

Ethnomedicinal *Cichorium intybus* Seed Extracts: An Impending Preparation against Multidrug Resistant Bacterial Pathogens

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Abstract

Background: The present study was undertaken to analyze the phytochemical content and biological activity of *Cichorium intybus* seeds traditionally used in Charsadda, Pakistan against multidrug resistant (MDR) bacterial pathogens.

Objectives: This study explored the qualitative and quantitative antibacterial potential of *C. intybus*. Further qualitative analysis of phytochemical content was performed.

Methods: *Cichorium intybus* seed extracts were prepared in aqueous, chloroform, ethanol, and hexane separately.

Results: All the extracts of *C. intybus* seeds were screened for antibacterial activity and phytochemical content. *Cichorium intybus* seed extract showed considerable activity against MDR pathogenic bacteria. In the well diffusion method, aqueous extracts showed a higher zone of inhibition against *Pseudomonas aeruginosa* (16 mm ± 0.7 mm) and *Acinetobacter baumannii* (13 mm ± 0.5 mm), whereas chloroform, ethanol, and hexane extracts showed activity against *P. aeruginosa* (11 mm ± 0.3 mm, 12 mm ± 0.5 mm, and 11 mm ± 0 mm, respectively) as compared to Imipenem, a broad spectrum antibiotic. Minimum inhibitory concentration and minimum bactericidal concentration values for aqueous and ethanol extracts indicate that they were more effective against MDR bacteria. Phytochemical analysis revealed that aqueous and ethanol extracts were rich in alkaloids, carbohydrates, gallotannins, and triterpenoids, whereas chloroform and hexane extracts were more concentrated with phenolics, pseudotannins, saponins, and tannins. *Cichorium intybus* seed extract demonstrated potential activity against MDR human pathogenic bacteria.

Conclusions: The undertaken study has for the first time reported the effects of *C. intybus* seed extracts against MDR bacterial pathogens. Findings of the current study will be helpful for further elucidation of bioactive molecules for therapeutic use against MDR bacterial pathogens.

Keywords: *Cichorium Intybus* Seed Extract, Antibacterial Activity, MIC, MBC, Phytochemical Analysis

1. Background

Medicinal plants are a documented source of traditional herbal medicine worldwide. These plants contain bioactive molecules offering a rich source of structural biodiversity. More than 60% of the population of developing countries uses plant-based medicines (1). The therapeutic uses of plant extracts are due to the presence of several active phytochemical substances (2). Investigations on the mode of action and potential uses of plant extract have gained momentum in recent decades due to high demand for drugs and dietary supplements derived from plants (3).

Antibiotic resistance has recently become a serious threat to public health. Moreover, infections caused by multidrug resistant (MDR) pathogens in hospitals are

more prone to antibiotic resistance and thus limit the antibiotic choices available (4). The MDR bacteria used in the current study are *Pseudomonas aeruginosa* and *Staphylococcus aureus*, the causative agents of skin infections; and *Escherichia coli* and *Acinetobacter baumannii*, common causes of urinary tract infections (UTIs). There is increasing interest in exploring the potential activity of medicinal plants against these pathogens.

Cichorium intybus seeds have been successfully used in the “ayurvedic” and “unani” system of medicine. In traditional medicine, all parts of the plant are used as diuretic, laxative, antibilious, and antipyretic medicine, as well as for blood purification and strengthening of the stomach. *Cichorium intybus* is a rich source of alkaloids, and the root extract of this plant has revealed antitumor potential in as-

cites carcinoma in mice; it also has immune-modulating properties (5), and it is used as an adulterant in coffee to reduce gastrointestinal problems, such as gastritis (6). In the undertaken study, the *C. intybus* seeds were checked for antibacterial activity against MDR, and minimum inhibitory concentration (MICs) and minimum bactericidal concentrations (MBCs) of plant extract were reported to evaluate the quantitative bacterial inhibitory and killing concentrations.

