg/ml (Figure 1E). There was a bivariate, nonlinear, cubic and significantly strong positive correlation between concentration level and inhibition zone for *S. epidermidis* (r=0.974, p=0.000001).

For *P. mirabilis*, the inhibition zone was lower with emodin concentration 0.5µg/ml, and higher with 4µg/ml (Figure 1F). There was a bivariate, nonlinear, cubic and significantly strong positive correlation between concentration level and inhibition zone for *P. mirabilis* (r=0.990, p=0.000001) (Figure 2).

For *S. pyogenes*, the inhibition zone was lower with emodin concentration 0.5µg/ml, and higher with concentration of 4µg/ml, and there was a bivariate, nonlinear, cubic and a significantly strong positive correlation between concentration level and inhibition zone (r=0.988, p=0.000001) (Figure 2A-G).

Table: Mean values of the inhibition zone in different emodin concentrations.

Bacteria isolate	n	emodin Inhibition Zone Concentration (mg/ml) (Mean ± SD) (M.R)				disc (30mg/ml)	Kruskal-Wallis Test	Post-hoc*	*p*-value
		0.5	1	2	4				
Escherichia coli	10	6.9 ± 0.57 (5.5)	11.3 ± 0.48 (15.5)	17.6 ± 0.52 (25.5)	23.5 ± 0.71 (43.0)	22.6 ± 0.97 (38.0)	K= 46.14 Sig. 0.000001	0.5 vs. 1 0.5 vs. 2 0.5 vs. 4 0.5 vs. disc 1 vs. 2 1 vs. 4 1 vs. disc 2 vs. 4 2 vs. disc 4 vs. disc	0.999 0.02 0.0001 0.0001 0.999 0.0001 0.005 0.066 0.540 0.999
Pseudomonas taeruginosa	10	0.00±0.00 (0.0)	11.2±0.42 (5.5)	19.5±0.85 (15.5)	23.3±0.82 (25.5)	0.00±0.00 (0.0)	K= 26.80 Sig. 0.000002	1 vs. 2 1 vs. 4 2 vs. 4	0.096 0.0001 0.096
Staphylococcus aureus	10	7.5±8.5 (5.5)	C11.2±0.42 (16.0)	12.5±0.53 (25.0)	21.0±0.94 (38.1)	21.8±0.42 (43.9)	K= 45.96 Sig. 0.000001	0.5 vs. 1 0.5 vs. 2 0.5 vs. 4 0.5 vs. disc 1 vs. 2 1 vs. 4 1 vs. disc 2 vs. 4 2 vs. disc 4 vs. disc	0.999 0.025 0.0001 0.0001 0.999 0.006 .0001 0.418 0.054 0.999
Staphylococcus epidermis	10	7.7±0.48 (5.5)	10.3±0.48 (15.5)	13.0±0.94 (31.7)	22.9±0.99 (45.5)	12.6±0.52 (29.3)	K= 45.56 Sig. 0.000001	0.5 vs. 1 0.5 vs. 2 0.5 vs. 4 0.5 vs. disc 1 vs. 2 1 vs. 4 1 vs. disc 2 vs. 4 2 vs. disc 4 vs. disc	0.999 0.001 0.0001 0.002 0.123 0.0001 0.329 0.329 0.999 0.123
Proteus mirabilis	10	8.7±0.48 (5.5)	10.6±0.52 (15.5)	12.8±0.63 (25.5)	21.9±0.88 (41.9)	21.6±0.52 (39.1)	K= 45.88 Sig. 0.000001	0.5 vs. 1 0.5 vs. 2 0.5 vs. 4 0.5 vs. disc 1 vs. 2 1 vs. 4 1 vs. disc 2 vs. 4 2 vs. disc 4 vs. disc	0.999 0.019 0.0001 0.0001 0.999 0.0001 0.003 0.110 0.349 0.999
Streptococcus	10	7.6±0.52 (5.5)	12.4±0.52 (15.5)	16.5±0.53 (25.5)	24,7±1.2 (43.35)	23.3±0.95 (37.65)	K= 46.28 Sig. 0.000001	0.5 vs. 1 0.5 vs. 2 0.5 vs. 4 0.5 vs. disc 1 vs. 2 1 vs. 4 1 vs. disc 2 vs. 4	0.999 0.020 0.0001 0.0001 0.999 0.0001 0.006 0.057

