Trop J Nat Prod Res, August 2021; 5(8):1354-1359
ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Inhibitory Effects of Radish Peeled Root Extracts against Some Pathogenic Bacteria

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ARTICLE INFO

Article history:
Received 05 March 2021
Revised 20 May 2021
Accepted 18 August 2021
Published online 02 September 2021

ABSTRACT

Several studies have shown the ability of extracts from different plant parts to suppress the growth of pathogenic bacteria, including drug-resistant strains. This study was aimed to investigate the antibacterial effectiveness of radish (*Raphanus sativus)* root peel extracts against selected pathogenic bacteria. Different clinical samples were collected in period between December 2019 - June 2020 in Tikrit city of Iraq. The Identification of isolates was conducted using microscopic, macroscopic cultural characteristics and biochemical tests. Ethanol and methanol extracts were prepared from radish root peels. The Kirby-Buer well diffusion assay was employed to test the antibacterial activities of the extracts, selected antibiotics, and interaction between both agents. Virulence factors were detected before and after exposure of test isolates to the extracts. The results indicated that *Pseudomonas aeruginosa, Klebsiella pneumoniae, and Escherichia coli* were the predominant isolates. The methanol extract showed more inhibitory effect than the ethanol extract against the test pathogenic bacteria. The concentration of 100 mg/mL of the methanol extract had higher inhibitory activity compared to other concentrations. A synergistic inhibitory effect between the extracts with test antibiotics was obtained against a wide variety of bacteria, thereby expanding the target spectrum. In addition, it was observed that biofilm production was the factor most affected, suggesting the possibility of the extracts reducing the virulence of the test bacteria. It can be concluded that the combination of radish peeled root methanol extract with ethanol extract or antibiotics inhibited bacterial growth synergistically.

***Keywords*:** Antimicrobial activity, Radish, Root peel extract, Virulence factor.

Introduction

The emergence of multiple drug-resistant bacteria has become a worldwide health concern due to the misuse of commercial antimicrobial drugs. Therefore, there is still a need to explore prospective alternative compounds capable of inhibiting bacterial pathogens.[1] There are more than 1,340 plants known to have potential sources of antimicrobial compounds, yet, few have been studied scientifically.[2] Antimicrobial properties of plant-derived compounds were demonstrated in the late 19th century.[3] Based on reports of the World Health Organization, more than 80% of the world's population rely on herbal medicines as a primary source of health care. Concerning the side effects of conventional medicine, the use of natural products as an alternate treatment in healing various diseases has risen during the last few decades.[4]

Raphanus sativus is a winter food crop that is commonly used as a salad ingredient in Asian countries, this plant comes in numerous types with common names such as black radish (English), *Daikon* (Japanese), and *Mooli* (Urdu), and it has long been used as a medicinal herb. It is used to treat poor digestion and liver dysfunction by acting as an appetizer and having laxative effects on the bowel.[5] Flavonoids, phenols, and other active substances present in radish result in a decrease in the cell wall, and cytoplasmic membrane permeability, denaturation of bacterial protein, and formation of hydrogen bond with protein, thereby damaging the protein structure. Furthermore, it creates macromolecular and micromolecular imbalances, as well as cell lysis.[6]

The present study was aimed at investigating the antibacterial activities of *R. sativus* extracts against pathogenic bacteria and detecting differences in virulence factor expression before and after exposure to the crude extracts.

Materials and Methods

Bacteria and cultural conditions

The pathogenic bacteria were collected from different clinical cases including urinary tract infection, pharyngitis, otitis media, wounds, and lower respiratory tract infection using cotton swabs or universal sterile cups depending on the specimens. These samples were immediately transported to the laboratory under the supervision of physicians. Bacteria were isolated from the clinical samples and identified using microscopic, and cultural characterization. Biochemical tests under aerobic conditions were performed which included Indole test to investigate the production of indole; methyl red test to investigate sugar fermentation with acid production; Vogus-Proscauer test for acetone compound detection; utilization of citrate as a sole source of carbon and formation of sodium carbonate and urease test to indicate the hydrolysis of urea and formation of ammonium. In addition, an oxidase test was used to investigate the production of cytochrome; catalase and fermentation tests for sugar fermentation; motility screening, and coagulase enzyme tests were carried out in two ways (slide and tube methods) as described by Mahmoodn, 2019. [7]