2. Objectives

The present study was carried out to explore the antibacterial activity of *C. intybus* seeds collected from the Charsadda District in Pakistan against MDR bacteria. Qualitative analysis of phytochemical content was also performed.

3. Methods

3.1. Collection of Plant Material and Processing and MDR Bacterial Culture

The *C. intybus* seeds were collected from Charsadda, Pakistan and identified at the Herbarium of the Botany Department of the Kohat University of Science and Technology (KUST), where the voucher specimen was deposited under reference number 10052/CIS. The grinding and filtration process was performed as described earlier (7). After grinding and filtration, plant dried powder was mixed in each selected solvent (aqueous, chloroform, ethanol, and hexane) and incubated for 21 days. Following filtration and evaporation of the solvent, 0.25 grams of each extract was added to 10 mL of DMSO, achieving a concentration of 25 g/L.

Multidrug resistant bacterial strains *P. aeruginosa*, *S. aureus*, *E. coli*, and *A. baumannii* were provided by the department of microbiology at KUST in Kohat, Pakistan (7).

3.2. Antibacterial Activity of *C. intybus* Seed Extracts

Activity of *C. intybus* seed extracts was evaluated against selected MDR bacteria by using the well diffusion method (8). Overnight, fresh cultures adjusted to McFarland (0.5) were inoculated on Mueller-Hinton (MHA) agar plates. Aqueous, chloroform, ethanol, and hexane extracts were poured in each well, and DMSO was incorporated as a negative control in one well. An antibiotic disc (Imipenem) was applied as a positive control. After incubation at 37°C for 24 hours, results were measured as zone of inhibition in millimeters (mm) around the wells, and the disc was measured as per CLSI (2007) guidelines (9).

3.3. Bacteriostatic Assay for Seed Extracts Against MDR Bacterial Pathogens

The bacteriostatic effects of *C. intybus* seed extracts were evaluated by measuring MICs, as described earlier (10). Minimum inhibitory concentrations were determined by adding plant extract to a nutrient broth to reach a concentration of 100 mg/mL. Following serial dilutions, 100 μ L of bacterial broth cultures equal to McFarland (0.5) were inoculated overnight in all the tubes. Parallel to this experiment, a positive control (nutrient broth and bacterial culture but without extract) and a negative control (nutrient broth and extract but without bacterial culture) were also run. The tubes were incubated at 37°C for 24 hours, after which results were recorded. The lowest concentration at which no visible bacterial growth was observed was considered as the MIC.

3.4. Bactericidal Assay for Seed Extracts Against MDR Bacterial Pathogens

Bactericidal activity of *C. intybus* seed extracts was evaluated by measuring MBCs of seed extracts, as described earlier (10). Briefly, tubes of MICs in which no bacterial growth was observed were processed for MBC. Each selected MIC tubes' contents were inoculated on nutrient agar plates. The plates were incubated at 37°C for 24 hours. After incubation, the lowest concentration at which no growth appeared was considered as the MBC of the plant extract against the specific bacterium.

3.5. Phytochemical Screening

Phytochemical analyses of secondary metabolites were carried out as previously described (11).

3.5.1. Test for Alkaloids and Flavonoids

For alkaloids, 500 mg of plant extract was dissolved in 5 mL of HCl (1%) and boiled. After boiling, the extract was filtered and processed for the addition of Mayer's reagent. For flavonoids, a few drops of NH₃ (1%) was added to the plant extract, after which the appearance of yellow color indicated the presence of flavonoids.

3.5.2. Test for Phenolic Compounds

A few drops of lead acetate were added to plant extract (2 mL), after which the appearance of a white precipitate reflected the presence of phenolic compounds.

3.5.3. Test for Bufadienolides

For bufadienolides, plant extract (3 mL) was mixed with acetic anhydride (3 mL) and then heated. H₂SO₄ was added to the mixture, and the change in color to blue indicated the presence of bufadienolides.