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Table-1: *continued from previous page*

Bacteria isolate	n	emodin Inhibition Zone Concentration (mg/ml) (Mean ± SD) (M.R)				disc (30mg/ml)	Kruskal-Wallis Test	Post-hoc*	*p*-value
		0.5	1	2	4				
Klebsiella	10	0.4±0.8 (5.5)	12.5±0.53 (15.5)	15.7±0.95 (25.95)	18.2±1.03 (36.75)	19.4±0.52 (43.8)	K= 45.94 Sig. 0.000001	2 vs. disc 4 vs. disc 0.5 vs. 1 0.5 vs. 2 0.5 vs. 4 0.5 vs. disc 1 vs. 2 1 vs. 4 1 vs. disc 2 vs. 4 2 vs. disc 4 vs. disc	0.601 0.999 0.999 0.016 0.0001 0.0001 0.999 0.010 0.0001 0.951 0.058 0.999

SD: Standard deviation.

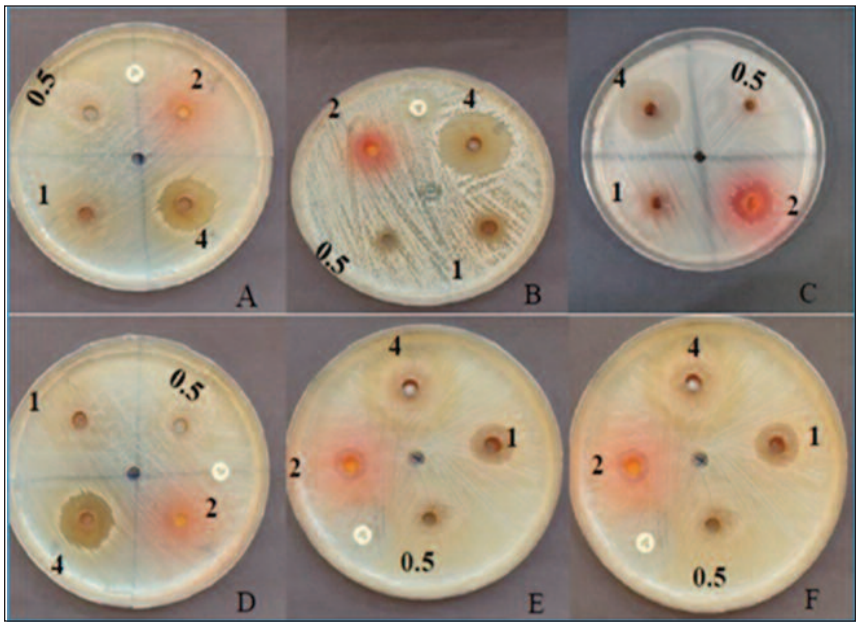

Figure 1: Antibacterial activity of emodin extract of Trichoderma longibrachiatum against (A) *Escherichia (E.) coli*, (B) *Klebsiella (K.)*, (C) P*seudomonas (P.) aeruginosa*, (D) *Staphylococcus (S.) aureus*, (E) *Staphylococcus (S.) epidermis* and (F) *proteus (P.)* mirabilis on Mueller-Hinton agar, after 24 hours at 37°C using agar well diffusion method (diameter of the well 5mm).