Confirmation of the identity of bacterial isolates

Vitek compact technique using automated Vitek device (BioMérieux, Marcy l'Etoile France) was used to confirm the identity of bacterial isolates. Each isolate was cultured on nutrient agar and incubated overnight at 37°C. Bacteria were suspended in 2.5 mL of a 0.45% sodium chloride solution. The suspension used was adjusted to a McFarland standard of 0.5 using a Densicheck [8]. The cards were automatically filled by a vacuum device, sealed, and inserted into the

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Citation: Abed SM, Yasin LQ, Mahmood WS. Inhibitory Effects of Radish Peeled Root Extracts against Some Pathogenic Bacteria. Trop J Nat Prod Res, Month 2021; 5(8):1354-1359. doi.org/10.26538/tjnpr/v5i8.5

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

reader-incubator module (incubated at 35.5°C) and subjected to a kinetic fluorescence measurement every 15 min. The results obtained were interpreted via the ID-GP and ID-GN database, and the final results were obtained automatically. All cards used were automatically discarded into a waste container. The bacteria were identified up to the species level and preserved on slants until further tests were required.

Preparation of radish plant material
Radish plant was obtained from the city of Sharqat at Salah el-Din Province in November 2019 and then identified in the Herbarium of the College of Science, the University of Tikrit, Tikrit, Iraq with the classification number 2419. The plant roots were washed with distilled water to remove dust particles from them and then dried at a temperature of 25℃. Root peels were collected from the dried plant samples, by stirring them to prevent the occurrence of rotting. After drying, these peels were ground into a fine powder, and the milled parts were kept in clean plastic containers under a moisture-free condition until required for extraction.[9]

Solvent extraction of radish root peel
Ethanol and methanol extracts of radish root peels were prepared as described,[5] using 80% of both solvents. The Radish root peel sample was weighed (1 g) and added to 50 mL of each solvent. After constant shaking at 150 rpm for 72 h at room temperature, the sample was filtered. The filtrate was incubated as recommended by Ogueke *et al* at 40°C until all the solvent was evaporated leaving behind the crude ethanol extract.[10] crude extracts (1 g) obtained was dissolved in 5 mL of 1% dimethylsulphoxide (DMSO) and then diluted to obtain 100 and 50 mg/mL concentrations. These were stored at 15^{o}C in amber-coloured bottles until required.[11]

Radish extract susceptibility test
Antimicrobial activity of radish root peel extracts was tested by the Kirby-Bauer agar diffusion method using Mueller Hinton agar (Himedia)**.** The inoculum size of each test bacterium was prepared using No. 0.5 McFarland tube to give a concentration of 1x10^{8} Colony Forming Unit (CFU).[8] Aliquot of 0.1 mL from the test bacterial culture was inoculated onto the plate, left to dry at room temperature for a while and then wells were made on top with a sterile stick, these wells were filled with approximately 100 µL of plant extracts (100 mg/mL). Then, bacterial cultures were incubated at 37°C and the diameters of the inhibition zones were measured in millimeters[1]

Antibiotic susceptibility test
Antibiotic susceptibility test was performed using Kirby-Bauer method following standards of Clinical and Labo-Standards Institute (CLSI) Guidelines.[12] The bacterial isolates were classified as being resistant (R)/ Intermediate (I)/ Susceptible (S)/ for each of the test antibiotics.[12]

Synergistic effects of alcoholic extracts with antibiotics
The Kirby-Bauer disc diffusion method was conducted using Mueller Hinton agar by adding 10 μL of methanol extracts of Radish peeled root onto the antibiotic disc.[13]

Detection of some virulence factors before and after exposure
Certain media were used to determine isolates' possession of the necessary enzymes to hydrolyze materials present in them. The virulence factors detection tests included: Hemolysin production which was tested using blood base agar; Urease production was detected using Urea agar base; Biofilm production test was conducted using Congo red agar procedure.[14] Gelatinase and protease production tests involving skim milk agar for the latter were conducted according to Birri *et al*.[15] Lecithinase production was detected using egg yolk; lipase production was conducted by dissolving 10 g of acacia in 400 mL of warm distilled water, mixed thoroughly, then 100 mL of olive oil was added and agitated vigorously for emulsification.[16] In exposing the isolates to the extracts, bacterial cells were transferred into 5 mL phosphate buffer saline pH7 and an equal volume of 100 mg/mL concentration of both extracts were added and incubated at 37^{o}C in a shaker incubator at 200 rpm for 24 h.[17]