3.5.4. Test for Proteins

Plant extract (1 mL) added to distilled water was mixed with a few drops of Millon's reagent. Formation of a white precipitate that changed to red upon heating showed the presence of proteins.

3.5.5. Test for Carbohydrates

Plant extract (2 mL) was mixed with a few drops of α -naphthol, and then 2 mL of concentrated H_2SO_4 was added, after which the appearance of a violet ring indicated the presence of carbohydrates.

3.5.6. Test for Saponins

Powdered plant extract (50 mg) was added to distilled water (4 mL), followed by shaking, after which the presence of saponins could be detected by persistent foam.

3.5.7. Test for Tannins

Plant extract (500 mg) was added to distilled water (10 mL) and then filtered. A few drops of ferric chloride (1%) were added to the filtrate (2 mL) after which a blue-black appearance indicated the presence of tannins.

3.5.8. Test for Gallotannins or Pseudotannins

2 mL of plant extract was mixed with 5% $FeCl_3$, after which appearance of a white-brown or green color indicated the presence of pseudotannins or gallotannins, respectively.

3.5.9. Test for Triterpenoids and Steroids

Plant extract (2 mL) was mixed with few drops of H_2SO_4 , after which the appearance of a red or yellow lower layer reflected the presence of steroids or triterpenoids, respectively.

3.5.10. Test for Resins

Plant extract (5 mL) was added to copper (5 mL), then vigorously shaken and allowed to separate into two phases. After phase separation, formation of a green precipitate indicated the presence of resin.

3.6. Statistical Analysis

All the experiments were carried out in triplicate. Experimental data was reported as standard error mean (SEM) values. A chi-square test was performed to check the level of the significance of differences between extracts and controls.

4. Results

4.1. Antibacterial activity of *C. intybus* Seed Extracts

The present study was conducted to evaluate the antibacterial activity and phytochemical content of *C. intybus* seed extracts (aqueous, chloroform, ethanol, and hexane), which exhibited significant activity ($P < 0.05$) compared to a negative control against MDR clinical bacterial isolates. Aqueous extract was more effective than the other three extracts. Aqueous extract showed significant potential activity against *P. aeruginosa* ($16 \text{ mm} \pm 0.7 \text{ mm}$) and *A. baumannii* ($13 \text{ mm} \pm 0.5 \text{ mm}$) compared to Imipenem ($10 \text{ mm} \pm 0.5 \text{ mm}$). Hexane, chloroform, and ethanol extracts showed a higher zone of inhibition ($14 \text{ mm} \pm 0.3 \text{ mm}$, $13 \text{ mm} \pm 0.3 \text{ mm}$, and $12 \text{ mm} \pm 0 \text{ mm}$, respectively) against *S. aureus*. Chloroform, ethanol, and hexane extracts against *A. baumannii*, *E. coli*, and *P. aeruginosa* showed a variable zone of inhibition (Table 1).

4.2. Bacteriostatic and Bactericidal Effect of *C. intybus* Seed Extracts

To evaluate the bacteriostatic effects of plant extracts on MDR pathogens, MICs were calculated. The MIC of aqueous extract against *A. baumannii*, *P. aeruginosa*, and *S. aureus* was 6.25 mg/mL, whereas the MIC against *E. coli* was 12.5 mg/mL. The MIC of chloroform extract against *A. baumannii* was 12.5 mg/mL, and the MIC against *E. coli*, *P. aeruginosa*, and *S. aureus* was 25 mg/mL. Ethanol and hexane extracts had MICs of 6.25 mg/mL and 25 mg/mL against selected MDR bacteria, respectively. To further evaluate the bactericidal effects of plant extracts on MDR pathogens, MBCs were determined. Aqueous and ethanol extracts of *C. intybus* seeds had an MBC of 100 mg/mL against *A. baumannii*, *P. aeruginosa*, and *S. aureus*. Chloroform and hexane extracts showed no bactericidal activity in any of the concentrations used (Table 2).