Discussion

The activity of emodin varied according to microbial species and strains. Studies have revealed that minimal emodin concentrations of 50µg/mL had bactericidal action, and bacteriostatic action against bacillus subtilis, and *S. aureus* at emodin concentrations of 7.8µg/mL and 3.9µg/mL, respectively.[17,18] The current findings disagreed with a study showing emodin was not active against *klebsiella (K.) pneumoniae* and *E. coli*.[19] and that the minimum inhibition concentration of emodin against these two bacterial species was 32µg/mL and 16µg/mL, respectively. A study determined the minimum inhibition concentration of emodin for bacillus cereus, bacillus subtilis and bacillus pumilus (0.5, 1.5, 2.0µg/mL), and found it to be higher for *P. aeruginosa* and *S. aureus* (70.0, 90.0µg/mL). *Listeria (L.) ivanovii* and *S. pneumoniae* were less sensitive, and trichoderma species produced antibiotics and other compounds damaging to pathogens and limiting development in plants (antibiosis).[20]

The considered mechanisms for biocontrol are antibiosis, lysis, competition and mycoparasitism. These may act alone or in combination. Trichoderma species are also current against various gram-negative and gram-positive bacterial species. They produce around 40 different metabolites of trichoderma, as well as norfloxacin and ciprofloxacin in cultures of trichoderma that are antibacterial in nature.[21] Anthraquinones' antibacterial actions are numerous, ranging from basic cell wall instability to changes in metabolic pathways or deoxyribonucleic acid (DNA) inclusions either directly or indirectly (caused by free radicals). The chemical properties of anthraquinone are linked to the efficiency of these mechanisms, including steric effect, potential of hydrogen (pH) and polarity of group substituents. To add to the difficulty of developing bacterial resistance, a single anthraquinone derivative may have many mechanisms of action.[22] A study demonstrated that emodin can attach to and insert into the bacterial cell membrane, resulting in cytoplasmic membrane integrity loss.[23] According to the study, emodin may block electron