Statistical analysis
Data were statistically analyzed using one-way ANOVA test and means were compared via Duncan multiple range test using Minitab version with a confidence level of 0.01.[18]

Results and Discussion

Identification of clinical bacterial isolates
Bacterial isolates collected from clinical cases (urinary tract infection, pharyngitis, otitis media, wounds, and lower respiratory tract infection) were cultured and identified using phenotypic, microscopic, and biochemical characteristics (Table 1). Further confirmatory tests were conducted using the Vitek2 technique to identify each isolate at the species level. Thirteen isolates were obtained, belonging to nine species. Among the isolates, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Escherichia coli* were dominant, having two isolates for each species.

Table 1: Biochemical test for identification of bacterial isolates

	Characteristics of bacterial isolate								
	Enterobacter clocae	*E. coli*	*Klebsiella pneumoniae*	*Proteus vulgaris*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Moganella morgani*	*Aeromonas hydrophila*	*Citrobacter freundii*
Biochemical test									
Catalase	+	+	+	+	+	+	D	+	+
Citrate	+	-	+	+	+	+	-	+	+
Coagulase	nt	nt	nt	nt	nt	+	nt	nt	nt
Gas Forming	-	+	+	+	+	-	+	+	+
Gelatin	-	-	-	+	+	+	-	+	-
Gram Stain	-	-	-	-	-	+	-	-	-
H2S	-	-	-	+	-	-	-	+	+
Indole	-	+	-	-	-	-	+	+	v
Motility	-	+	-	+	+	-	+	+	+
Methyl red	-	+	-	+	-	+	+	-	+
Oxidase	-	-	-	-	+	-	-	+	-
Urease	-	-	+	+	-	+	+	+	v
Glucose	+	+	+	+	-	+	+	+	+
Lactose	-	+	+	-	-	+	-	v	+
Lipase	+	-	-	+	+	+	-	-	-

V: Variable; nt: Not tested

Inhibitory effect of radish root peel alcoholic extracts and their interactions with antibiotics
Comparing the effects of radish root peel ethanol and methanol extracts on test bacterial isolates, it was observed that the methanol extract was more efficient than the ethanol extract at 100 mg/mL concentration (Table 2). This result is in agreement with Ahmad's study, he was observed that the highest antibacterial effects were observed in ethanol and methanol extracts, followed by ethyl acetate, chloroform, benzene, aqueous hot and aqueous cold.[19] The

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

antimicrobial activity of the methanol extract on the bacterial isolates was significantly different. The maximum inhibitory activity of the extract was obtained against Gram-negative bacteria (*E. coli*, *K. pneumonia*, and *M. morgani*).

The mechanism of action of plant extract is based on their ability to bind proteins, thereby inhibiting cell protein synthesis, cell wall degradation, destruction of the plasma membrane and membrane proteins, intracellular content leakage, interference with metabolic enzymes or active transport, coagulation of cytoplasm, dissipation of cellular energy in ATP form, proton motive force (PMF) depletion and electron flow, which can ultimately result in cell death. The highest inhibitory activity against test bacteria was observed in the 100 mg/mL concentration of methanol extract compared to the other concentrations. It was revealed that the potential capacity of the extract increased with increasing concentration. The methanol plant extract may contained tannins, anthocyanins, polyphenols, terpenoids, saponins, xanthoxyllines, totarol, quassinoids, lactones, flavones, and phonons.[20] Research published by Nagappan *et al*, indicated that the presence of flavonoids, phenolic compounds, and carotenoids enhanced antibacterial activities comparable to the standard antibiotics such as gentamicin sulphate, ofloxacin, tobramycin, and ciprofloxacin.[21]

Inhibition zones obtained when a combination of both extracts was tested ranged from 29 to 45±1 mm, suggesting a synergistic effect between the two extracts due to their increased inhibitory effectiveness (Table 2). The diversity of active substances contained in extracts can be an efficient way to increase their effectiveness against pathogenic microorganisms and the expansion of the target spectrum. A mixture of alcoholic extracts increases their inhibitory activities compared to when the extracts were used individually. This observation is due to the combination of active substances such as polyphenolic compounds, thymoquinone, and trigonelline contained in them.[22] The results of the current study agree with the findings of Al-Ani, who reported that the synergistic action between both natural materials was more effective and had a distinctive effect on bacterial resistance to test antibiotics. [23]