4.3. Phytochemical Analysis

After evaluation of antibacterial activity, the *C. intybus* seeds were processed. Aqueous extract of *C. intybus* showed the presence of alkaloids, carbohydrates, flavonoids, and gallotannins in higher amounts. The chloroform extract exhibited relatively high levels of phenolics, pseudotannins, and tannins. When ethanol extract was studied, it exhibited relatively high levels of alkaloids, carbohydrates, gallotannins, tannins and triterpenoids. Hexane extract contained relatively high levels of phenolics, pseudotannins, and tannins (Table 3).

Table 1. Antibacterial Activity of *Cichorium Intybus* (Seeds) Extracts

MDR bacteria	Aqueous (mm)	Chloroform (mm)	Ethanol (mm)	Hexane (mm)	Positive control ^a	Negative control ^b
<i>A. baumannii</i>	13 ± 0.5	9 ± 0.3	10 ± 0.3	9 ± 0.3	10 ± 0.5	0
<i>E. coli</i>	14 ± 0.7	11 ± 0.3	12 ± 0.5	12 ± 0.3	23 ± 0.7	0
<i>P. aeruginosa</i>	16 ± 0.7	11 ± 0.3	12 ± 0.5	11 ± 0	10 ± 0	0
<i>S. aureus</i>	15 ± 0.7	13 ± 0.3	12 ± 0.3	14 ± 0.3	15 ± 0.7	0

Abbreviation: ±, Standard error of three replicates.

^aPositive control (Imipenem).

^bNegative control (DMSO).

Table 2. MICs and MBCs of *Cichorium intybus* (seeds) extracts

MDR Bacteria	Aqueous Extract (mg/mL)		Chloroform Extract (mg/mL)		Ethanol Extract (mg/mL)		Hexane Extract (mg/mL)		Positive Control ^a	Negative Control ^b
	MICs	MBCs	MICs	MBCs	MICs	MBCs	MICs	MBCs		
<i>A. baumannii</i>	6.25	100	12.5	> 100	6.25	100	25	> 100	+	-
<i>E. coli</i>	12.5	> 100	25	> 100	6.25	> 100	25	> 100	+	-
<i>P. aeruginosa</i>	6.5	100	25	> 100	6.25	100	25	> 100	+	-
<i>S. aureus</i>	6.5	100	25	> 100	6.25	100	25	> 100	+	-

Abbreviations: >100, Concentration greater than 100 mg/mL; +, Bacterial growth observed; -, No bacterial growth observed.

^aPositive control (no antibiotic added).

^bNegative control (no bacterial cells added).

5. Discussion

Plants have been used in traditional medicines for the treatment of different types of infections since ancient times. Medicinal plants are the richest bio-resources of traditional systems of medicine, as well as the basis for many synthetic drugs (12). Modern medicine has evolved from the traditional system only after thorough chemical and pharmaceutical screening (13).

In our previous study, *A. baumannii*, *E. coli*, *P. aeruginosa*, and *S. aureus* were confirmed as multidrug resistant (7). In the present work, *C. intybus* seed extracts (aqueous, chloroform, ethanolic, and hexane) were evaluated for activity against *A. baumannii*, *E. coli*, *P. aeruginosa*, and *S. aureus*. Phytochemical analysis was conducted separately for each extract. The selection of *C. intybus* seeds was based on their traditional use in the Charsadda district of Pakistan. A previous study from Cameroon documented the use of medicinal plants against MDR bacteria (14).

When *C. intybus* seeds were investigated, they exhibited variable zones of inhibition. Aqueous extract appeared to be more effective than the other three extracts against all selected MDR bacteria. Interestingly, aqueous extract showed higher activity against *P. aeruginosa* and *A. baumannii* compared to Imipenem, which is a broad-spectrum antibiotic used to limit infections caused by multidrug resistant pathogens.