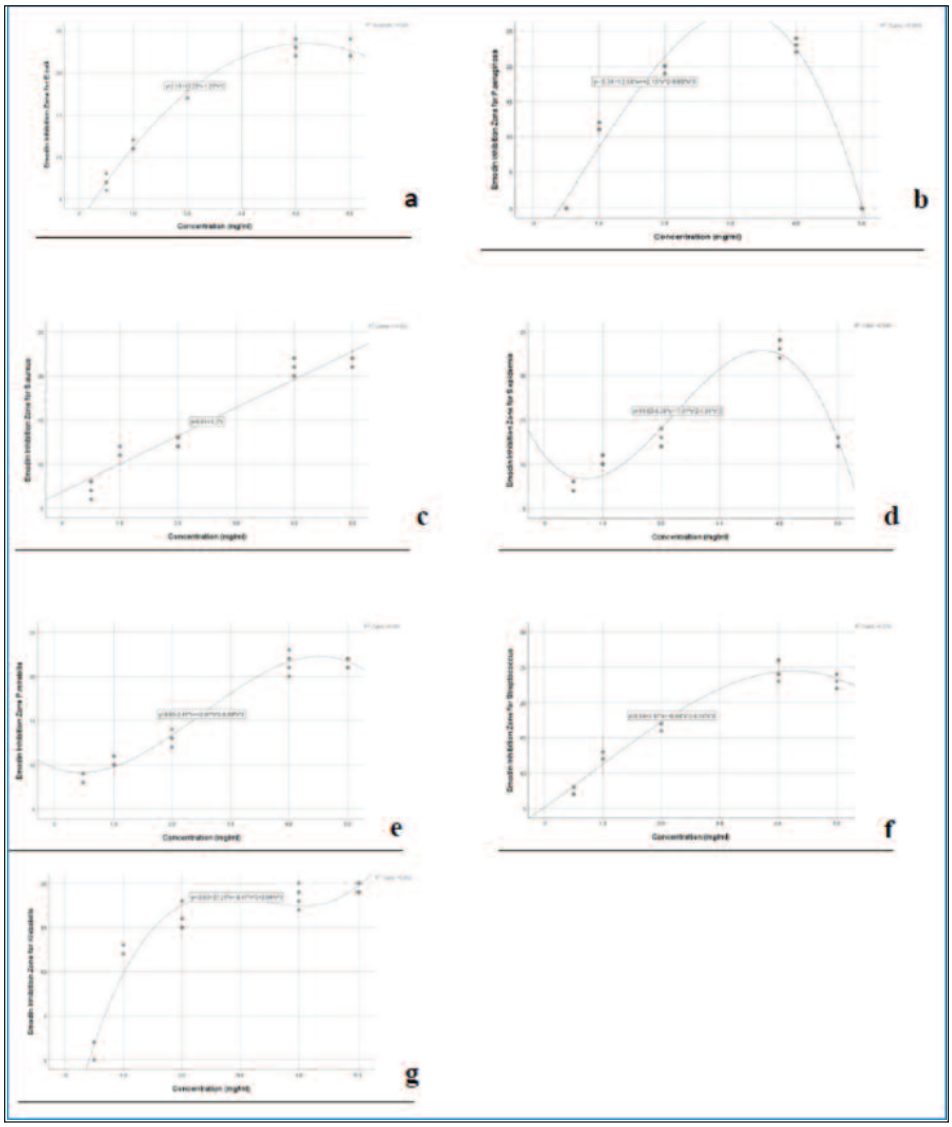

Figure 2: Scatterplot showing the relationship between concentration and inhibition zone (n=70) of a) E*scherichia (E.) coli*, b) *Pseudomonas (P.) aeruginosa*, c) *Staphylococcus (S.) aureus*, d) *Staphylococcus (S.) epidermis*, e) *Proteus (P.) mirabilis*, f) *Streptococcus (S.) pyogenes*, and g) *Klebsiella species*.

transport in the respiratory chain, substrate oxidation and dehydrogenation processes in bacteria. There was also speculation that causing DNA damage and inhibiting internal processes in bacteria would be the way to kill them rather than membrane permeabilisation.[23] A study showed that trichoderma isolates' crude extract had high antibacterial activity, and *S. aureus* exhibited higher sensitivity than other pathogenic bacteria. The growth zone inhibition increased when concentrations increased, but *E. coli, acinetobacter (A.) baumannii* and s*almonella (S.)* typhi showed resistance to all concentration of the trichoderma isolates.[24] A study showed the *aspergillus (A.)* chevalieri and *trichoderma (T.)* harzianum metabolites has good antimicrobial activity against *E. coli, P. aeruginosa*, and methicillin-resistant *S aureus* (MRSA).[25]

Bioactive chemicals harm bacterial cells through a variety of processes. By forming complexes with soluble bacterial cell proteins, including essential enzymes, emodin can block them or act as a DNA-intercalating agent. It can also coagulate soluble bacterial cell proteins, including important enzymes. Others are in charge of membrane disruption as well as the inhibition of cell wall and nucleic acid synthesis. Lysozyme damages bacterial cell walls and the cell membrane, causing membrane breakdown and the release of intracellular contents, as well as bacterial cell death.[26]

Despite the fact that emodin had no effect on genes involved in cell wall construction and lysis, as well as lactamase activity and drug accumulation, it reduced membrane fluidity and degraded membrane integrity, resulting in monoatomic monocation from sodium (Na+) and potassium ion (K+) leakage from the bacteria.[27]

Emodin's capacity to connect with the phosphate group of DNA and intercalate into the base pairs of the DNA helix is the basis of its antibacterial effects. Replication and transcription are affected, expression is repressed, and the cells will potentially die (inhibition of nucleic acids synthesis).[27] Emodin has high antibacterial action against gram-positive bacterial strains, including those that are medication-resistant. Halo emodin expedites the rate at which potassium ions can pass across the plasma membrane of bacteria. More crucially, halo emodin binds to proteins more strongly and stably than emodin. These findings help to explain why halo emodin has stronger antibacterial properties than its parent nucleus, emodin.[11]

Conclusion

Emodin extract of trichoderma longibrachiatum showed high antimicrobial activity against all the human pathogenic bacteria studied. Emodin from trichoderma

longibrachiatum can be used as an effective treatment to eliminate human pathogenic bacteria rather than the use of chemical antibiotics.

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