The synergistic effects of alcoholic extracts with antibiotics against test bacteria

Antibiotics susceptibility testing revealed that the best inhibitory results on test pathogenic bacteria are in the order of Amikacin > Doxycycline > Ceftriaxone > Trimethoprim > Chlorteracycline > Cefexime in relation to the other antibiotics. The synergistic effect of methanol extract and antibiotics was reported in (Table 3), which demonstrated a considerable difference in antibacterial activity against target species. This observation is in accordance with the findings of Fatma and coworkers, where they discovered that a synergy occurred with Oxacillin antibiotics, a synergistic potential also observed with Ampicillin, Chlorteracycline, Trimethoprim, Augmentine, and Doxicycline.[24] Furthermore, our results agreed with the discovery of Ennacerie *et al*.,[25] who published that the association between extracts and antibiotics showed an increased level of antibacterial potency compared with lone antibiotics. The antibiotics susceptibility test results revealed that *S. aureus* was the isolate most affected by the test antibiotics, while *E. coli* showed the highest changes among the test microorganisms. For both the Gram-positive and Gram-negative bacteria, the extract might have increased permeability of the cell by interacting with the cell membrane and/or layer of lipopolysaccharide, thereby allowing the antibiotics to have access to the cytoplasmic targets. Meanwhile, the synergy indicated a broad-spectrum activity with a decreased risk of resistant strains.[26]

Detection of virulence factors

Virulent factor production was evaluated using traditional culture-based methods. The results were recorded as (+) for present and (–) for absent, before and after exposure to a concentration of 50% (v/v) of extract (Table 4). The current study revealed that biofilm production was the factor most affected by the active substances of the extracts. This indicates the possibility of the extract reducing the virulence of the test bacteria and this observation is in agreement with a previous study, where antimicrobial agents of radish extract affected biofilm activities. Previous study discussed the use of various compounds to inhibit pathogenic bacteria, such as the direct influence of Ag and TiO_2 nanoparticles on pathogenic bacteria.[28] On the other hand, several studies have found that using physical forces therapy, such as Audible Sounds and Magnetic Fields, can reduce pathogenic bacteria resistance.[29]

Table 2: Inhibitory effects (inhibition zone diameter in mm) of ethanol extracts and methanol extracts on bacteria and the combined effect of both extracts

Bacterial Isolate	Ethanol	Methanol	Equal volume of both extracts (v/v 50 µl)
Staphylococcus aureus	22 CD b	20 C b	30 C a
Citrobacter frunedii	16 F c	27 B b	31 C a
Proteus vulgaris	25 BC b	30 B a	33 C a
Klebsiella pneumoniae	20 DE c	37 A b	43 A a
Pseudomonas aeruginosa	18 EF b	21 C b	29 C a
Enterobacter clocae	28 AB b	37 A a	39 B a
E. coli	28 AB c	40 A b	45 A a
Moganella morgana	30 A b	38 A a	38 B a
Aeromonas hydrophila	20 DE b	23 C b	29 C a

Same lower-case alphabet indicate values that are significantly different at P< 0.01, Different letters in a vertical column indicate significant difference between means of extract in relation to its effect on the bacterial isolates

Conclusion

The findings of this study revealed that the methanol radish root peel extract at 100 mg/mL concentration showed more inhibitory activity than the ethanol extract against the test pathogenic bacteria. In addition, a synergistic inhibitory effect was obtained between the extracts and the test antibiotics against a wide variety of bacteria. Biofilm production was detected to be the most affected factor.

Conflict of Interest

The authors declare no conflict of interest.