Hexane, chloroform, and ethanol extracts exhibited a

higher zone of inhibition against *S. aureus*. In a previous study, the antibacterial activity of chloroform and hexane extractions of *C. intybus* against *E. coli* and *S. aureus* was investigated (15), and the results of this study are in accordance with our results. It was found that ethanol extract was more effective against *P. aeruginosa* and *S. aureus*, which is also consistent with a previous study (16). In comparing the results for inhibitory zone, bacteriostatic concentration, and bactericidal concentration, it was observed that aqueous and ethanol extracts have more potent activity against selected MDR bacteria.

Phytochemical analysis revealed that aqueous and ethanol extracts of *C. intybus* seeds exhibited relatively high amounts of alkaloids, carbohydrates, gallotannins, and triterpenoids. A previous complementary study identified phytochemical components such as alkaloids, flavonoids, saponins, tannins, and steroids in *C. intybus* seeds (17). Several studies have analyzed the antibacterial and phytochemical activity of *C. intybus*, the results of which are in line with our findings (18, 19).

The present work demonstrated for the first time that *C. intybus* seeds have significant biological activity against MDR bacterial pathogens, and they are also rich in phytochemicals. Future studies on the pharmacokinetic properties of *C. intybus* seeds would be helpful for the development of new antibacterial drugs.

Table 3. Phytochemicals Analysis of *Cichorium Intybus* (Seeds) Extracts

Phytochemicals	Aqueous Extract	Chloroform Extract	Ethanol Extract	Hexane Extract
Alkaloids	+++	++	+++	+
Bufadienolides	+	+	++	-
Carbohydrates	+++	-	+++	-
Flavonoids	+++	-	-	-
Gallotannins	+++	-	+++	-
Phenolics	-	+++	-	+++
Proteins	+	+	+	+
Pseudotannins	-	+++	-	+++
Resins	+	++	++	++
Saponins	+	++	++	++
Steroids	-	-	-	-
Tannins	-	+++	+++	+++
Triterpenoids	++	+	+++	+

Abbreviations: -, Not detected; +, Compound present in small amount, ++, Compound present in moderate amount, +++, Compound present in higher amount.

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Footnotes

Authors' Contribution: Hazir Rahman and Usman Ali Khan contributed equally; Hazir Rahman and Usman Ali Khan designed the study, performed the experiments, interpreted the results, and drafted the manuscript; Muhammad Qasim, Noor Muhammad, and Muhammad Daud Khan analyzed the data and helped in drafting the manuscript; Muhammad Asif and Azizullah Azizullah contributed to results interpretation and discussion; Muhammad Adnan and Waheed Murad contributed to plant identification and in manuscript revision; All authors read and approved the final manuscript.

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References

- Alviano DS, Alviano CS. Plant extracts: search for new alternatives to treat microbial diseases. *Curr Pharm Biotechnol*. 2009;**10**(1):106-21. [PubMed: [19149593](#)].
- Kushad M, Masiuyamas J, Smith M, Kalt W, Eastman K. Health promoting phytochemicals in vegetables. *Hortic Rev*. 2003;**28**:125-85. doi: [10.1002/9780470650851.ch3](#).
- Abourashed EA, Koetter U, Brattstrom A. In vitro binding experiments with a Valerian, hops and their fixed combination extract (Ze91019) to selected central nervous system receptors. *Phytomedicine*. 2004;**11**(7-8):633-8. doi: [10.1016/j.phymed.2004.03.005](#). [PubMed: [15636177](#)].
- Flaherty JP, Weinstein RA. Nosocomial infection caused by antibiotic-resistant organisms in the intensive-care unit. *Infect Control Hosp Epidemiol*. 1996;**17**(4):236-48. [PubMed: [8935732](#)].
- Hazra B, Sarkar R, Bhattacharyya S, Roy P. Tumour inhibitory activity of chicory root extract against Ehrlich ascites carcinoma in mice. *Fittoterapia*. 2002;**73**(7-8):730-3. [PubMed: [12490244](#)].
- Bremness L. 4 ed. London, UK: Darling Kindersley; . The complete book of herbs of joanna chisholm. 1998.
- Khan UA, Rahman H, Qasim M, Hussain A, Azizullah A, Murad W, et al. Alkanna tinctoria leaves extracts: a prospective remedy against multidrug resistant human pathogenic bacteria. *BMC Complement Altern Med*. 2015;**15**:127. doi: [10.1186/s12906-015-0646-z](#). [PubMed: [25902854](#)].
- Valgas C, de-Souza SM, Smania EFA, Smania A. Screening methods to determine antibacterial activity of natural products. *Braz J Microbiol*. 2007;**38**:369-80. <http://dx.doi.org/10.1590/S1517-83822007000200034>.
- Clinical Laboratory Standards Institute. . Performance standards for antimicrobial susceptibility testing; 16th informational supplement. U.S.A: CLSI M100-S16. Wayne, PA: Clinical and Laboratory Standards Institute; 2007.
- Petrus EM, Tinakumari S, Chai LC, Ubong A, Tunung R, Elexson N. A study on the minimum inhibitory concentration and minimum bactericidal concentration of Nano Colloidal Silver on food-borne pathogens. *Inter Food Res J*. 2011;**18**:55-66.
- Tiwari P, Kumar B, Kaur M, Kaur G, Kaur H. Phytochemical screening and extraction: a review. *Internationale Pharmaceutica Scientia*. 2011;**1**(1):98-106.
- Ncube NS, Afolayan AJ, Okoh AI. Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends. *African J Biotechnol*. 2008;**7**(12):1797-1806.
- Alagesaboopathi C, Sivakumar R. Phytochemical screening studies on the leaves and stem of *Andrographis neesiana* Wight-An endemic medicinal plant from India. *World Appl Sci J*. 2011;**12**:307-11.

14. Tchana ME, Fankam AG, Mbaveng AT, Nkwengoua ET, Seukey JA, Tchouani FK, et al. Activities of selected medicinal plants against multi-drug resistant Gram-negative bacteria in Cameroon. *Afr Health Sci.* 2014;**14**(1):167-72. doi: [10.4314/ahs.v14i1.25](https://doi.org/10.4314/ahs.v14i1.25). [PubMed: [26060474](https://pubmed.ncbi.nlm.nih.gov/26060474/)].
15. Mehmood N, Zubair M, Rizwan K, Rasool N, Shahid M, Uddin Ahmad V. Antioxidant, antimicrobial and phytochemical analysis of cichoriumintybus seeds extract and various organic fractions. *Iran J Pharm Res.* 2012;**11**(4):145-51. [PubMed: [24250548](https://pubmed.ncbi.nlm.nih.gov/24250548/)].
16. Shaikh T, Rukhsana A, Rub A, Sasikumar S. Antimicrobial screening of Cichorium intybus seed extracts. *Arab J Chem.* 2012;**10**:10-6. <http://dx.doi.org/10.1016/j.arabjc.2012.04.012>.
17. Morton JF. Search for carcinogenic principles. In: Swain T, Kleiman R, editors. Recent Advances in phytochemistry. Florida, USA: Plenum Press; 1980. pp. 53-73.
18. Nandagopal S, Kumari BDR. Phytochemical and antibacterial studies of Chicory (Cichorium intybus L.)-A multipurpose medicinal plant. *Adv Biol Res.* 2007;**1**(1-2):17-21.
19. Pratibha N, Sushma D. Screening for antioxidant and antibacterial potential of common medicinal plants in the treatment of acne. *Int J Drug Dev Res.* 2012;**4**(1):65-71.