Author's Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Table 3: Antibiotics susceptibility results before and after exposure

Antibiotic	**Ox**		**E**		**TE**		**AM**		**CH**		**AUG**		**AK**		**SXT**		**Do**		**CTR**		**CT**		**AZM**	
(CON)	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**
Isolate																								
Pseudomonas	14	30	14	28	18	25	10	12	0	0	14	20	0	0	0	0	0	0	0	0	0	0	8	13
	Ab	Aa	Bb	Aa	Ab	Aa	Ba	Ba	Ba	Da	Ab	Aa	Ba	Ba	Ca	Da	Cb	Ba	Ca	Ca	Ca	Ea	BCb	Ca
E. coli	12	15	13	17	10	17	6	9	8	11	16	20	0	0	11	18	6	18	0	0	12	30	6	12
	ABb	Ca	Bb	Ca	Bb	Ba	Cb	Ca	Ab	ABa	Ab	Aa	Ba	Ba	Ab	Ba	Bb	Aa	Ca	Ca	Ab	Aa	Cb	Ca
Morganella	11	18	6	9	0	0	-	0	8	10	-	0	0	0	0	0	0	0	18	26	0	9	6	8
	BCb	Ba	Db	Ea	Da	Da		E	Aa	Ba		C	Ba	Ba	Ca	Da	Ca	Ba	Ab	Aa	Cb	Ca	Ca	Da
Aeromonas	9	12	10	12	6	9	6	6	6	10	10	16	0	0	6	10	11	19	8	8	9	6	11	16
	Cb	Da	Ca	Da	Cb	Ca	Ca	Da	Ab	Ba	Bb	Ba	Ba	Ba	Bb	Ca	Ab	Aa	Ba	Ba	Ba	Db	Bb	Ba
klebsiella	10	12	17	21	0	0	-	0	0	0	8	15	0	0	0	0	0	0	0	0	0	0	-	-
	BCa	Da	Ab	Ba	Da	Da		E	Ba	Da	Bb	Ba	Ba	Ba	Ca	Da	Ca	Ba	Ca	Ca	Ca	Ea		
Staphylococcus	6	8	0	0	0	0	0	0	6	6	-	0	0	0	0	0	0	0	0	0	0	0	-	-
	Da	Ea	Ea	Fa	Da	Da	Da	Ea	Aa	Ca		C	Ba	Ba	Ca	Da	Ca	Ba	Ca	Ca	Ca	Ea		
Citrobacter	0	0	0	0	0	0	13	30	-	13	-	0	10	12	0	0	0	0	0	0	0	0	15	30
	Ea	Fa	Ea	Fa	Da	Da	Ab	Aa		A		C	Aa	Aa	Ca	Da	Ca	Ba	Ca	Ca	Ca	Ea	Ab	Aa
Proteus	6	9	6	12	0	0	15	30	-	0	-	0	0	0	6	9	0	0	0	0	13	17	-	-
	Db	Ea	Db	Da	Da	Da	Ab	Aa		D		C	Ba	Ba	Bb	Ca	Ca	Ba	Ca	Ca	Ab	Ba		
Enterobacter	0	0	0	0	0	0	-	0	-	0	-	0	0	0	10	27	0	0	0	0	0	0	-	-
	Ea	Fa	Ea	Fa	Da	Da		E		D		C	Ba	Ba	Ab	Aa	Ca	Ba	Ca	Ca	Ca	Ea		

(-): Means the antibiotics worked properly without addition of extract
B: Before exposure to extract
A: After exposure to extract
Lower-case alphabet indicates significant different at P< 0.01; Different letters in a vertical column indicate significant difference between means in relation to the bacterial isolates.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Table 4: Virulence factors before and after exposure to the combined extracts

Virulence factor	**Hemolycin**		**Urease**		**Biofilm**		**Gelatinase**		**Lecithinase**		**Protease**		**Lipase**	
Bacterial Isolates	B	A	B	A	B	A	B	A	B	A	B	A	B	A
S. aureus	+	+	-	-	+	-	+	+	-	-	-	-	-	+
Citrobacter	+	+	+	+	+	-	-	-	-	-	-	-	-	+
Proteus	-	-	+	+	-	-	+	+	-	-	+	+	-	-
klebsiella	+	-	+	+	+	-	-	-	-	-	+	+	-	-
Pseudomonas	-	-	_	-	+	-	+	+	-	-	-	-	-	-
Enterobacter	-	-	+	+	+	-	-	+	-	-	-	-	+	+
E. coli	+	-	+	+	-	-	-	-	-	-	+	+	-	-
Morganella	-	-	-	+	-	-	-	-	-	-	-	+	-	+
Aeromonas	+	-	-	+	-	-	-	-	-	+	+	+	-	+

+: Positive; -: Negative; B: Before exposure to extract; A: After exposure to extract

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